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Review

Exploring the mesenchymal stem cell niche using high throughput screening

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ABSTRACT

In the field of stem cell technology, future advancements rely on the effective isolation, scale-up and maintenance of specific stem cell populations and robust procedures for their directed differentiation. The stem cell microenvironment – or niche – encompasses signal inputs from stem cells, supporting cells and from the extracellular matrix. In this context, the contribution of physicochemical surface variables is being increasingly recognised. This paradigm can be exploited to exert control over cellular behaviour. However, the number of parameters at play, and their complex interactions, presents a formidable challenge in delineating how the decisions of cell fate are orchestrated within the niche. Additionally, in the case of mesenchymal stem cells (MSC), more than one type of stem cell niche has been identified. By employing high throughput screening (HTS) strategies, common and specific attributes of each MSC niche can be probed. Here, we explore biological, chemical and physical parameters that are known to influence MSC self-renewal and differentiation. We then review techniques and strategies that allow the HTS of surface properties for conditions that direct stem cell fate, using MSC as a case study. Finally, challenges in recapturing the niche, particularly its three dimensional nature, in surface-based HTS formats are discussed.

1. Stem cells and the stem cell niche

Harnessing the potential of stem cells to proliferate and form a range of differentiated cell types is critical to the application of these cells in the regenerative medicine and cell therapy arenas. Specification of cell fate is a paramount goal and the stem cell niche microenvironment is central to the regulation of stem cell division and fate allocation. The niche is a specialised microenvironment consisting of stem cells, differentiating progenitors, non-stem support cells and extracellular matrix (Fig. 1). The niche structure conspires to integrate signalling from soluble and surface-bound factors, cell-cell contacts and mechanical properties of the surrounding environment [1].

Tissue reservoirs of stem cells are retained into adult life as a cell source for tissue regeneration and repair in the face of injury. In the absence of a regenerative need, stem cells typically remain quiescent within the niche, limiting the accumulation of genetic damage in this cell pool [4]. When required, the sequestered cells are able to rapidly respond to cues to produce differentiating progeny. To compensate for cell turnover and differentiation, cell division to form replacement stem cells must also occur. Hence, stem cell division may be symmetric (producing two stem cell or two differentiating daughter cells) or asymmetric (producing one differentiating cell and one stem cell) cell division [5]. While its structure may vary, the niche must be capable of specifying symmetric and asymmetric cell division as needed.

Our understanding of how the niche structure is able to specify both symmetric and asymmetric outcomes from a single cell population relies on well-studied *in vivo* systems such as the *Drosophila* testes and haematopoietic bone marrow compartment [4,6]. The positioning of the daughter cells within the niche determines whether cells remain as stem cells or begin differentiation by different levels of exposure to fate determinants. The plane of cell division is the critical instrument of this differential exposure, facilitating whether cells remain with the signalling range of stem cell determinants [7]. Contact with basal lamina and short range diffusible molecule gradients are potential mechanisms to drive asymmetric division [4,6,8].

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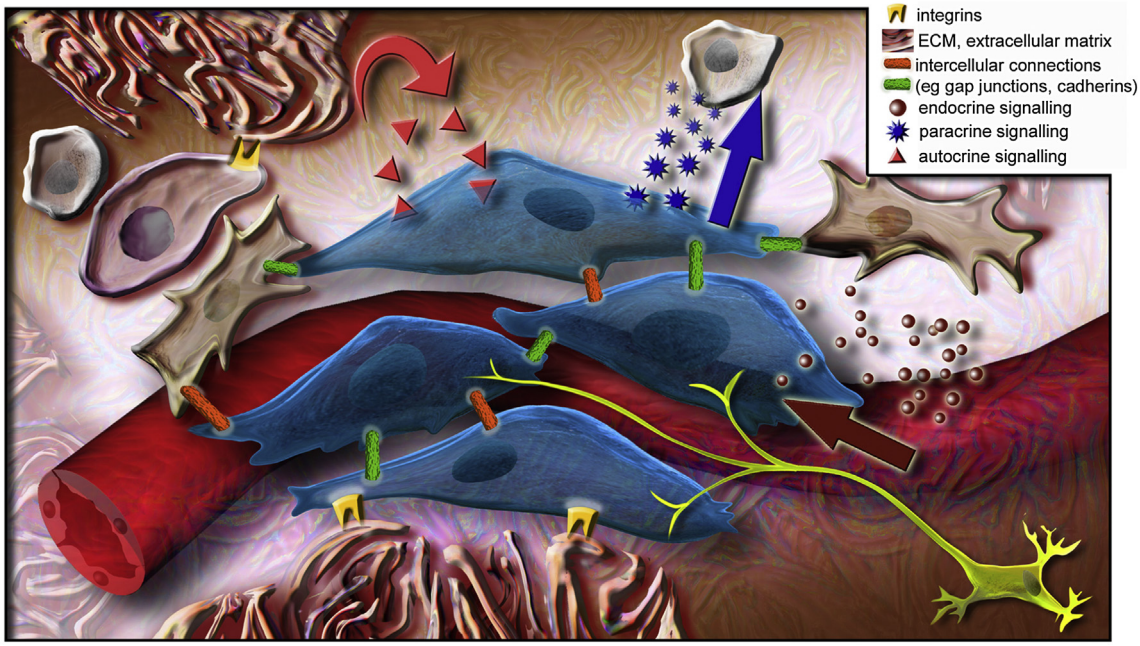

Fig. 1. Mesenchymal stem cell niche. MSC are shown in their putative perivascular niche, interacting with (1) various other cells by means of cell-adhesion molecules, such as cadherins, (2) other MSC (3) extracellular matrix (ECM) deposited by the niche cells mediated by integrin receptors, and (4) signalling molecules. Signalling molecules may include neurotransmitters released from neurons; hormones, growth factors, nutrient levels and oxygen saturation signals transmitted through the vascular system autocrine and paracrine factors produced inside the niche by stem cells and neighbouring cells, including the vasculature. Adapted from Refs. [2,3].

In the majority of cases, adult stem cells are limited to forming tissue-specific cells. However, in some instances, differentiation potential is broader and encompasses multiple tissue types. Mesenchymal stem cells (MSC) are defined by their chondrogenic (cartilage), osteogenic (bone) and adipogenic (fat) fate potential [9]. Other tissues, including neural cells, cardiomyocytes, hepatocytes, and endothelial cells have also been claimed to be derived from MSC or MSC-like cells [10]. MSC are able to be derived from multiple tissues [11], including bone marrow, bone tissue, fat and umbilical cord. Several populations can be isolated from dental tissues [12].

While the MSC is known to act as a key player in the haematopoietic stem cell niche, the characteristics that define its own stem cell niche are far less studied. Initially, MSC were proposed to emanate from the bone marrow as required to replenish mesenchymal tissues throughout the body [13]. Inefficient long-distance homing capability, coupled with variable detection in circulation, tends to rule out this hypothesis [14]. In its place, a perivascular location for the MSC niche has been suggested [15,16]. This niche position explains why MSC can be derived from several vascularised sites [17]. A close relationship between MSC and pericyte cell populations has been confirmed by gene profiling [18]. However, this does not easily explain the presence of MSC in non-vascularised tissues such as articular cartilage [19,20]. Using a model system of rodent semi-deciduous incisors, where dentine and underlying tissue is ground down and must be continuously replaced, Feng and colleagues recently revealed that a second source of MSC are harboured within the dental pulp, distinct from that associated with the vasculature (Fig. 2) [21]. Although pericytes contributed to dental tissue repair, they do so in concert with MSC of other origins. This raises the possibility of the existence of several different MSC populations and supportive niches.

The consensus definition of MSC, built to encompass stem cells derived from diverse tissues, relies on a suite of both positive and negative markers to define the MSC population [9,22]. However, these criteria are limited, since these markers are not specific to

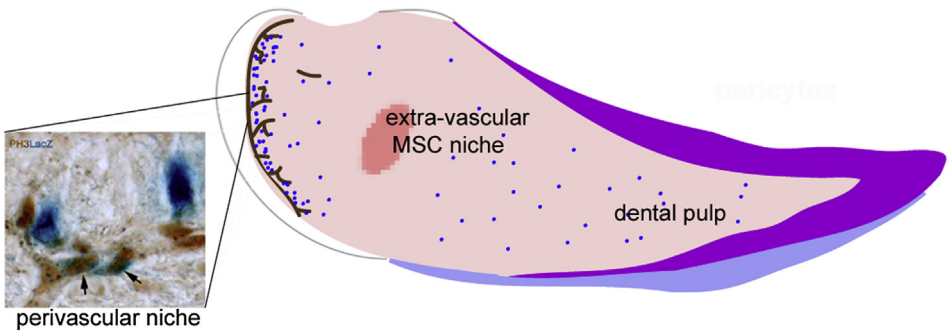

Fig. 2. Two distinct MSC sources exist within rodent incisors. Tracing of odontoblast generation revealed a pericyte derived odontoblast population (blue) associated with blood vessels in the cervical loop (brown) and found sporadically within the dental pulp (pink). A second, non-pericyte population of stem cells was located approximately 400 μm from the cervical opening (red). In the inset showing the typical histology, enamel is stained blue, dentin purple. Adapted from Ref. [21]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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MSC. Additionally, the criteria do not distinguish between multipotential MSC and committed progenitors: defining the MSC population possessing the capacity to self-renew on the basis of surface marker expression remains elusive. Whilst no single marker has so far been identified for MSC across all anatomical sites of derivation [2,22,23], the markers STRO-1 and CD146 have been reported to mark the MSC population residing within a subset of tissue sources [15,16,18]. Differences exist in differentiation bias and functional properties between MSC derived from different sites (Table 1). Likewise, variations in transcriptome profile and surface marker expression have also been reported [23,24]. It is not clear whether this reflects innate differences in the various MSC cell populations at each site, or whether differences in the stem cell environment prime the stem cell population to produce slight variations in outcome. The predilection of MSC to differentiate into cells from their tissue of origin may reflect the presence of committed progenitors within the pool of cells isolated as MSC (for example [25,26]). The majority of studies of MSC differentiation potential are carried out *ex vivo*, and the culture conditions are poised to play a role in directing MSC behaviour [27]. MSC in culture are known to be highly sensitive to environmental cues, and a large body of work has arisen detailing the effect of surface and cell culture materials parameters on this cell type.

Given the difficulties on accessing the putative niche, *in vitro* experimental systems incorporating surface-displayed biological factors present a methodology for probing the cues governing MSC renewal and differentiation. Considering the number of possible cues and their interactions, high throughput screening (HTS) methodologies are particularly attractive for studying the construction of the MSC niche. In this review, we use MSC as a case study to showcase the potential of surface-based HTS to explore biological, chemical and physical parameters acting within the stem cell microenvironment.

2. Parameters controlling MSC fate decisions

External stimuli that control MSC fate can be broadly categorised as biological, chemical and physical. These external inputs activate complex signalling pathways, which in turn modulate intrinsic regulatory pathways to promote self-renewal or the generation of specialised cells. Fig. 3 summarises factors, signalling pathways and master transcription factors associated with the three canonical MSC differentiation pathways of osteogenesis (bone formation), chondrogenesis (cartilage formation) and adipogenesis (fat cell formation), together with MSC self-renewal. Control of MSC maintenance and differentiation has been reviewed elsewhere in detail [2,43–50], including from a biomaterials-centric perspective [51]. Notably, there is overlap and cross talk between cohorts of signalling molecules associated with self-renewal and differentiation pathways. For instance, FGF-2 (fibroblast growth factor-2) is critical to MSC proliferation [52], but also synergises with BMP-2 (bone morphogenic protein-2) to promote osteogenic differentiation [53]. This example emphasises the notion that the presence of a single factor may be insufficient to predict cell response. Likewise, the timing and context of the signalling pathway activation with respect to the stage of cell maturation informs the specific action of the signal. For example, activation of the canonical Wnt pathway stimulates differentiation of MSC committed to osteogenic lineages, but inhibits their terminal differentiation as mature osteoblasts [44].

Differentiation of MSC in culture is also strikingly sensitive to the surface chemistry of the cell culture substrate: human MSC can be differentiated towards osteogenic, adipogenic and chondrogenic lineages in the absence of morphogenic factors through functionalisation of the substrate surface [54–57]. These reports indicate that MSC maintain their phenotype in contact with CH_3 -functionalised surfaces under basal conditions. NH_2- and SH-modified surfaces induce osteogenic differentiation but are not supportive of chondrogenesis. In stark contrast, OH- and COOH-surface functionalisation promotes chondrogenesis. Other work suggests that COOH-surface functionalisation upregulates osteogenic differentiation [58]. Differentiation bias observed in MSC in response to surface chemistry is thought to be mediated by adsorption of proteins, both those present in the culture medium formulation and those produced endogenously, through hydrophobic and electrostatic interaction. The role played by protein content in the cell culture milieu in specifying stem cell–material interaction is demonstrated by the change in surface COOH-group density required to support mouse embryonic stem cell self-renewal when conventional medium formulation is replaced by a low serum containing medium variant [59]. Surface chemistry also modulates the presentation of ECM proteins on the surface, modifying in turn integrin binding and focal adhesion kinase signalling [60,61], and resulting in the activation of pathways that also receive inputs from the biological and physical properties of the niche.

The 3D shape of tissues and organs during embryogenesis is influenced by the mechanical forces and environmental stimuli that they experience [62,63]. Mechanical forces regulate cell proliferation, haemostasis, migration and differentiation. *In vitro*, these mechanical forces arise from cell-cell contacts, extracellular matrix secreted by the cell population and are also modulated by characteristics of the culture substrate. The influence of physical properties of materials on MSC differentiation and self-renewal is well documented. Mediated through mechanotransducive pathways that leverage cell contractility to process external stimuli [64,65], strong responses to surface topography, including feature size shape and spacing of patterned substrates, have been described [66–69]. Substrate stiffness, or elastic modulus, also biases MSC differentiation through changes in focal adhesion formation and cytoskeletal tension [70]. The particular cell fate engendered by the elasticity of the *in vitro* culture substrate correlates with known elastic properties tissues *in vivo*. For example, MSC are more likely to commit to osteogenic fates when cultured on stiff substrates

Table 1
MSC site of derivation biases differentiation trajectory and functionality.

Site	Property	Ref
Bone marrow MSC	Efficient osteogenic and chondrogenic differentiation	[26,28–31]
	Limited capacity for self-renewal (comparatively low proliferation rate, early senescence)	[32–34]
Adipose derived MSC	Enhanced immunomodulation	[35,36]
	Capacity to differentiate to cardiomyocyte fate	[32–34]
	High capacity for self-renewal (high proliferation rate)	[32]
Umbilical cord blood derived MSC	Poor osteogenic, adipogenic and chondrogenic differentiation yields	[29,37,38]
	High proliferation rate	[33]
	Low derivation efficiency (few MSC colonies arising from tissue)	[29,38]
Synovium derived MSC	Differentiation bias towards osteogenic, but not chondrogenic fate	[39]
	High proliferation rate	[39]
	High derivation efficiency	[39]
Dental pulp derived MSC	Enhanced odontogenic and neural potential	[26,40,41]
	Poor adipogenic and chondrogenic potential	[42]

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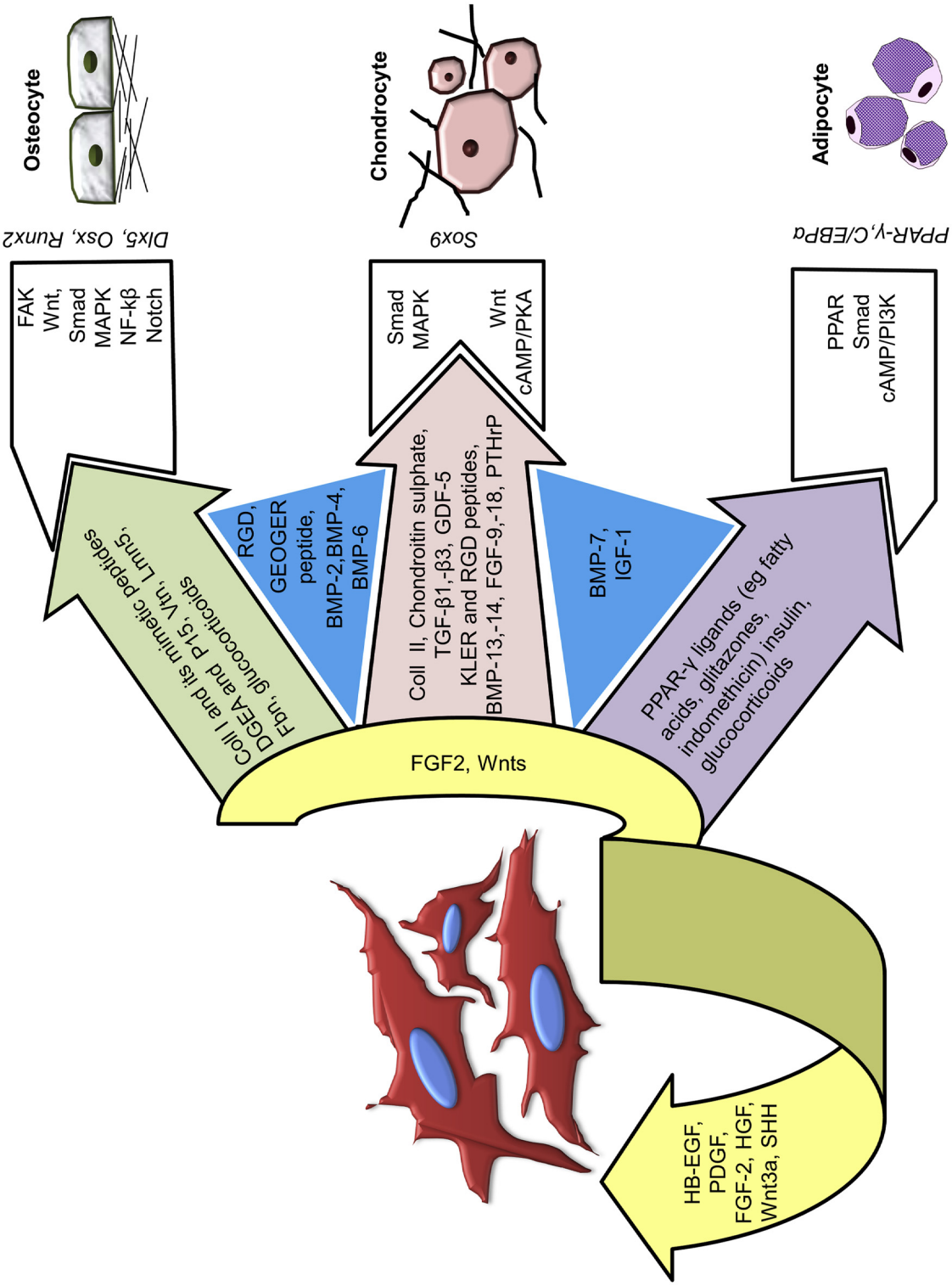

Fig. 3. Soluble, surface associated and ECM factors influencing MSC fate. MSC maintenance and proliferation molecules shown in yellow arrow; specific biomolecules associated with directed differentiation shown in green and red arrows and blue polygons, respectively; downstream signalling pathways activated shown in white polygons. Master transcription factors for each differentiation pathway shown in italics. Coll, collagen; Fbn, fibronectin; Lmn, laminin; PThrP, parathyroid hormone-related protein; Vtn, vitronectin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

similar in elasticity to the bone environment, whilst softer substrates elicited increased myogenic or neurogenic expression. Surface stiffness strongly influences the lineage commitment of MSC. It has been shown that human MSC grown on substrates with varying stiffness express diverse cell lineage markers [70,71]. Other chemical and physical properties of cell culture materials and biomaterials that influence MSC fate have been recently reviewed [51].

3. High throughput technologies that allow screening of the stem cell niche

The complexities of stem cell niches create a tremendous challenge when attempting to gain a profound understanding of the mechanisms underlying cell fate regulation. Several *in vitro* systems have been designed in order to screen the natural and synthetic biological factors affecting stem cell behaviour. Cell microarrays and gradient surfaces allow the surface parameters that contribute to the niche to be examined in a high throughput manner. Cell microarrays consist of a library of distinct material or molecular entities, displayed on a single substrate at specific addressed locations, so that comparative cell response to each test entity can be assessed. Surface gradients incorporate a gradual variation of a surface property across the length of a substrate. Using these formats, large combinatorial spaces can be screened on a single surface. These techniques speed up analysis and decrease cell experimental variability by allowing data that would usually require a number of discrete experiments to be directly compared using the same cell population. Importantly for stem cell populations that may be difficult to obtain in large numbers, miniaturisation of the screening platform and reductions in the number of control replicates necessary for statistical analysis maximise the amount of data that can be obtained from limited numbers of cells. Each methodology has its particular advantages: microarray technologies allow for the simultaneous analysis of many combinations of surface variations, capturing a large combinatorial space to on a single surface. Gradient surfaces permit only one or two parameters to be investigated at a time, but the ability to screen variables continuously across a range may reveal subtle changes and interactions that may be overlooked under the discrete, specified combinations necessitated under microarray screening. This allows us to determine the limits by which each surface characteristic is effective, allowing specific tailoring of a surface to deliver an optimum cell response [72].

3.1. Cellular microarrays

By adapting the technology used to fabricate DNA microarrays, in which the expression of hundreds of genes can be measured simultaneously, cellular microarrays can be constructed to analyse cell response to a combinatorial library of biomolecules or materials surface features (Fig. 4A–C). A broad range of analytes, including ECM components, cadherin adhesion molecules (CAMs), growth factors (GFs), peptides, antibodies, nucleic acids, siRNA, monomers and polymers have been patterned onto the microarrays [73,74]. Thousands of spots can be printed on to a single array surface the size of a microscope slide [75]. Spatial separation of spots allows each spot to be considered as an independent experimental replicate. Spot size can be tuned to capture cell numbers ranging from a single cell to thousands of cells. In addition to the advantages of analytical speed, the array format requires only a small fraction of the analyte material compared to conventional testing methods [76].

Generally, a cell microarray is based on a solid support substrate. Typical substrates include glass, polymers including Thermanox and nitrocellulose, gold or silicon, and sometimes feature thin coatings such as poly-L-lysine, agarose or polyacrylamide [77–79]. Hydrogel-based arrays offer an alternative to solid substrates [80]. Hydrogels allow the elastic modulus (stiffness) of the screening platform to be tuned. Using a hydrogel platform, the modularity of the biomolecule-spotting approach can be augmented with stiffness modulation [81–83], capturing the interaction of physical, chemical and biological contributions on a single surface.

The surface chemistry of the base substrate must be considered as part of the array printing strategy: it plays a significant role in immobilisation of biomolecules through presentation of functional groups. Some commonly incorporated functional groups are N-hydroxy succinimide esters, aldehydes, isocyanates, epoxides, and oxyamine (-O-NH_2) groups [78]. Cell microarray substrates can be functionalised by dip or spin coating [84,85], plasma treatment [85,86], surface grafting [84], self-assembled monolayer (SAM) formation [77] and microfluidics [73] approaches. Target molecules are able to be adsorbed onto functional groups of the base substrate via electrostatic interaction, ligand-receptor pairing such as biotin-avidin, and covalent attachment [87]. Whilst simple to implement, adsorption of biomolecules onto the surface runs the risk of inactivation through steric hindrance or denaturation [88,89] and of desorption during the cell culture period. Covalent immobilisation is hence desirable for studying cell behaviour across long-term culturing. Other immobilisation strategies involve the generation of tagged fusion proteins with coordinate anchors on the surface (for example His-tag/Ni^{2+}, biotin/avidin, Fc/protein A [90,91]). Surface tethered growth factors retain activity and the ability to induce a functional cell response [92,93].

Printing of biomolecules and polymeric materials onto the array can be performed by contact printing, ink-jet printing, photolithography or soft-lithography [94] (Fig. 4A). Contact printing is achieved using a robotic spotter by dipping a solid pin into a solution of the target molecule and robotic spotting at defined locations on the substrate. Ink-jet printing, also known as non-contact printing, is based on the ejection of nanoliter volumes of the desired solution from a micro-capillary onto the substrate. The ink-jet printing technique produces homogenous spot geometry with precise control over the dispensed volume [95]. Photolithography involves the irradiation of a surface with high energy beams, such as UV light, through a photomask. This results in the ablation of photoresist layer, initiation of polymerisation or formation of chemical bonds between the surface and the grafted molecule [96]. Soft lithography is a technique developed to pattern surfaces that uses stamps or channels fabricated in an elastomeric (`soft') material for pattern transfer. The stamp is generally prepared by casting the liquid prepolymer of an elastomer (such as mostly poly (dimethylsiloxane) (PDMS)) against a master that has been created via photolithography. The stamp is then "inked" with the molecule to be printed [97].

The base substrate of the final microarray construct must be inert, limiting protein adsorption and cell adhesion between spots in order to avoid interaction between independent experimental replicates. This can be achieved by passivation of the background after array printing with cell repellent polymers such as polyethylene glycol (PEG) [98], polyacrylamide [76] and agarose [79,99], or proteins such as bovine serum albumin (BSA) [84,100]. The high fluorescence generated by some of these agents can impact downstream analysis, limiting the use of fluorescence microscopy based techniques such as immunofluorescence [101]. Alternatively, passivated array backgrounds can be modified to produce an adhesive substrate suitable for biomolecule attachment. Poly(-hydroxyethyl methacrylate) (PHEMA) polymeric coatings [102,103] have been described to resist cell attachment and provide a matrix into which printed macromonomers can interpenetrate to become

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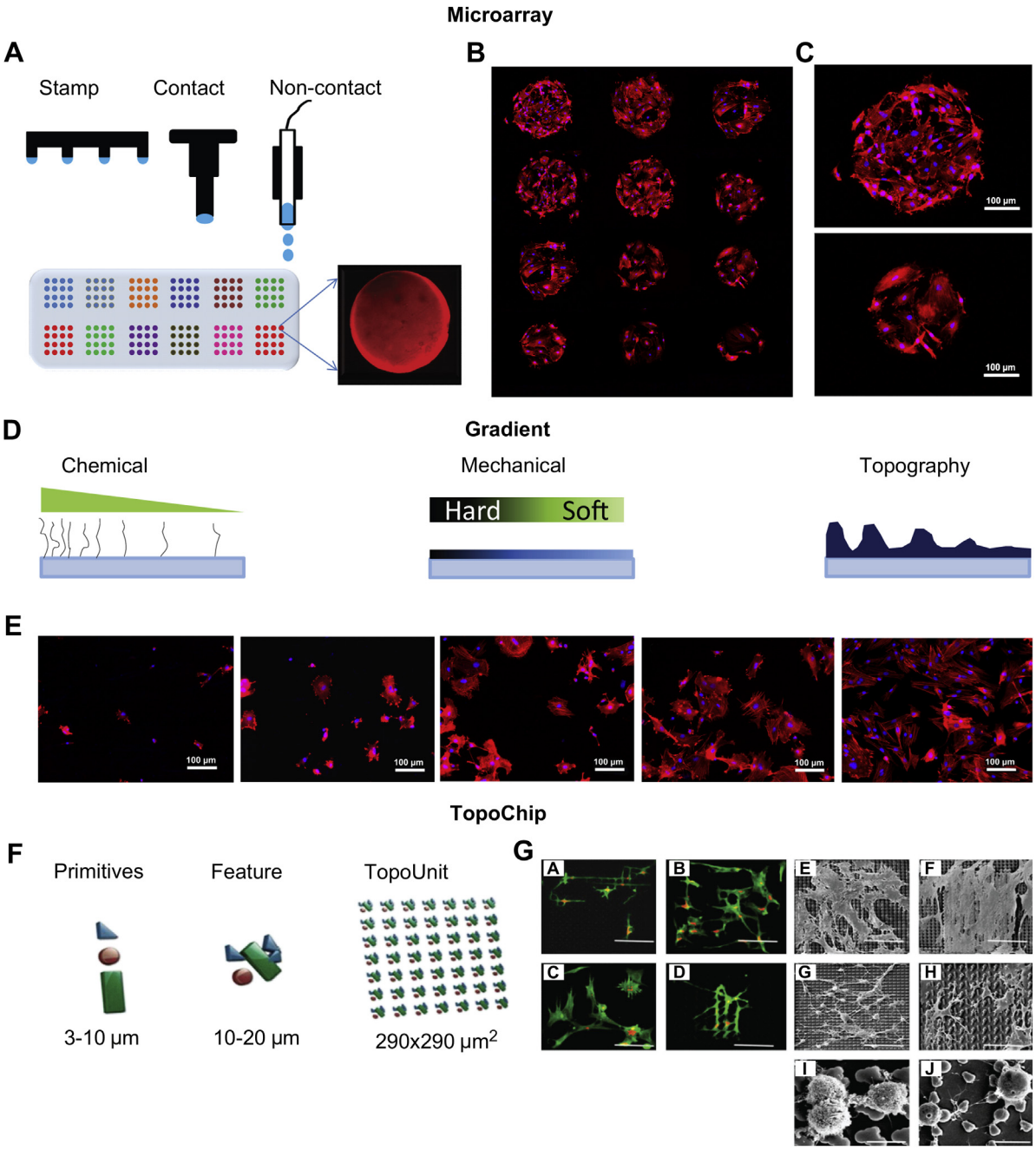

Fig. 4. Interrogation of artificial stem cell niches by high throughput screening technology, (A) Schematic presentation of a cell microarray platform generation by stamping, contact and non-contact printing of pico/nano-microliter volumes of biomolecules on an activated substrate. (B) MSC captured on ECM protein arrays (collagen I, laminin, collagen II and fibronectin (top to bottom) printed with solution concentrations of 200, 150 and 100 μg/ml from left to right, respectively) spots after 4 h. Nucleus was stained with Hoechst in blue. (C). MSC captured on collagen I (top) and fibronectin (bottom) printed at concentration of 150 μg/ml. The cytoplasm was stained by phalloidin in red and the nucleus by Hoechst in blue. (D) Attributes of surface gradient based on chemistry, mechanical and topography. (E) Change in the morphology of MSC grown on a nanowire to nanoparticle gradient surface, small, rounded cell on wires to branched, spindle or polygonal shapes on nanoparticle decorated surfaces. (F) The design of the TopoChip is based on three types of primitives: circles, triangles and lines. Repeated features constitute a TopoUnit. (G) Morphology of MSC on different TopoUnits revealed by fluorescence imaging (pseudocolored green: actin stained with Alexa Fluor 488 phalloidin; red: nuclear staining with TOTO-3; scale bar: 90 μm) and SEM (Scale bar: 10 μm). Images F and G have been adapted from Ref. [107]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

crosslinked. Allylamine plasma polymer coated glass slides spin coated with copolymer poly(ethylene glycol)methyl ether methacrylate (PEGMA) and glycidyl methacrylate (GMA) in dioxane [85,86] provide reactive epoxy polymer groups from the GMA component to immobilise biomolecules, while the display of poly(ethylene glycol) on the coating, provided by the PEGMA component, inhibits non-specific cell attachment. Polymers arrayed onto a non-fouling surface, such as a PEG-based coating, can also be covalently attached by modifying the polymer with a UV-reactive crosslinker or incorporating epoxy groups into the PEG layer [98].

Given the long period of culture time required to investigate stem cell behaviour, especially in the case of terminal differentiation (up to four weeks for MSC), a particular consideration for the application of cell microarrays to this purpose is the long-term stability of the array coating. The delamination of polymer or hydrogel layer from the underlying substrate under cell culture

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conditions remains a challenge to be overcome [98,104] since the retention of the cellular pattern relies on the continued integrity of the passivating layer. Covalent binding of the passivating layer to the underlying substrate appears to improve its longevity [105,106]. A compromise may be required when selecting a passivating strategy for longer-term use. For example, whereas BSA is excellent for blocking short-term cell attachment between spots [100], the BSA passivating layer degrades within the first week of culture, requiring an alternative PEG-based strategy to be devised (S. Rasi Ghaemi, unpublished data).

3.2. Gradient surfaces

Gradient surfaces allow the investigation of cell responses to one or two specific surface parameters, such as functional group density or factor concentration, to be investigated in depth over a defined range (Table 2, Fig. 4D,E). The position along the gradient axis correlates to a particular value or level of the variable of interest. The influence of a particular surface parameter can be inferred from trends in cell response across the surface. As noted above, gradient surfaces allow in depth investigation of response to a specific parameter within a set range of value, but are constrained in the number of variables that can be tested. This lends the format to studies aimed at the optimisation of conditions needed to induce a certain outcome, rather than screens to identify new biomaterial "hits". The development of 2D gradients, whereby two gradients run in different directions of the surface plan, allows the interaction of two parameters to support or drive stem cell self-renewal or differentiation to be probed. In the case of orthogonal 2D gradients, the two parameters are graded in a 90° angle to one another. Incorporating three components into a surface pattern by printing binary stripes of two molecules over a surface gradient of third entity heralds the creation of gradient surfaces with additional complexity [108].

Table 2
Gradient surfaces generated to screen cell behaviour.

Gradient type	Gradient parameter	Example references
Surface chemistry gradients	Polymer thickness gradients	[109,110]
	COOH-group density gradients	[59,121,122]
	NH_2-group density gradients	[123]
	Polymer graft density gradients	[124,125]
	Co-polymer composition gradients	[126,127]
	Wettability gradients	[128–130]
Surface immobilised biomolecule gradients	Biomolecular gradients created by diffusion	[119,120,131]
	Biomolecular gradients created by printing	[132,133]
	Electrospun nanofibre based gradients	[134–136]
Physical property gradients	Elasticity gradients	[137–140]
	Topological gradients	[111,112,141]
	Pore size gradients	
	Micro- and nanoparticle gradients	[142,143]
	Roughness gradients	[144,145]
Orthogonal gradients	Orthogonal density gradients of two biomolecules	[117]
	Orthogonal gradient of surface chemistry and topography	[146]
	Orthogonal gradient of polymer molecular weight/thickness	[113]
	Orthogonal gradient of copolymer composition and crystallinity	[147]
	Orthogonal gradient of substrate elasticity and ECM surface density	[148]
	Orthogonal gradient of elasticity and roughness	[144]
	Orthogonal gradient of pore size and peptide density	[149]

Physical and chemical transport phenomena can be harnessed to create a range of parameter values across a surface. For example, the diffusion of monomers species under a fixed mask in a plasma reactor results in the formation of polymer thickness gradients [109,110] and differential current density generated via asymmetric electrode positioning during silicon etching allow the generation of pore size gradients in porous silicon [111,112]. Other gradient-generating techniques are based on changing the level of exposure to the modifying chemistry by movement of the substrate relative to a source, e.g. by means of dip-coating [113] or by moving a substrate under a mask with changing feed gas in a plasma polymer reactor [114]. Surface-bound gradients have recently been reviewed in depth [115].

Surface gradients of biomolecules can be built by exploiting a gradient of underlying surface physicochemical properties. For example, protein density gradients can be fabricated via differential adsorption across surface gradients of wettability or roughness [116,117]. Biomolecular gradients can be grafted onto hydrogel substrates by harnessing the diffusion of a chemical modifier through the polymer to form a gradient of chemical modifications available for biomolecule coupling [118]. Alternatively, microfluidic approaches, in which the repeated mixing and dilution of two solutions inside a network of microchannels generates the gradient can be used to create biomolecular gradients on top of [119] or within [120] the hydrogel structure. In contrast to diffusion based gradients, adjustment of reagent flow and network structure offer flexibility in gradient size and shape [89].

3.3. Modular topographical screening

The tuning of micro- and nanoscale surface topography for specific applications in regenerative medicine has not yet been fully realised because the number of possible topographies using micro- and nanoscale features is practically unlimited. The rational design of biomaterial topographies is thus best tackled using combinatorial screening approaches. Recently, Unadkat et al. [107] reported on technology capable of analysing MSC behaviour in response to microscale shapes in high throughput. The so called TopoChip is a library of 2178 randomly generated surface topographies based on a mathematical algorithm. Topographies are designed using three types of primitive shapes, triangles, circles, and rectangles to generate different types of patterns (Fig. 4F,G). A 'feature' is fabricated by first randomly selecting parameter values for feature size, the number of primitives and the distribution over the different primitive types, the size of the primitives, and the degree to which the primitives are aligned. After designing the chip, a chromium mask is used to pattern a silicon wafer by conventional photolithography. The micropatterned silicon wafer then forms a master for hot embossing generating arrays of topographies in a thermoplastic chip substrate. To avoid artefacts caused by differences between the edge and center of the chip, patterns are randomly replicated twice per chip. Cell response to each feature of the TopoChip was noted to be independent of the surrounding features. The power of generating random shaped topographical features was demonstrated by discovering an unexpected correlation between abstract mathematical measures of shape and cell behaviour. A machine learning algorithm was employed to find relationships between 35 topographical parameters that predict high MSC proliferation and osteogenic differentiation.

4. Surface based HTS of biological and physicochemical signals active within the MSC niche

Stem cell behaviour within the niche microenvironment is governed by interactions between stem cells and soluble proteins,

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immobilised factors, small molecules and neighbouring cells [6]. In this section, we discuss how HTS can be used to parse out the influence of biological, chemical and physical factors in mimicking the MSC microenvironment.

4.1. Screening of surface mediated biological signals regulating MSC fate

Localisation of stem cells within the niche requires adhesive interactions with ECM proteins and supporting cells [150,151]. The ECM component of the niche plays a key role as an adhesive anchor to retain stem cells within the niche. High throughput technology provides a platform to study the effect of a library of different ECM molecules over a wide concentration range on one single chip. Using a microarray designed for MSC culture in a serum-free environment allowed the determination of the concentration of surface-presented fibronectin optimal for cell adhesion to microarray array spots [100]. High density ECM arrays, capable of analysing over 750 ECM combinations on a single hydrogel coated slide, have recently been described [75]. The development of an "adhesion profile" to differentiate metastatic adenocarcinoma from primary tumour demonstrates the potential of applying cell microarrays to probe and separate the identity of cell sub-populations.

ECM proteins also provide a platform for the presentation of cell instructive GFs. An example is the modification of neural differentiation by tethering soluble sonic hedgehog (SHH) protein anchored to specific domains of vitronectin [152]. GFs may act synergically with ECM, through a mechanism of cross talk between different intracellular domains of signalling receptors [153]. A cell microarray screen of adipose derived MSC, aimed at optimising primary hepatocyte culture conditions, revealed that co-presentation of HGF with collagen I increased albumin secretion, a measure of hepatocyte function, almost two-fold compared to replicates where GF was supplemented in solution [154]. In a cell microarray assay designed to screen for interactions between ECM proteins and GF molecules relevant to the neural stem cell niche, combinations of matrix molecules and GFs were scored semi-quantitatively to identify conditions conducive to neural stem cell (NSC) self-renewal and differentiation to astrocytes and glial cells [155]. Similarly, Soen et al. [156] developed an array system combining ECM and CAMs with signalling molecules to examine the effect of those factors on the human neural precursor cell differentiation. Analysis of one, two and three factor combinations allowed conditions supportive of glial cell formation, neural cell formation and neural progenitor maintenance to be categorised. Application of signalling factors and ECM components in combination significantly altered cell fate compared to exposure to single factors at the same concentration, revealing synergies in GF/ECM signalling. The characteristics of the neural stem cell niche have also been probed with cell microarrays focussed on immobilised growth factors only [91].

In contrast to the microarray platform introduced by Soen and colleagues, which immobilised both ECMs and signalling factors, Flaim et al. [76] arrayed 32 mixtures of five ECM proteins (collagen I, collagen III, collagen IV, laminin and fibronectin) on a polyacrylamide surface then added GFs in the medium to investigate the proliferation and differentiation of mouse embryonic stem (ES) cells toward early hepatic cells and maintenance of primary rat hepatocyte phenotype. This platform was further developed to allow multiple soluble signalling environments to be interrogated on the one chip by the incorporation of separation gaskets [157]. The format, which permits cross talk between soluble growth factors and ECM to be elucidated, revealed both synergistic (such as Activin A/BMP-4 and collagen I/collagen III) and antagonistic (Wnt3/BMP-4 and collagen I/BMP-4) factor combinations regulating cardiomyocyte cells from ES cells.

This body of work arraying ECM and surface-bound proteins was further extended by Lutolf and co-workers [81] by the development of a PEG hydrogel-based microwell substrate. Robotic spotting enabled an array of candidate proteins supportive of NSC proliferation to be printed within the microwell structure. Protein immobilisation to surface was performed through two complementary strategies: a non-specific maleimide linkage to attach proteins amine groups to the PEG hydrogel and affinity based attachment of Fc-tagged proteins to protein A included in the hydrogel matrix. The hydrogel microwell construct expanded the application of ECM cell microarrays to non-adherent cell types and permitted real-time tracking of the behaviour of single cells. In addition, the elastic modulus of the underlying PEG hydrogel was also tunable, so that the interaction of biological and physical inputs into the niche could be probed. The utility of this platform was demonstrated by a systemic analysis of the contributions of basal lamina (Jagged-1, fibronectin and laminin) and soluble epidermal growth factor (EGF) to NSC self-renewal. Little synergy between presentation of tethered Jagged-1 and laminin was detected in the presence of high concentrations of EGF, but combination of Jagged-1 and laminin improved survival at low EGF concentration compared to each factor alone. The same hydrogel-based microwell system has been used to define proteins active within the haematopoietic stem cell niche [158].

Similar to the stem cell systems interrogated in the HTS studies described above, MSC fate decisions are also influenced by both soluble GF and ECM molecules (Fig. 2). However, the power of the multifactorial analysis made available in the cell microarray format has not been fully exploited to characterise MSC differentiation and self-renewal. A microarray of printed HGF (hepatocyte growth factor) and collagen I has been constructed for hepatic differentiation of adipose derived MSC. The analysis performed was confined to simple factorial analysis of surface-immobilised vs. soluble GF presentation [154].

Gradient surfaces also have potential applications in defining optimal GF concentrations that bias MSC differentiation. MSC are known to respond to GF concentrations in a dose-dependent manner. For instance, IGF-1 (insulin-like growth factor −1) promoted MSC proliferation at concentrations below 10 ng/mL, but triggered differentiation at concentrations above this threshold [159]. Immobilised GF gradients on COOH-functionalised self-assembled monolayers have been used to analyse the threshold surface density of the integrin binding peptide GRGDS and BMP-2 derived peptide necessary to support osteogenesis of MSC [58]. FGF-2 covalently immobilised on photo-crosslinkable PEG hydrogels in a gradient fashion, a construct used to measure chemotaxis of smooth muscle cells, has direct relevance for studying MSC self-renewal [160]. More complex patterned hydrogels, containing independent orthogonally positioned or diametrically opposed gradient patterns, have been constructed using microfluidics using biotinylated and/or Fc-tagged GFs, captured on the surface NeutrAvidin or protein A displayed on the hydrogel surface [119].

4.2. Directing stem cell fate using synthetic chemistry

Suitable methods to screen biomaterial chemistries that are supportive of particular MSC differentiation pathways would enable the engineering of effective scaffolds for regenerative medicine. Categorisation of cellular response to polymer characteristics has been performed using combinatorial high throughput analysis of polymer libraries printed in a microarray format [79,102,161–163], including a screening of polymer materials supportive of MSC adhesion and proliferation [103,164]. By performing *in situ* polymer synthesis on the spots of the microarray, the range of polymers can be expanded to include crosslinked polymers that

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cannot be directly printed [74]. To take full advantage of the high throughput format, both polymer characterisation and cell response also need to be quantified using high throughput technology, preferably on the fabricated chip itself. Incorporation of automated high content fluorescence microscopy together with high throughput surface characterisation (atomic force microscopy, nanoindentation, contact angle and time of flight - secondary ion mass spectrometry (TOF-SIMS)) into the polymer microarray analysis system is conducive to this goal [79,161,163]. The power of the polymer microarray format to facilitate a thorough understanding of stem cell–biomaterials interactions is epitomised in the work of Mei et al. [161,165], in which HTS of a polymer library is used to develop a comprehensive model of human embryonic ES cell colony formation on acrylate-based copolymers. The construction of structure-function relationships between wettability, topography, chemistry and elastic modulus culminate in the predictive mapping of ES cell clonal growth on acrylate polymers outside the training set used to construct the model. A similar strategy could be employed to probe aspects of MSC behaviour, including proliferation, clonogenicity and differentiation to a particular cell fate, thus yielding basic information about the relevant characteristics that inform fate decision making that would otherwise remain obscure when examined using conventional factor-by-factor *ad hoc* analysis techniques.

4.3. Screening physical properties influencing stem cell fate

The physical characteristics of a biomaterial, such as surface elasticity, hardness, stiffness, shape, and topography, have been well documented to strongly influence stem cell behaviour. However, few studies have so far examined these parameters using an HTS approach.

4.3.1. Substrate viscoelasticity

Microenvironmental physical cues that originate from bulk properties of the surrounding material, including elastic modulus (stiffness), can drive stem cell differentiation. Whilst existing studies recapitulate the potential of substrate stiffness as a regulatory factor controlling cell behaviour and cell fate, it is important to note that the majority of studies investigated cell responses on substrates with uniform mechanical properties. Over the last decade, several approaches have been described to fabricate matrix substrates with a defined gradient modulus of elasticity in order to study the interactions of cells with their biophysical microenvironment. The elasticity of polymer matrix substrates, such as hydrogels, can be changed by adjusting the relative concentrations of monomer and crosslinker [138,166], by incorporation of photolithographically patterned photoactivated initiators [120,137,139], or through microfluidics setups [139]. We have recently reported a novel and simple fabrication procedure for PDMS stiffness gradients [138]. The proposed technique is based on a temperature gradient along the length of a planar PDMS substrate during siloxane crosslinking. Application of these stiffness gradients to the screening of osteogenic differentiation of MSC demonstrated that accumulation and mineralisation of MSC is strongly dependent on the PDMS substrate stiffness. Another approach for the fabrication of stiffness gradient surfaces, presented by Engler and Tse [167] is based on differential photopolymerisation of a polyacrylamide hydrogel by exposure of the substrate to UV light source through a photomask. MSC were cultured on the stiffness gradient hydrogels with and without collagen I coating. Accumulation of cells was observed on the stiffest region rather than softest region. MSC durotaxis occurred in the absence of collagen I coating, suggesting stiffness alone, without the mediation of biomolecular stimuli such as GFs or ECM, is sufficient to dictate cell behaviour. Similarly Lutolf and co-workers [81] used PEG hydrogels with modular stiffness ranging from 6 to 26 kPa as the substrate to test interactions between substrate stiffness and ECM presentation. Increasing the stiffness of the substrate resulted in increasing the osteogenic differentiation of MSC independent of fibronectin fragment (FN9-10) surface density (Fig. 5). However, others have reported that ECM molecules presented to MSC have the potential to modulate or override signals from underlying substrate stiffness directing differentiation [71,168].

4.3.2. Topography

As described in Section 2, the MSC response to a surface does not only depend on the chemistry of the biomaterial but also on the geometry of the micro- and nanoscale microenvironment. Based on what has have been achieved through conventional low throughput approaches, high throughput methods facilitate deeper insight via the systematic investigation of the exposure of cells to diverse topographical cues.

Surface porosity can be an effective regulator of cell behaviour [169,170]. Porosity can be introduced into a silicon surface by using it as an anode in an electrochemical etching process based on hydrofluoric acid (HF). The average pore size and the size distribution can be adjusted by varying the HF concentration in the electrolyte and the current density on the anode [171]. Using asymmetric anodisation, gradients with pore sizes ranging from tens to hundreds of nanometers in diameter have been generated in silicon to screen MSC attachment and differentiation to osteogenic lineage [141]. Cell adhesion was observed to increase gradually towards the large pore size end of the porous silicon gradients. Although cell proliferation was not influenced by surface topography, osteogenesis of MSC was enhanced by a porous topography with a ridge roughness lower than 10 nm, and adipogenesis was enhanced across the entire pSi gradient compared with flat silicon substrates. Orthogonal gradient surfaces incorporating pore size variation with a gradient of the adhesive peptide cyclic RGD (c(RGDfK)) allowed the comparative impacts of topography and chemistry on MSC behaviour to be deconvoluted (Fig. 6, [149]). In this case, the contribution of pore size to MSC substrate adhesion was overshadowed in short-term cultures by the influence of cyclic RGD density, allowing cell attachment to occur in the presence of the ligand even in pore size regions that did not previously support cell adhesion.

3D electrospun nanofiber mats incorporating a gradient of amorphous calcium phosphate nanoparticles with different composition have been created using an offset 2-spinnerette device [172]. Osteogenic cells (MC3T3-E1 murine pre-osteoblasts) attached and proliferated more vigorously on the region of the gradients that contained a higher content of calcium phosphate nanoparticles, suggesting that osteoblast cells sense and respond to content of calcium. A similar method has been used to generate graded meshes by co-electrospinning of nanohydroxyapatite/polycaprolactone (nHAP-PCL) and poly(ester urethane) urea elastomer solutions [127]. The incorporation of gradients of GF-loaded microparticles into alginate hydrogels permits temporal changes in the gradient profile to develop over the culture period. This method was used to investigate the interaction of IGF-1 and BMP-2 on MSC osteogenic and chondrogenic differentiation, revealing IGF-1 to synergise with BMP-2 [136].

As discussed above, screening of geometric surface patterns using the TopoChip format provides another means to investigate topographical control of MSC behaviour, including differentiation and self-renewal [107]. This work builds on previous studies using 2D adhesive islands of different shapes to bias differentiation MSC towards osteogenic or adipogenic fate via mechanotransducive influence of Wnt and MAPK pathways [66–68]. Initial studies of

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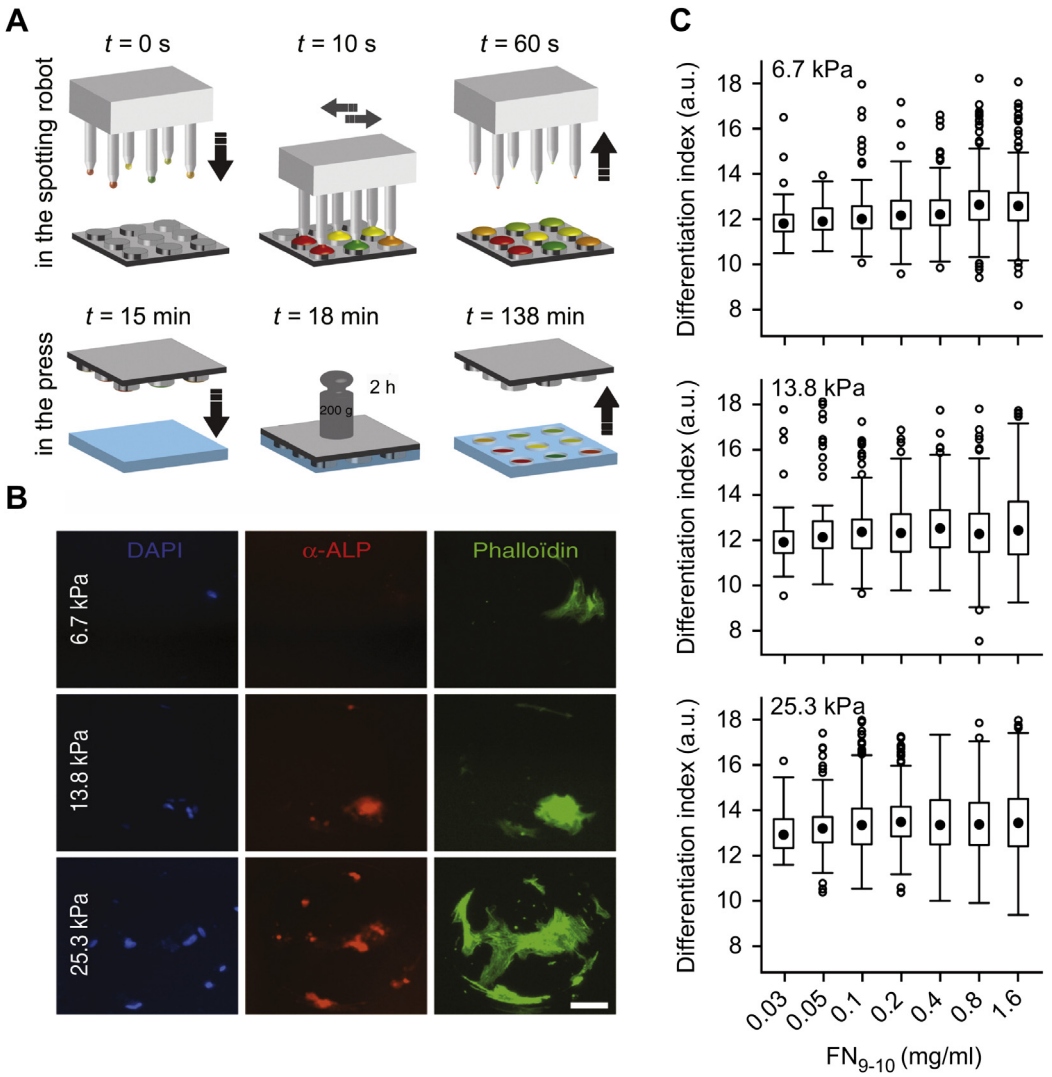

Fig. 5. Combinatorial screening of elastic modulus and fibronectin surface density inputs on MSC osteogenesis using an artifical niche microarray. (A) Construction of niche microarray. Protein solutions are spotted on a microfabricated silicon stamp with a DNA microarray spotter. The protein-coated pillars of the stamp are then pressed into a partially crosslinked PEG hydrogel layer. t = time. (B) α-Alkaline phosphatase (α-ALP) expression in MSC seeded on microarrays of various elastic moduli is monitored after 11 days of culture. DAPI and phallodin are used to determine cell location in the microwells. (C) The correlation of osteogenic differentiation with matrix stiffness and fibronectin surface density is assessed using an index incorporating intensity of α-ALP staining normalised to cell number within the microwell. Adapted from Ref. [81].

cell proliferation confirmed that MSC mitogenicity can be controlled through surface topography. More surprisingly, the shape analysis of the HTS data revealed a mathematical abstract measure of shape distribution as the parameter that best correlated topography with cell proliferation. Osteogenic differentiation was associated with significantly smaller cell area and a higher major axis length [107]. Recent developments in the fabrication of topographically patterned microwell containers from the same group present the possibility of screening surface-immobilised and soluble factors in conjunction with surface topography [173].

5. Challenges facing the *in vitro* screening and exploration of the MSC niche

Whilst the goal of HTS of components that contribute to stem cell decision making in the niche has been recently realised, it is still difficult to construct a screening platform that encompasses the complexity of molecular and cellular geometries that occurs within the niche. For example, the roles played by neighbouring stem cells and supportive cells, known in some niche systems to be critical mediators of fate, are challenging to define in a conventional cell microarray format. Given that cell density influences the trajectory of MSC differentiation (high cell density being associated with adipogenic cell generation [68,81]), intercellular signalling is likely to be active in the MSC niche. Some aspects of cell-cell interactions can be mimicked by screening for cell adhesion molecules [164]. However, this requires some prior understanding of the mechanisms by which cells communicate within the niche system. In order to deconstruct the signalling occurring between cells, control of cell number and juxtaposition is required, necessitating micropatterning at single cell resolution. On occasion, this has been possible by close observation of cells seeded on ECM islands at dilutions low enough to produce single cell seeding [174]. Specific positioning of single cells on a surface is possible with a so called "BioFlipChip". This microwell system enables a single cell to be isolated within each well of the chip, and, by inverting the microwell construction, subsequently seeded on any substrate [175]. The distance between cells can be adjusted through the pitch of the

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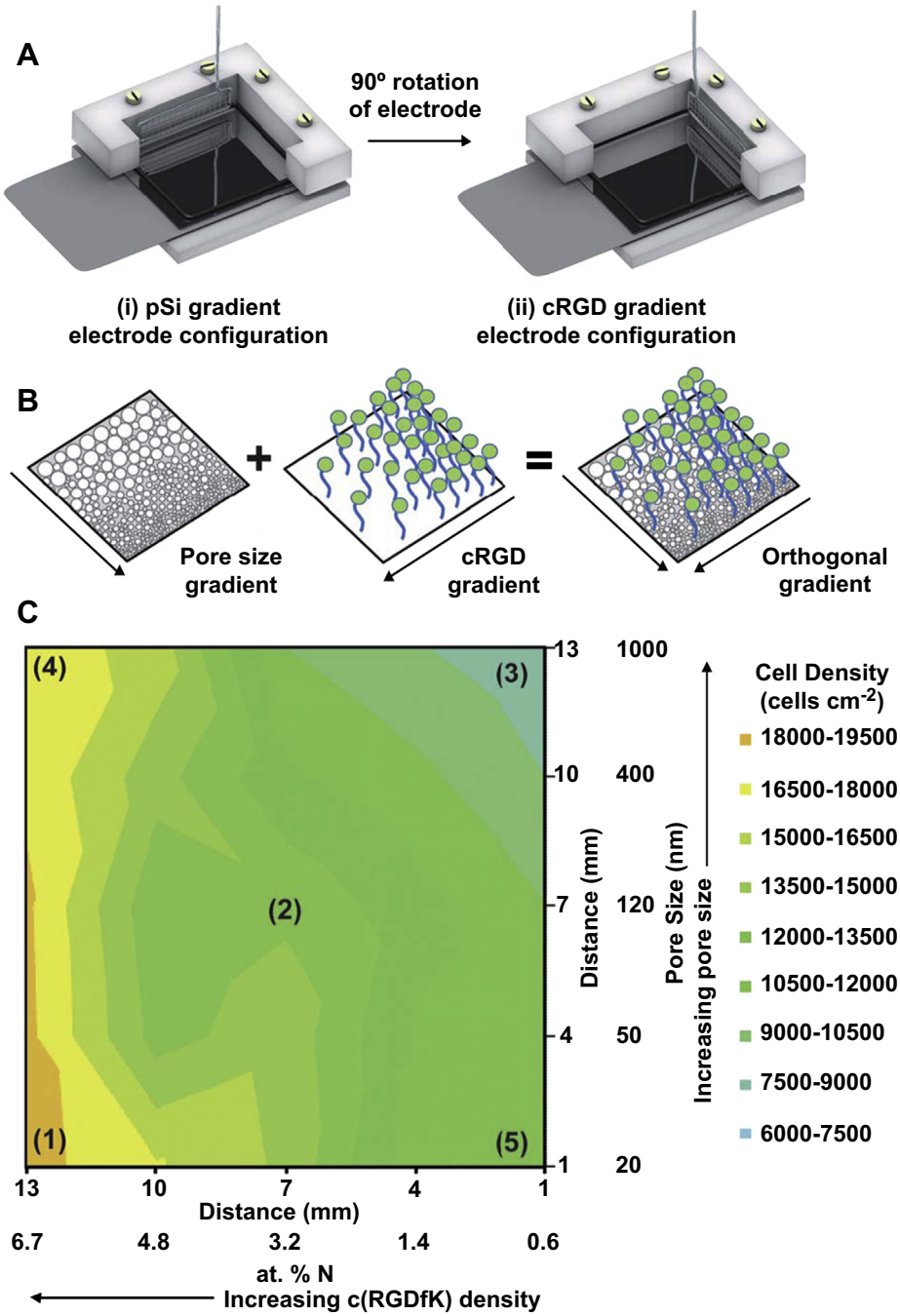

Fig. 6. Orthogonal gradient screening surface chemistry and topography in tandem (A) Orthogonal 2D gradien is prepared by first generating a pore size gradient by the anodisation of silicon in a Teflon reaction cell with the electrode perpendicular to the substrate. To create the second gradient, the platinum electrode is then rotated by 90° to create a gradient of reactive COOH functional groups via electrografting. A gradient of peptide encoding the integrin receptor motif RGD (amino-terminatedcyclic arginine-glycine-aspartic acid-D-phenylalaninelysine, c(RGDfK)) is formed using this functional group gradient via an EDC/NHS coupling reaction. (B) Schematic of the surface showing orthogonal pore size and c(RGDfK) density (C) Contour plot of 6 h MSC attachment onto an orthogonal gradient of pore size and c(RGDfK) density. Adapted from Ref. [150]. Reproduced by permission of The Royal Society of Chemistry.

microwell array. As proof-of-concept, the platform was used to probe the influence of cell-cell interaction on the efficiency of mES cell colony generation. Poor colony generation from single ES cells was not improved by printing increased numbers of cells per colony. However, very high efficiency was achieved by patterning cells in close proximity (45 μm separation), but not touching. Subsequently, the rescue of colony formation by incubating cells in blocking antibody linked E-cadherin, expressed on the surface of ES cells, to the negative feedback regulatory framework.

A major shortcoming of conventional microarray and gradient formats is that their 2D nature does not capture the 3D nature of the *in vivo* niche. The incorporation of nano- and microparticles capable of controlled growth factor release into fibrous polymer scaffolds allows for the creation of spatial and temporal gradients across a 3D scaffold [136,176]. An alternative system builds on microfluidics-generated biomolecular gradients within crosslinked hydrogels to encapsulate cells within the hydrogel structure, generating convective gradients within the hydrogel. Adaption of

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cellular microarrays to a 3D format has been attempted by encapsulation of mES cells in alginate hydrogel, with subsequent spotting onto a rigid borosilicate substrate [77]. However, without a method to subdivide the array so that multiple soluble factor combinations can be tested on a single chip surface, the power of the HTS approach is lost. Inkjet printing has recently been reported as a method to fabricate micrometer sized 3D tissue mimics in an array format. Layer-by-layer construction, sequential printing of cells and ECM printing in a 440 well microdish, allowed up to five layers of cells to be built [177]. Interface of screening chips with a perfusion bioreactor reactor setup increases cell viability, potentially extending the period that cell differentiation can be monitored [178].

Harnessing the full potential of surface-based screening techniques to probe cell fate also requires effective strategies to mine the data generated to uncover hidden correlations. A particular challenge faced during the investigation of the MSC microenvironment using HTS is that the consensus definition relies on multiple inclusion and exclusion markers. This increases the number of experimental replicates required for HTS assays in order to encompass multiple readouts, and potentially clouds interpretation of data if inconsistencies arise between the readouts employed. Adaptations of surface characterisation techniques to complement the miniaturised HTS format have been reported [161]. Immunocytochemical and cell morphological readouts common to the assessment of cell response to biomaterials can also be adapted to an HTS regime, facilitated by automated high content imaging instrumentation (such as Perkin–Elmer Operetta® and Opera®, GE IN Cell Analyzer and BD Pathway™systems). Critical to the interpretation of the extremely large data sets generated in HTS studies is the development of mathematical algorithms and statistical strategies [179].

The ability to direct or modulate the behaviour of stem cells will underpin the development of further tissue engineering and regenerative medicine applications and aid in clinical translation. However, analysing the complex regulatory mechanisms acting within the niche and the interactions between them on an *ad hoc* basis constitutes a truly herculean task. In this regard, miniaturised and highly parallelised surface-based formats, such as cell microarrays, surface-based gradients and topological libraries, provide an ingenious solution to interrogate the construction of the stem cell niche. The platform techniques we described in this review possess an intriguing capacity to screen cell-material surface interaction in depth, positioning those techniques to contribute in the research effort of optimising differentiation and stem cell maintenance protocols, discovering and understanding of molecules and pathways controlling stem cell fate, and designing biomaterials that incarnate elements of the stem cell niche.

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