Modulation of human mesenchymal and pluripotent stem cell behaviour using biophysical and biochemical cues: a review[†]

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Abstract

In vitro manipulation of human stem cells is a critical process in regenerative medicine and cellular therapies. Strategies and methods to maintain stem cells and direct them into specific lineages are ongoing challenges in these fields. To date, a number of studies have reported that besides biochemical stimulation, biophysical cues in the form of surface patterning and external stimulation also influence stem cell attachment, proliferation and differentiation, and can be used in cell reprogramming and the maintenance of pluripotency. While biochemical cues generally are effective and easy to deliver, biophysical cues have many other advantages for scalability as they are cost-efficient, have a longer lifetime, and can be easily well-defined. However, different protocols and cell sources utilized in a variety of studies have led to difficulties in obtaining clear conclusions about the effects of the biophysical environment on stem cells. In addition, the examination of different types of external stimulation is time consuming and limited by available fabrication techniques, resulting in a delay in commercialisation and clinical applications. In this review, we aim to summarise the most important biophysical cues and methods for the culture of human stem cells, including mesenchymal and pluripotent stem cells, to facilitate their adoption in stem cell biology. The standard classical protocols of using biochemical cues will also be discussed for comparison. We believe that combining biochemical and biophysical stimulation has the greatest potential to generate functionally mature cells at a scalable and inexpensive rate for diverse applications in regenerative medicine and cell therapy.

Keywords: human stem cells, biophysical stimulation, differentiation, cell reprogramming, tissue engineering

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1. Introduction

Stem cells have the ability to self-renew indefinitely and differentiate into other cell types (Kosovsky, 2013), offering numerous potential applications in regenerative medicine and as research models (de Peppo and Marolt, 2013; Porcellini, 2009). Developing methods to expand stem cells, maintain their phenotype, and direct cell lineage differentiation with artificial materials is a vital step towards realising this potential (de Peppo and Marolt, 2013).

Pluripotent stem cells (PSCs) include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). PSCs have the ability to differentiate into cells of any of the three germ layer lineages – ectoderm, mesoderm and endoderm – using chemical and/or biochemical methods (Efthymiou et al., 2014), and have immense potential in cell therapy, drug screening

and disease modelling (Hazeltine et al., 2013; Kingham and Oreffo, 2013). ESCs are derived from the early embryo, while iPSCs are adult somatic cells artificially reprogrammed to become pluripotent and self-renewing (Takahashi and Yamanaka, 2006). Some differences do exist between ESCs and iPSCs, such as the presence of epigenetic “memory” in iPSCs (Kosovsky, 2013), but in general, they are extremely similar (Kingham and Oreffo, 2013). There are fewer ethical issues concerning iPSCs compared to ESCs, and they could revolutionise regenerative medicine with the possibility of patient-specific treatments (Kingham and Oreffo, 2013). Overall, PSCs have a promising future in research and medicine owing to their unique properties.

Multipotent stem cells, such as mesenchymal stem cells (MSCs) derived from bone marrow or adipose tissue, have a more restricted differentiation ability compared to PSCs. However, they are easier to harvest and expand without ethical issues around their provenance. MSCs derived from mesodermal tissues can spontaneously differentiate or be directed to differentiate into mesodermal cells like osteoblasts, chondrocytes, or adipocytes (Cook and Genever, 2013). Though still a controversial finding, it has been reported that in addition to these mesoderm lineages, MSCs can be transdifferentiated into nerve cells, hepatocytes, beta-pancreatic islets and certain epithelial cell types using specific induction media (Damien and Allan, 2015; Porcellini, 2009). Therefore, it appears that biochemical stimulation in the form of induction media can essentially break the barriers between cell lineages (Cho et al., 2012; Thiruvikraman et al., 2014), although many uncertainties still exist about this method, such as the underlying cellular signalling pathways involved (Wang et al., 2016c). Nevertheless, MSCs can vary depending on their tissue of origin (Damien and Allan, 2015), and the source of cells must be considered in clinical use to ensure safety and efficacy (Hoogduijn et al., 2014). MSCs have also been found to modulate the behaviour of other cells such as neural stem cells through paracrine signalling, with important implications for the clinical uses of

MSCs (Haragopal et al., 2015; Yang et al., 2015). Multipotent stem cells may have some limitations compared to pluripotent stem cells, but research into these cells still has great potential.

While biochemical modulation of stem cell growth and differentiation using small molecules and growth factors have been the focus of stem cell biology for years (Efthymiou et al., 2014), less attention has been paid to biophysical cues but recent research is proving to be very promising (Downing et al., 2013; Hao et al., 2015; Kshitiz et al., 2012; Wang and Tsai, 2015). Studies have proposed that biophysical cues can direct stem cell behaviour by either maintaining their phenotype or improving lineage commitment, and they are of great interest because they are cost-effective, longer-lasting, easily characterised and can be manufactured with high reproducibility (Villa-Diaz et al., 2013). However, limited fabrication techniques, which present problems with scale up and fabrication of large scale substrates for systematic studies, have led to a delay in clinical or industrial applications of these technologies. On the other hand, biochemical modulation (e.g. adding different growth factors to culture media) are easy to handle but expensive and difficult to control or define over the long term, because the functional half-life of biochemical factors is generally short and varies depending on the molecule (Johnson et al., 2015; Lee and Blaber, 2009; Mitchell et al., 2016). The majority of studies nowadays is using static culture where the period of medium change and diffusion of growth factors are varying between laboratories. Uncertainties in using these biomolecule approaches, such as safety issues, still exist. Methods of biochemical and biophysical manipulation of stem cells each have their advantages and disadvantages.

This review explores what is currently known about biophysical manipulation of stem cells, and how it compares to biochemical methods, and how the combination of both methods could provide advantages compared to established biochemical methods alone.

Differentiation and maintenance of the multi- or pluripotency of stem cells will be the focus of this review. An overall summary of topics covered are presented in **Illustration 1**.

Illustration 1. A summary of various strategies to control human stem cell expansion and differentiation. Culture media and substrates are both critical for stem cell manipulation including cellular reprogramming, lineage reprogramming, expansion, differentiation. While biochemical methods are easy to handle but hard to control over the long term, biophysical methods are harder to handle but can be easily characterised and controlled. Stem cells must be cultured under a fully defined system before using in clinic. A combinational protocol of biochemical and biophysical cues may be needed for the effective generation of functionally mature adult cells.

2. Biophysical stimulation

The effects of a wide variety of biophysical cues on stem cell culture have been extensively investigated by many groups (Cha et al., 2013; Dalby et al., 2007; Engler et al., 2008; Geuss and Suggs, 2013; Hirt et al., 2014; Lee et al., 2010; Li et al., 2015; McMurray et al., 2011; Rao et al., 2013; Sridharan et al., 2009; Tandon et al., 2009; Wang et al., 2011; Wang et al., 2015d; Wittenbrink et al., 2015). Biophysical cues known to have an effect on stem cell behaviour include substrate patterning (Downing et al., 2013; Villa-Diaz et al., 2013), matrix stiffness (Engler et al., 2006), external stress and strain (Hazeltine et al., 2013; Nava et al., 2012), electrical stimulation (Chan et al., 2013), hydrostatic pressure (Nava et al., 2012), electromagnetic fields (Kim et al., 2015), ultrasound (Kang et al., 2013a), and photostimulation (Wilner, 2010). However, the effects of individual types of biophysical

stimulation have not yet been completely elucidated. It is important to note that many outcomes reported using biophysical perspectives have also been based on combinational protocols which contain both biophysical and biochemical cues (e.g. soluble molecules in culture media). The true biophysical effects on stem cell culture in the absence of any additional chemical factors is an issue in stem cell culture that needs clarifying. In this section, we discuss the effects of biophysical effects in terms of surface topography (particularly at the submicron scale), substrate stiffness, and external forces on stem cell culture, as listed in **Table 1**.

Many systems have been developed to biophysically stimulate cells during *in vitro* culture. Several methods are available for producing various patterned substrate topographies, such as photolithography (Edalat et al., 2012), electron beam lithography (Luna et al., 2011; Wang et al., 2010; Wang et al., 2012d), or self-assembled colloidal crystals (Ji et al., 2012; Wang et al., 2015a; Wang et al., 2015b; Wang et al., 2016b); however, methods of production such as lithography may be time-consuming or expensive (Wang et al., 2015b). Polyacrylamide hydrogels, which can serve as ECM-like substrates *in vitro*, can be produced at different stiffnesses to study the effects of matrix stiffness on cell behaviour (Macrí-Pellizzeri et al., 2015). Mechanical forces such as tension, compression, shear stress and fluid shear stress are often applied using bioreactor systems, which may rotate culture flasks, physically stretch substrates during cell growth, or pump fluid at a constant rate through the culture, while maintaining an environment conducive to cell growth (Jin et al., 2015). Electrical and magnetic stimulation is applied to cells via electrodes attached to the cell substrate (Jin et al., 2015; Tandon et al., 2009). A variety of methods exist for biophysical stimulation of stem cells.

2.1. Surface topography

Stem cell differentiation and phenotype can be highly sensitive to substrate topographical features (Dalby et al., 2014). Several reviews have summarised the effects of topographical cues, especially microtopography, on stem cell behaviour (Dalby et al., 2014; Griffin et al., 2015; Koegler et al., 2012; Nava et al., 2012; Nikkhah et al., 2012; Park and Im, 2015; Teo et al., 2010); thus, we only summarise the key outcomes of topographical stimulation, particularly at the submicron scale.

The effects of surface topography on stem cell phenotype are dependent on cell type, the type of topographical pattern, feature sizes, and substrate composition (Dalby et al., 2007; Lapointe et al., 2013; Murphy et al., 2014; Wang et al., 2012a; Wang et al., 2012b; Wang et al., 2012c; Wang et al., 2013a; Wang et al., 2015c). In general, substrates with nanoscale topographical features have been found to assist cell attachment (Bacakova et al., 2011). Due to the large variety of surfaces studied so far, identifying general trends in how cells react to topographical cues is a difficult endeavour (De Luca et al., 2015). For example, random nanotopographical patterning may either promote or inhibit cell-matrix adhesion depending on cell type, pattern type, and other parameters such as surface chemistry or substrate elasticity (Cha et al., 2013; Dalby et al., 2007; Dalby et al., 2014). Despite these uncertainties, a number of groups have reached important conclusions about the effects of some substrate topographies on stem cells. In a study comparing ordered and random feature distribution, highly ordered nanoscale pitted patterns were found to inhibit cell-matrix adhesion (Dalby et al., 2007). Multipotency and MSC markers in human bone marrow- and adipose-derived MSCs are retained by culture on surfaces consisting of 100 nm-deep “pits” with 120 nm diameter arranged in a square lattice with a spacing of 300 nm between each pit (McMurray et al., 2011). Small “nanoislands”, 10 – 20 nm in height, have been found to promote cell adhesion and cell spreading in several cell types, while relatively larger features of the same type (100 nm in height) inhibited cell spreading and differentiation (Dalby et al.,

2014). The geometric shape of features at the micrometre scale also plays a role in influencing MSC behaviour (Nava et al., 2012), as several studies have identified shape-specific effects on cell proliferation and differentiation (Kilian et al., 2010; Luo et al., 2008; Wan et al., 2010). There are many factors to consider when examining the effects of substrate topography on stem cells.

Culture of stem cells on nanogrooves is able to direct differentiation towards neural cell lineages, even in the absence of conventional growth factors (Kingham and Oreffo, 2013). These substrates generally require some form of surface treatment to support cell attachment, such as oxygenated plasma treatment and gelatin coatings (Kingham and Oreffo, 2013). For example, hESCs were found to differentiate into neural lineages on a gelatin-coated nanoscale groove patterned substrate (350 nm width and 500 nm height) (see **Figure 1**) (Lee et al., 2010). hESCs cultured on nanofibrils made of a collagen-carbon composite material in the absence of retinoic acid, a key component of neural induction media, differentiated towards ectodermal lineages (Sridharan et al., 2009). Nanotopographical groove (300 nm/300 nm width/depth) or pillar (300 nm height, 300 nm spacing) substrate patterning promoted differentiation of human NSCs into neurons and astrocytes (Yang et al., 2013). However, these promising results are contested by the findings of several groups which report that ESCs default to neural differentiation pathways when there is insufficient signalling towards other lineages (Kingham et al., 2013). The influence of such substrates on neurogenesis requires further exploration.

Figure 1. Morphology and mesodermal, endodermal and neuronal differentiation of hESC on gelatin-coated oxygen plasma-treated nanogrooved surfaces (500 nm height, 350 nm width) without induction media, compared to flat control surfaces. The grooved surfaces caused cell

elongation and alignment, and were found to promote neuronal differentiation in the absence of induction media (image adapted from Lee et al. (Lee et al., 2010) and reprinted with permission from Elsevier Ltd.). Blue (DAPI) = nuclei. Red (Nestin) = early differentiation marker. Green = cell markers as specified. Brachyury = mesoderm marker; Pdx1 = endoderm marker; Tuj1 = early neuronal marker.

Disordered nanoscale features, which create “nanoroughness” on the culture substrate, promote osteogenesis (Dalby et al., 2007; Dalby et al., 2014; Kingham et al., 2013; Nava et al., 2012; Wang et al., 2012b; Wittenbrink et al., 2015). When human bone marrow-derived MSCs are cultured on surfaces with 120 nm diameter pits with an average spacing of 300 nm randomly offset by 50 nm, differentiation is promoted and targeted towards osteoblasts (Dalby et al., 2007). Completely random arrangement of the pits further promoted osteoblast differentiation, as detected by the presence of bone-specific matrix components and osteoblast adhesion molecules (Dalby et al., 2007). The results of the studies by Dalby *et al.* are shown in **Figure 2**. In hESCs, disordered nanotopographies encourage differentiation towards a mesodermal lineage resulting in osteogenesis (Dalby et al., 2014; Kingham et al., 2013). Random nanopatterning may also improve osteoblast maturation, as disordered polystyrene nanopillars and nanopores of 200 nm diameter induced a more stretched osteoblast morphology with increased proliferation and somewhat enhanced cell-substrate attachment (Cha et al., 2013), and osteoblasts cultured on substrates with shallow rippled patterning of 11.5 nm feature height increased proliferation and developed pseudopodia which aligned along the ripples, although the cells themselves were not aligned (Wittenbrink et al., 2015). These studies demonstrate that a disordered substrate nanotopography can assist osteogenesis.

Figure 2. Maintenance of human MSC multipotency on ordered 120 nm diameter, 100 nm depth pit square lattice patterns in standard media. Multipotency is maintained on highly ordered patterns (SQ) compared to patterns with a random 50 nm offset (NSQ50) (image adapted from McMurray et al. (McMurray et al., 2011) and reprinted with permission from Nature Publishing Group). Green = ALCAM or STRO-1 (MSC markers), red = actin (indicating cell morphology), blue = nuclei.

Substrates patterned with microscale grooves promote differentiation and maturation of stem cells into cardiomyocytes. Fibronectin-coated microgrooves with dimensions of 4 μm /10 μm (width/depth) and 10 μm spacing improved the phenotype of hiPSC-derived cardiomyocytes without significant changes in gene expression (Rao et al., 2013). The cardiomyocytes aligned longitudinally along the grooves, had elongated morphology and showed improved sarcoplasmic reticulum organisation, visible as aligned cell striations (Rao et al., 2013), making them more similar to mature native heart cells (Hartman et al., 2015). Additionally, there were improvements in calcium cycling (Rao et al., 2013), the changes in intracellular Ca^{2+} concentration necessary for cardiomyocyte beating and other Ca^{2+} -dependent cellular processes (Marks, 2013). These iPSC-derived cardiomyocytes, cultured on microgrooved substrates, were faster to reach maximum concentrations of Ca^{2+} transients during spontaneous beating and had a more synchronised release of Ca^{2+} from the sarcoplasmic reticulum (Rao et al., 2013). These results indicate that microgrooved substrate patterning improves the maturation and function of cardiomyocytes differentiated from iPSCs. While nanogrooved substrates have been used for maturation of cardiomyocytes (Wang et al., 2011), it has not been widely used for the PSC differentiation.

For some time, systematic analysis of the effects of nanotopographical patterning on stem cells and rational surface pattern design has been a major challenge (Unadkat et al., 2011), due to a lack of understanding of many underlying mechanisms and the huge range of topographical features and pattern types possible (Hulsman et al., 2015). To address this issue, “screening” approaches such as the TopoChip have been developed to investigate the cellular response to over 2000 different surfaces at a time (Unadkat et al., 2011). These approaches have contributed to the discovery of several interesting trends in how nanotopography can influence stem cell behaviour. For example, one study examining feature size, density and “roundness” found that smaller topographical features arranged at higher density promoted hiPSC proliferation and pluripotency (Reimer et al., 2016). Correlations between cell morphological features and more complex cell behaviours, such as human MSC differentiation, have also been found using these techniques (Unadkat et al., 2013). The invention of high-throughput methods to examine how substrate topography modulates stem cell behaviour has been a major development in this area.

Investigation into the effects of surface topography on cell behaviour needs to take surface protein adsorption into account (De Luca et al., 2015). The process of cell adhesion to substrates first requires the adsorption of proteins from the surrounding fluid (culture media *in vitro* or interstitial fluid *in vivo*), to which cells then attach (Koegler et al., 2012; Nune and Misra, 2014). The initial surface properties of these substrates is an important factor in determining the amount and characteristics of the proteins that are adsorbed, and therefore the interactions between the substrate and cells (Koegler et al., 2012). Surface patterning is known to affect protein adsorption from culture media (Koegler et al., 2012; De Luca et al., 2015; Pegueroles et al., 2010), and the process of contact guidance, by which cells detect and respond to contact-dependent environmental signalling molecules, has been well-studied (Rørth, 2011). Despite this, it is still uncertain whether surface topography directly affects

cell attachment mechanisms, or whether it in fact influences protein adsorption from solution to indirectly control cell behaviour. The interaction between surface protein adsorption and substrate topography requires further study (Koegler et al., 2012).

2.2. Substrate stiffness

One major development in the area of biophysical cell manipulation has been the discovery that substrate stiffness is able to direct cell behaviour (Wang et al., 2011; Zhang and Kohn, 2012) and human stem cell lineage specification (Bacakova et al., 2011; Engler et al., 2006; Tse and Engler, 2011; Wang et al., 2012e). A matrix reflecting the stiffness of a particular native tissue will direct human MSC differentiation towards that tissue type (Engler et al., 2006). Soft brain-mimicking surfaces (0.1 – 1 kPa) were neurogenic, harder muscle-like surfaces (8 – 17 kPa) were myogenic, and very stiff bone-like surfaces (25 – 40 kPa) were osteogenic, as shown in **Figure 3** (Vidane et al., 2013). Adipogenesis of human adipose-derived MSCs on soft substrates similar to native adipose tissue (2 kPa) can occur even in the absence of inductive soluble chemical factors (Young et al., 2013). Non-muscle myosin II activity was found to be the mechanism behind matrix stiffness-determined human MSC differentiation (Bacakova et al., 2011; Engler et al., 2006). Regulation of MSC behaviour is influenced not only by the degree of matrix stiffness, but also by gradients or variation in stiffness. Human MSCs cultured on hydrogel with an elasticity gradient of around 1 kPa/mm showed significant migration towards stiffer areas, and tended to differentiate towards myogenic lineages (Tse and Engler, 2011). The mechanism behind modulation of MSC behaviour by substrate stiffness has been investigated. There are a number of known mechanosensitive cell molecules expressed in MSCs, including type A lamin, found at the nuclear membrane, which is important in maintaining nuclear integrity and is able to influence gene transcription (Kulangara et al., 2014). Expression of type A lamin has been

found to increase in cells cultured on substrates with increased stiffness, and therefore may play a role in mediating the effects of substrate elasticity on MSC differentiation (Swift et al., 2013). Overall, it is clear that substrate stiffness can have a profound effect on MSC behaviour.

Figure 3. MSC differentiation is influenced by matrix stiffness: in “identical media conditions”, soft (0.1–1 kPa) matrices are neurogenic, matrices of intermediate stiffnesses (8–17 kPa) are myogenic, and rigid (25–40 kPa) matrices are osteogenic. The expression of $\beta 3$ tubulin (cytoskeletal neural marker), MyoD (muscle transcription factor) and CBF $\alpha 1$ (osteoblast transcription factor) in green mark cell differentiation into neural, muscle, or osteoblast-like cells respectively, as indicated by the arrows (image adapted from Engler et al. (Engler et al., 2006) and reprinted with permission from Cell Press). Blue = nuclei; green = $\beta 3$ tubulin, MyoD, or CBF $\alpha 1$, as indicated in each row.

The generation and behaviour of hiPSCs is also influenced by matrix stiffness. Very soft (< 0.1 kPa) matrices appear to enhance reprogramming of human fibroblasts into hiPSCs by increasing expression of pluripotent markers (Higuchi et al., 2014b). This finding could contribute to development of more efficient methods of forming iPSCs (Higuchi et al., 2014b), and agrees with earlier observations of the correspondence between cell type and native-like matrix stiffness, as the native environments of stem cells *in vivo* tend to have a lower tissue modulus than the surrounding tissue (Akhmanova et al., 2015). The neurogenic potential of low stiffness matrices was further characterised in a study finding that hPSC culture on soft matrices (0.1 – 0.7 kPa) for five days promoted formation of early neural

ectoderm and increased differentiation into neurons and dopaminergic cells, with no effect on proliferation (Keung et al., 2012). As with MSCs, iPSC differentiation appears to be guided in part by matrix stiffness.

The influence of matrix stiffness on cardiac cell differentiation and development has been the subject of significant research (Engler et al., 2008; Forte et al., 2012; Hazeltine et al., 2014; Tallawi et al., 2015), partly due to its potential in clinical and scientific applications. Again, the native environment should be taken into consideration. Elasticity of the ECM is a crucial factor in heart development in both foetal and postnatal stages. The development of the thicker left ventricle wall occurs in response to physiological increase of mechanical load during foetal development (Zimmermann, 2013). Matrix elasticity varies during human development, from around 10 kPa in embryonic myocardium (Engler et al., 2008) to 20 – 500 kPa in adult myocardium (Tallawi et al., 2015). *In vitro*, cardiomyocytes grown on substrates with a similar stiffness to native myocardium were most well-developed, as they had a larger calcium cycling amplitude, better-defined sarcomeres, and increased twitch power, all resulting in a more mature phenotype (Tallawi et al., 2015). Matrix stiffness and other external forces were found to affect these physiological features of cardiomyocyte function by influencing the organisation and assembly of contractile protein fibres (Tallawi et al., 2015). Biomimetic substrate stiffness could help produce functional, mature cardiomyocytes from stem cells.

2.3. Compression and stretching

Mechanical forces are an important factor in embryonic tissue development *in vivo* and PSC phenotype *in vitro* (Nsiah et al., 2014). Some types of forces maintain pluripotency of stem cells by enhancing proliferation and inhibiting differentiation, while other forces stimulate differentiation into certain lineages (Geuss and Suggs, 2013). Cyclic strain appears to help

maintain stem cell phenotype, as application of 10% strain at 10 - 30 cycles/min has been found to inhibit hESC differentiation (Saha et al., 2006). It has been hypothesised that a constantly applied shear stress of under 8 dynes/cm² could help maintain pluripotency in hiPSCs (Geuss and Suggs, 2013). Additionally, “dynamic culture” in the form of orbital shaking at 100 rounds per minute significantly improved the efficiency of mouse fibroblast reprogramming into iPSCs (Sia et al., 2016). However, the exact effects of such mechanical forces, and how they might relate to events during native embryonic development, are generally still unclear (Ahsan and Nerem, 2010).

Mechanical forces also influence the phenotype and morphology of PSC-derived cells. For example, hiPSC- and hESC-derived vascular smooth muscle cells, were found to align perpendicular to the axis of strain and increase ECM gene expression when cultured under uniaxial tensile strain of 7% elongation for 24 hours, as shown in **Figure 4** (Wanjare et al., 2015). When cultured under 1 Hz cyclic 5% uniaxial strain, hiPSC-derived neural crest stem cells aligned perpendicular to the axis of strain and expressed higher levels of the smooth muscle cell lineage marker calponin-1 (Li et al., 2012). Circumferential strain of 7% elongation, mimicking bloodflow, promoted expression of collagen III and elastin (largely responsible for blood vessel strength) in mature hPSC-derived vascular smooth muscle cells (Wanjare et al., 2015). In the native cardiovascular system, fluid shear stress influences endothelial cell lineage specification, morphological alignment, and phenotype to different degrees depending on the magnitude and duration of the stress (Levesque and Nerem, 1985). Mechanical forces could be used to further modulate the phenotype of cells generated from PSC differentiation to improve their maturity and function.

Figure 4. Uniaxial strain of 7% elongation applied for 24 hours to human pluripotent stem cell-derived vascular smooth muscle cells, cultured in normal smooth muscle cell growth media, cause cell alignment perpendicular to the direction of strain (image adapted from Wanjare et al. (Wanjare et al., 2015) and reprinted with permission from the American Physiological Society). Direction of strain is indicated by arrows. Con-vSMCs = contractile vascular smooth muscle cells; hPSC = human pluripotent stem cell; mSMLCs = mature smooth muscle-like cells; vSMCs = vascular smooth muscle cells. Blue = nuclei; green = actin; red = calponin, early vascular smooth muscle marker.

Compressive and tensile forces have various effects on MSCs, distinct from those found in ESCs. Mechanical forces, such as those driven by fluid flow, cell-cell contact and cell-matrix interaction, are part of the complex regulatory signals controlling MSC behaviour *in vivo* (Hao et al., 2015; Liu and Lee, 2013). Human bone marrow-derived MSCs with high intracellular tension differentiate towards osteoblasts, while low intracellular tension or low actin/myosin interaction skews differentiation towards adipocytes (McMurray et al., 2011). Additionally, cyclic strain appears to promote myogenesis in human bone marrow-derived MSCs (Gong and Niklason, 2008). $0.5 \pm 4 \text{ dyn/cm}^2$ oscillating shear stress was found to promote the differentiation of human bone marrow-derived MSCs towards adipogenic and osteogenic lineages, depending on the time duration of force, via changes in actin arrangement which modulate Wnt/ β -catenin signalling (Kuo et al., 2015). Cyclic uniaxial tension was found to upregulate osteogenic gene expression in human bone marrow-derived MSCs while dynamic compression upregulated chondrogenesis-associated genes; these changes were again mediated by Wnt/ β -catenin signalling (Haudenschild et al., 2009). Shear stress, which plays a major role in endothelial cell behaviour in the form of bloodflow *in vivo*,

also appears to stimulate human MSCs from various tissue origins to differentiate into endothelial-like cells (Dan et al., 2015). The effects of mechanical strain differ from one cell type to another.

2.4. Electric or magnetic fields

Electrical stimulation and conduction is of particular interest in cardiac cell lineage differentiation because of its importance in embryonic cardiac development (Cameron et al., 1993; Chan et al., 2013) and maintenance of native heart function (Cutler et al., 2011). Cardiomyocytes cultured under electrical field stimulation have a greater resemblance to native cardiac tissue in terms of cell elongation, organisation, alignment/orientation, and some aspects of metabolic activity (Tandon et al., 2009). The timing of electrical stimulation during cardiomyocyte development is important, as applying an electrical field too early in development causes protein accumulation and inhibits development of contractile behaviour, while applying it when cells are already mature means there is no effect on cell assembly (Tandon et al., 2009). Electrical stimulation applied during development of hiPSC-derived cardiomyocytes to pace beating has been found to significantly improve the resulting cell phenotype, as *in vitro* culture under a pulsed biphasic electrical field of 2 V/cm every 4 ms (2 ms per polarity) caused hiPSC-derived cardiomyocytes to become more similar in structure to native cardiomyocytes (Hirt et al., 2014). The resulting cells were more anisotropically aligned, longer and slimmer in morphology, and had a higher cytoplasm-to-nucleus ratio, greater contractile mass and generation of force, and more organised cross-striation compared to cells grown without electrical stimulation (Hirt et al., 2014). Electrical stimulation during stem cell culture therefore has an important influence on cardiomyogenesis.

Electrical stimulation is also of interest in neurogenesis, due to the role of electrical conduction in embryonic development, normal neuron function, and regeneration after neuron damage (Park et al., 2012). External electrical fields can be used to guide neural precursor migration, neuron migration, and neurite outgrowth, which are essential steps in neurogenesis (Yao et al., 2011). Neurogenic behaviour is highly sensitive to electrical stimulation; neural stem cells derived from hESCs were found to migrate towards the positive charge in a small direct current electrical field of only 16 mV/mm (Feng et al., 2012). Human neural stem cells became elongated in morphology and showed increased neural differentiation when cultured under an electrical field of 1 V/cm applied to an electrically conductive polymer substrate (Pires et al., 2015). Electrical fields of 100 mV/min applied for 10 min/day have been found to induce neuron-like filopodia morphology and increased neural gene expression in human bone marrow-derived MSCs, without the addition of induction media (Thrivikraman et al., 2014). Neurogenesis is greatly influenced by electrical cues.

The effects of electromagnetic stimulation on stem cells has been examined by several groups (Abbasnia et al., 2015; Cho et al., 2012; Kang et al., 2013b; Ma et al., 2014), with some controversial results (Fayol et al., 2013) likely due to differences in experimental conditions (Kang et al., 2013b). For example, there have been conflicting studies into the effect of superparamagnetic iron oxide (SPIO) labelling of human MSCs for magnetic resonance imaging (MRI), as some indicate a negative effect on chondrogenesis (Kostura et al., 2004) while others report no significant changes in cell phenotype (Arbab et al., 2005). Osteogenic differentiation in human adipose-derived MSCs has been found to either increase or decrease depending on electromagnetic field frequency and magnetic flux density: osteogenic marker expression was promoted at 30 – 45 Hz, 1 mT but inhibited at 7.5 Hz, 1 mT (Kang et al., 2013b). In one study, human bone marrow-derived MSCs showed decreased proliferation and

increased expression of neural cell markers when cultured with maintenance culture media in extremely low-frequency electromagnetic fields (sinusoidal, 1 mT, 50 Hz) (Cho et al., 2012). These results have implications for procedures like MRI used in tissue engineering, which involves magnetic labelling of stem cells (Fayol et al., 2013). Additionally, research into how magnetic fields affect stem cells could reveal the mechanisms behind treatments like repetitive transcranial magnetic stimulation for neurological disease, or low-frequency electromagnetic fields for bone fractures (Abbasnia et al., 2015; Kang et al., 2013b). Overall, the effects of electromagnetic stimulation are still unclear.

2.5. Ultrasound stimulation, photostimulation, and combined biophysical cues

In addition to cell adhesion-dependent forces such as surface topography, substrate stiffness, and mechanical forces, the effects of other non-contact-dependent forms of biophysical stimulation on stem cells have been studied. These include low-intensity ultrasound, and light of varying wavelengths. Additionally, some studies have investigated how different forms of biophysical stimulation influence stem cells when applied in combination.

Ultrasound frequencies can modulate stem cell behaviour, with important implications for present and future practical applications. Low-intensity ultrasound enhances stem cell differentiation into osteoblasts and is used in the clinical treatment of bone fractures and bone defects (Claes and Willie, 2007; Kang et al., 2013a). These osteogenic effects of ultrasound persist in microgravity environments, suggesting that ultrasound could be used as treatment for osteoporosis caused by disuse (Uddin and Qin, 2013). A study examining the influence of ultrasound frequency on effectiveness found that, when applied continuously, 1.5 MHz was most effective for promoting osteogenesis in human adipose-derived MSCs (Kang et al., 2013a). The mechanisms behind the effects of ultrasound stimulation on human MSCs have been found to involve soluble receptor activator of nuclear factor kappa B ligand (sRANKL)

(Chiu et al., 2015). Low-intensity ultrasound applied at 500 mW cm^{-2} was found to increase proliferation and differentiation in hiPSC-derived neural crest stem cells, raising the possibility of using ultrasound in nerve cell regeneration or tissue engineering (Lv et al., 2013). Ultrasound stimulation has been a particular success in the area of clinical biophysical stem cell manipulation.

Light stimulation alone can also influence stem cell behaviour. Helium-neon lasers emitting light at 632.8 nm, used clinically to promote wound healing, were found to promote human epidermal stem cell proliferation and migration, which are processes involved in skin self-renewal and regeneration (Liao et al., 2014). In one study, blue light inhibited human bone marrow-derived MSC proliferation at wavelengths of 455 nm and 470 nm (Wilner, 2010). In other species such as mice, MSC osteogenesis and bone formation was improved by application of blue light at 405 nm, which improved extracellular calcification (Kushibiki and Awazu, 2009). Photostimulation at different wavelengths can have various effects on stem cells.

Some studies have also examined the effects of combining different types of biophysical stimulation. Human neural stem cells cultured on semiconductive reduced graphene oxide nano-meshes under near-infrared lasers at 808 nm had a skewed differentiation towards neurons rather than glial cells (Akhavan et al., 2015). Experiments that combined electrical stimulation and topographical cues showed that they both appear to affect the same cell elongation pathways that assist cell alignment and organisation, although topography had a more significant effect than electrical stimulation (Tandon et al., 2009). Different forms of biophysical stimulation may be used together, with interesting insights.

3. Biochemical stimulation

Current protocols for directed stem cell differentiation primarily involve the addition of growth factors and chemical reagents (Efthymiou et al., 2014; Trounson, 2006). More recently, new reagents such as small molecules which target specific intracellular pathways have been proposed for stem cell differentiation protocols, and are now widely used in a soluble form in culture media (Xu et al., 2013). At the same time, studies have focused on immobilisation or pre-coating bioactive signals on cell culture substrates or the surface of implantable scaffolds (Bacakova et al., 2011; Chen et al., 2011; Chuah et al., 2015; Duffy et al., 2014; Kang et al., 2014; Kuddannaya et al., 2013; Lu et al., 2006; Nagaoka et al., 2010; Yamada et al., 2014). This has the potential to establish more defined culture systems and be used in future clinical applications, such as commercialised cell culture tools (Higuchi et al., 2014a; Wang et al., 2016a) and tissue engineered implants (Salerno et al., 2013). This section discusses the effect of soluble factors in medium and surface-immobilised biosignals on stem cell culture, and lists the remarkable outcomes in **Table 2**.

3.1. Soluble factors in media

The development of methods using soluble biochemical signals to fully differentiate stem cells into different cell types is the subject of extensive research and has been successful to varying degrees (Budiatzky and Gepstein, 2014; Efthymiou et al., 2014; Mallanna and Duncan, 2013). In general, an optimised recipe and a longer the culture time improve the maturation of differentiated stem cells. However, there are often limitations in producing fully mature and functional adult cells (e.g. cardiomyocytes, hepatocytes and pancreatic cells) from current protocols, which use various cocktails of growth factors at different concentrations (Efthymiou et al., 2014). The efficiency of protocols and purity of differentiated cell populations may also be limited. Here, we will explore some current

techniques for MSC and PSC differentiation into several key lineages, with a focus on particular achievements and difficulties in these methods.

MSC differentiation is traditionally induced with the addition of soluble growth factors and other chemicals to culture media. For instance, in osteogenesis, these are generally ascorbate, β -glycerophosphate, and bone morphogenetic proteins (BMPs) or dexamethasone (DEX) (Cook and Genever, 2013). BMPs such as BMP-2 stimulate the expression of major osteogenic genes such as osteopontin, osteocalcin and Runx2 (Runt-related transcription factor 2) (Sun et al., 2015). Adipogenesis requires DEX, isobutylmethylxanthine, indomethacin and insulin (Cook and Genever, 2013). DEX, a synthetic glucocorticoid, is thought to influence Hedgehog signalling in MSCs (Ma et al., 2013). Transforming growth factor beta (TGF β) is added to human adipose-derived or bone marrow-derived MSCs cultured at a high density or as “micromass pellets” for chondrogenesis (Cook and Genever, 2013; Peltari et al., 2008). In addition to these mesodermal lineages, MSCs may be transdifferentiated into ectodermal and endodermal lineages by certain soluble growth factors (Brzoska et al., 2005; Gao et al., 2014; Li et al., 2013). While these differentiation methods are widely used, biochemical induction of MSC differentiation still has its limitations, particularly in the maturity, functionality, cell phenotype, and tissue structure of the resulting cells (Somoza et al., 2014). MSCs also play an important role in paracrine signalling to other stem cells. For example, MSC co-culture or MSC-conditioned media have been found to regulate neural stem cell differentiation via the secretion of soluble signals (Bai et al., 2007; Yang et al., 2015). Overall, the types of chemicals that are applied in MSC differentiation depend on the cell lineage being targeted.

Biochemical differentiation of PSCs *in vitro* is traditionally achieved by uncontrolled spontaneous differentiation, or by directed differentiation towards specific cell lineages.

Spontaneous differentiation can be induced by culture of PSCs as embryoid bodies (EBs), with various medium compositions to “bias” differentiation towards certain lineages (Hazeltine et al., 2013). Spontaneous differentiation produces a mixed population of many cell lineages from all germ layers (Sridharan et al., 2009), and the efficiency of differentiation and percentage of specific cells is uncontrollable and generally low. These differentiation methods are inefficient, with a yield of 1 – 25% depending on the cell type in question (Budniatzky and Gepstein, 2014). Spontaneous differentiation of PSCs produces a large range of ectodermal, mesodermal and endodermal cell types but cannot yet be used for large-scale applications. Directed differentiation with soluble factors has been successful to various degrees in generating many cell types, such as cardiomyocytes (Hartman et al., 2015), neural cells (Ross and Akimov, 2014), pancreatic β cells (Agulnick et al., 2015), and hepatocytes (Schwartz et al., 2014). However, these protocols generally involve multiple steps with many different expensive biochemical factors to mimic *in vivo* development (Tabar and Studer, 2014), which can result in loss of cells, inefficient differentiation, or low cell type purity (Budniatzky and Gepstein, 2014). In particular, production of mixed cell populations can pose a problem, since efficient methods of cell sorting are required before these stem cell technologies can be used in tissue engineering and other applications. Clinical applications of stem cell differentiation would require either a differentiation efficiency of close to 100% or a large-scale method to purify selected cell types from a mixed population (Budniatzky and Gepstein, 2014). Spontaneous differentiation and directed differentiation of PSCs using soluble biochemical factors has been successful in many respects, but some disadvantages still exist.

Cardiomyocyte differentiation from PSCs is one area of great interest that has seen significant advances in soluble chemical differentiation methods (Hartman et al., 2015; Mummery et al., 2012). Spontaneous differentiation and growth factor-based protocols have

been established for generating myocytes (Laflamme et al., 2007; Yan et al., 2009; Yang et al., 2008). Functional cardiomyocytes can be produced by culture of EBs in differentiation media containing non-essential amino acids, L-glutamine, β -mercaptoethanol and 20% foetal bovine serum (FBS), followed by micro-dissection of beating areas (Zhang et al., 2009). Co-culture systems have also been established for the generation of cardiomyocytes from human iPSCs (hiPSC) and human ESCs (hESC) (Yoshida and Yamanaka, 2010), in which these human PSCs (hPSC) are cultured with other cell types such as mouse visceral-endoderm-like cells (Mummery et al., 2012). However, these methods are generally difficult to scale up and do not achieve the efficiency or cell purity required for potential clinical applications unless cell sorting methods are applied (Budnietzky and Gepstein, 2014). More recently, differentiation protocols using small molecules have been developed, and are able to generate cardiomyocytes in well-defined, scalable conditions with greater speed and differentiation efficiency (Fonoudi et al., 2015). These protocols primarily modulate Wnt signalling, which has an important role in native cardiac development (Lian et al., 2012). The main ingredients of these protocols are small molecule Wnt pathway inhibitors or activators, BMP inhibitors, shRNA, and/or other molecules, each added at specific time points (Aguilar et al., 2015; Fonoudi et al., 2015; Zhang et al., 2013). With the development of more efficient and well-defined cardiomyocyte differentiation protocols, routine use of stem cell-derived cardiomyocytes in research and the clinic could soon be possible.

Neurogenesis from hPSCs is another major area of study, owing to its potential clinical applications for treating neural disease or providing models for research into human brain development and disease (Ross and Akimov, 2014; Stoll et al., 2014). Many neurogenic protocols have been developed to date, with various advantages and limitations (Ambasudhan et al., 2014; Srikanth and Young-Pearse, 2014; Suter and Krause, 2008). Common protocols for human neurogenic differentiation involve the differentiation of ESCs grown as EBs into

neural stem cells (NSCs), which form a mixture of neurons, oligo-dendrocytes and astrocytes in *in vitro* culture under the influence of retinoic acid (Wesselschmidt and McDonald, 2006). Populations of astrocyte-like cells with relatively high purity (>90%) have been produced using retinoic acid, the growth factors sonic hedgehog (SHH), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and ciliary neurotrophic factor (CNTF), and 10% FBS (Krencik et al., 2011). A serum-free culture system, dubbed the “neurosphere assay”, uses EGF to maintain NSCs as undifferentiated, proliferating neurospheres which later differentiate into neuroglial cells when removed from EGF media and allowed to attach to a substrate (Chojnacki and Weiss, 2008). These methods for *in vitro* neural cell generation are based on mimicry of embryonic *in vivo* neural development via major signalling pathways including the Notch, Wnt- β -catenin, FGF2 and BMP pathways (Suter and Krause, 2008). Current methods based on small molecules rather than growth factors generate neural cells from PSCs by dual SMAD inhibition and Wnt activation (Chambers et al., 2009; Chambers et al., 2016). These small molecules can be used in combination with growth factors to induce differentiation into specific cell types in greater proportions, such as posterior neural crest cells, melanocytes (Mica et al., 2013), and sensory neurons (Chambers et al., 2012). There has been significant success in the area of hPSC neurogenesis, with numerous clinical trials using human iPSC and ESC technology to treat spinal cord injuries and retinal diseases (Kimbrel and Lanza, 2015). However, generating mature neural cells from PSCs remains a challenge. Though further research is still needed, neurogenic stem cell therapy is well on the way to realising its potential in many applications.

The derivation of several pancreatic cell types from stem cells has had highly promising results. Clinical trials by the company ViaCyte Inc. are now testing a Type I diabetes treatment based on an implant containing hESC-derived pancreatic endoderm (Agulnick et al., 2015; Kimbrel and Lanza, 2015). These functional insulin-producing pancreatic β -cells

were generated from hESCs by mimicking *in vivo* pancreatic development through the addition and removal of the growth factors activin, Wnt, fibroblast growth factor 10 (FGF10), KAAD-cyclopamine (CYC), all-*trans* retinoic acid, the γ -secretase inhibitor DAPT, exendin-4 (Ex4), insulin-like growth factor 1 (IGF1) and hepatocyte growth factor (HGF) over five stages of growth (D'Amour et al., 2006). Other more recently developed methods involve addition of Activin A, fibroblast growth factors (FGFs) and retinoic acid, the small-molecule BMP inhibitor LDN, and certain gene inhibitors such as Smoothened antagonists (SANT) (Pagliuca et al., 2014; Rezania et al., 2014). However, despite the success of these methods, there are still some problems to overcome: the complexity of the multistep protocols used, the cost of production, and ensuring that the cell generation processes remain reliable after scaling up (Schulz, 2015). Differentiation of PSCs into pancreatic cells has proven to be a successful and promising area in tissue engineering, but there are still limitations to be overcome.

Several methods have been established for the differentiation of PSCs to hepatocyte-like cells and pancreatic-like cells (Mallanna and Duncan, 2013; Rostovskaya et al., 2015; Schwartz et al., 2014). Hepatocytes perform hundreds of functions, including metabolic, synthetic, immunologic, and detoxification processes (Bhatia et al., 2014). Generation of hepatocyte-like cells from hPSCs primarily involve sequential addition and removal of activin-A, BMP-4, bFGF, HGF, oncostatin M (OSM) and, in some protocols, DEX at specific time points (Mallanna and Duncan, 2013; Schwartz et al., 2014). However, these cells are more similar to foetal than adult hepatocytes (Baxter et al., 2015), and current protocols are still unable to generate fully functional, mature hepatocytes (Huang et al., 2011). Overall, while there have been some successes in this area, several challenges still exist and the derivation of hepatic cells from PSCs is an area that requires more research.

3.2. Surface-immobilised biosignals

Surface coatings, such as ECM proteins or protein-enriched mixtures like Matrigel[®], are routinely used in stem cell culture to mimic the *in vivo* extracellular matrix (ECM). The importance of the ECM in controlling cell behaviour via presentation of both soluble and insoluble factors is well-known (Gattazzo et al., 2014; Walters and Gentleman, 2015), and taking advantage of this as a cell culture tool has been a significant area of research (Minardi et al., 2016). Matrigel, a gelatinous mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, consists of a large number of key proteins and growth factors which are known to influence many cellular processes, including stem cell self-renewal (Villa-Diaz et al., 2013). Gelatin is another relatively cheap option and is often used to improve cell attachment to culture substrates (Bacakova et al., 2011). While Matrigel is still most commonly used in cell culture, it has some insurmountable disadvantages such as containing undefined components and being itself an exogenous murine product, which is an obstacle to clinical applications (Villa-Diaz et al., 2013). Thus, non-xenogeneic and well-characterised alternatives are being continuously explored (Burridge et al., 2014; Hazeltine et al., 2013; Rowland et al., 2010). There are a number of surface protein coatings available for use in cell culture.

The reasons for using substrate coatings may differ depending on cell type. Surface protein coatings are frequently used in hPSC culture because they are anchorage-dependent cells and require some form of matrix to serve as an ECM (Villa-Diaz et al., 2013). In contrast, feeder-free human MSC culture does not require protein coatings on substrates. Instead, 10% FBS is usually added to culture media to support cell attachment and growth (Jung et al., 2012). FBS contains many cell adhesive or non-adhesive biomolecules, including, fibronectin, vitronectin and albumin as well as growth factors, hormones, amino acids, sugars and lipids which are

important in stem cell culture. The adsorption of proteins and biomolecules from FBS onto the substrate surface, rather than prior surface modification, is hypothesised to promote cell adhesion (Dänmark et al., 2012). However, very little is known about the time-dependent composition of the protein layer and the role it plays. While surface modifications (e.g. protein or polymer coatings) are not strictly necessary for MSC culture, they can still affect MSC behaviour (Kang et al., 2014). For example, when used alongside neuronal induction media containing 20% FBS, Matrigel was found to promote neuronal differentiation of human bone marrow-derived MSCs and, at higher coating densities of $50 \mu\text{g}/\text{cm}^2$ (compared to $5 \mu\text{g}/\text{cm}^2$), increase proliferation efficiency (Qian and Saltzman, 2004). Polyacrylate-based hydrogel substrates have also been developed to maintain the multipotency and self-renewal of human adipose-derived MSCs in long-term culture, as well as allow thermally modulated, enzyme-independent cell harvesting (Duffy et al., 2014). Polyacrylamide and polyacrylate hydrogels can not only serve as ECM-like substrates *in vitro* but can also be produced with specific elasticities and biochemical compositions (Macrí-Pellizzeri et al., 2015). Different substrate coatings may be applied for various effects on human PSC and MSC culture.

Direct surface modification is another method that has been used to influence cell behaviour. Protein coatings, such as Type I collagen (Chuah et al., 2015; Kuddannaya et al., 2013) or fibronectin (Kuddannaya et al., 2013; Wang et al., 2012e; Yamada et al., 2014), can be applied to facilitate attachment of MSCs and iPSCs to hydrophobic surfaces like polydimethylsiloxane (PDMS). Different surface properties provided by these modifications can affect the density and configuration of protein adsorption prior to cell culture or from media, which in turn affects cell adhesion and growth (De Luca et al., 2015). In addition to supporting cell attachment, immobilised ligands may be used to influence cell signalling. Immobilised biomolecules such as signalling proteins can stimulate cell pathways by mimicking *in vivo* juxtacrine signalling which usually occurs at cell-cell or cell-matrix

interfaces (Alberti et al., 2008). Several studies have examined the effect of immobilised ligands on cell signalling in human stem cells (Re'em et al., 2010; Russo et al., 2015; Santiago et al., 2006). Grafting bioactive peptides on topographical patterned surfaces can further alter the mechanism (Wang et al., 2012d) and speed of cell adhesion as well as the overall performance (Wang et al., 2013b; Wang et al., 2013c). Air plasma treatment of substrates improves cell attachment by adding functional groups that enhance surface hydrophilicity (Epifanio et al., 2015). However, this treatment alone cannot support stem cell growth as it lacks biochemical functions, so culture media or a protein coating is still required. Surface modification techniques can therefore be used to influence cell-substrate interactions.

4. Combining chemical and physical stimulation

Often, novel methods of stem cell culture involving biophysical stimulation are studied in combination with established biochemistry-based protocols. The effects of mechanical cues on stem cell differentiation in conjunction with soluble chemical factors, particularly those found in induction media, has been examined by many groups (**Table 3**). More importantly, increasing evidence is showing that a combination of chemical and physical stimulation could be a solution to generate functionally matured cells *in vitro* through synergistic effects (Engler et al., 2006; Kabiri et al., 2012; Li et al., 2015), and physical stimulation could potentially even replace some forms of chemical stimulation (Ahsan and Nerem, 2010).

Experiments that combine conflicting biophysical and biochemical cues may help ascertain the level of interaction or competition between these factors. For example, in the presence of both adipogenic and osteogenic induction media, stiffer matrices promote osteogenesis despite the presence of adipogenic media (mixed with osteogenic media), whereas softer

matrices promote adipogenesis despite the presence of osteogenic media (mixed with adipogenic media) (Guvendiren and Burdick, 2012). Human MSCs cultured on stiffer matrices (4.47 kPa) with adipogenic induction media had lower adipogenic ability compared to those cultured on softer matrices (1.37 kPa) (Hwang et al., 2015). In another study, human bone marrow-derived MSCs cultured on nano-patterned topography shown to maintain multipotency were able to partially maintain stem cell phenotype even in differentiation media, as these MSCs had lower differentiation levels compared to MSCs cultured solely in differentiation media (McMurray et al., 2011). These and similar studies may reveal in greater detail how cells respond to physical and chemical cues.

Several groups have examined the combined effects of biochemical and biophysical stimulation. For example, hESC-to-cardiomyocyte differentiation is promoted by cell culture on native heart-like substrate stiffness in the presence of the Wnt secretion inhibitor IWP-4 and a glycogen synthase kinase-3 (Gsk3) inhibitor (Hazeltine et al., 2014). Electrical stimulation has been shown to improve the maturation of cardiomyocyte-like cells derived from hESCs differentiated in cardiomyocyte induction media, as shown in **Figure 5** (Chan et al., 2013). Cell aggregation and three-dimensional culture combined with the soluble growth factors IWR1 (a Wnt pathway inhibitor), BMP-4, lithium chloride, and microRNA-206 inhibitor was able to induce cardiomyocyte differentiation from skeletal muscle-derived stem cells (Tchao et al., 2014). Ultrasound stimulation has also been studied in conjunction with biochemical stimulation. Low-intensity ultrasound pulses applied to human bone marrow-derived MSCs cultured on RGD-grafted oxidised sodium alginate/N-succinyl chitosan hydrogel promoted osteogenesis and differentiation towards endothelium (Wang et al., 2014). 5 MHz ultrasound has been reported to promote chondrogenesis in human bone marrow-derived MSCs when used alongside chondrogenic induction media containing TGF β 3 (Guha Thakurta et al., 2015). Photostimulation has also been shown to affect stem cell

differentiation in induction media, as blue light at 455 nm and 470 nm maintained human bone marrow-derived MSC multipotency while red light at 656 nm promoted osteogenesis (Wilner, 2010). Biochemical and biophysical cues can be combined to modulate stem cell behaviour.

Figure 5. A: Electrical stimulation was delivered using carbon electrodes in an eight-channel “C-Pace EP” cell culture stimulator (IonOptix, Milton, MA, USA) (image adapted from C-Pace EP system brochure (Unknown, 2011)). B: Combined with differentiation media, unidirectional electric stimulation (6.6 V/cm, 1 Hz, 2 ms alternating polarity) produces hESC-derived cardiomyocytes with improved Ca^{2+} cycling, cytoskeletal organisation, elongated growth aligned with electrical stimulation direction (indicated by arrows), and cardiac-specific gene expression (image adapted from Chan et al. (Chan et al., 2013) and reprinted with permission from Springer). Red = human cardiac troponin-T; Green = sarcomeric alpha-actinin; Blue = nuclei.

The consequences of this combinational approach can be useful, but such studies may pose issues in establishing the role of biophysical factors in regulating stem cell behaviour. For example, growth factors found in Matrigel, commonly used to facilitate PSC attachment, have an influence on cell behaviour which may need to be considered when interpreting experiment results where Matrigel is used (Hughes et al., 2010; Vukicevic et al., 1992). Components of Matrigel, such as laminins, collagens and various other proteins including signalling molecules, dissolve at different rates from the solidified layer into the surrounding fluid, where they may act as soluble biomolecules to influence cell behaviour (Zhang et al.,

2006). Additionally, it has been reported that biophysical cues such as substrate topography can trigger cell signalling pathways distinct from those triggered by chemical signals, relating to a wide range of cellular processes (Dalby et al., 2007) but it is still unclear whether the pathways overlap or interact when biophysical and biochemical cues are used in combination, and to what extent. It is important to distinguish between the effects arising from biochemical cues and those from biophysical cues in order to clarify the exact influence these factors have on stem cells.

The effect of patterned chemical surfaces is another area of interest in manipulating stem cell morphology and phenotype. Kilian *et al.* compared differentiation of human bone marrow-derived MSCs on sharp- and soft-edged two-dimensional fibronectin star shapes in the presence of a mixture of adipogenic and osteogenic soluble growth factors (Kilian et al., 2010). They found that cells grown on soft-edged patterns had lower intracellular tension and primarily differentiated into adipocytes, while cells on sharp-edged patterns had higher tension and mainly formed osteoblasts (Kilian et al., 2010). Adsorption of proteins like fibronectin to patterned or micro-textured PDMS surfaces has been used in biomaterial development for musculoskeletal tissue engineering from MSCs (Peterson and Papautsky, 2006). These studies have provided insights into cell signalling pathways and potential improvements in bioengineering.

5. Conclusion and future prospects

While biochemical stimulation has been firmly established as a method of culturing and manipulating stem cells, biophysical stimulation from substrates, such as topographical patterning and matrix stiffness, and external stimulation, such as mechanical loading and

electrical stimulation, have the potential to vastly improve current stem cell differentiation techniques and tissue engineering technologies. Since these factors are all present in combination with chemical stimulation in native stem cell environments, they could be applied to create more advanced biomimetic environments *in vitro* to generate more mature and native-like cells for artificially engineered tissues. Physical stimulation like surface topography could improve prosthetics and artificial tissue replacement surgeries, where conventional biochemical approaches may be inaccessible *in vivo*. These biophysical cues could also be used as a tool to study cell behaviour *in vitro*, where the environment and surrounding factors can be more tightly controlled than in studies *in vivo*. Additionally, biochemical and biophysical cues could be combined to better manipulate stem cell behaviour, such as complete differentiation into mature somatic cell types. New cell culture tools using both these methods need to be developed. Biophysical stimulation and its effects on stem cells is an area of great potential for future practical applications.

Major challenges in the development of new regenerative therapies using stem cells include the difficulty of scaling up stem cell self-renewal and differentiation processes to sufficient levels, and the fact that current *in vitro* differentiation techniques are limited in efficiency and functional maturation of resultant cells. These existing methods can also be expensive, time-consuming and difficult to control in terms of defining exact media chemical compositions, difficulties in sequential addition and removal of growth factors, and isolation or purification of desired cell types (Kingham and Oreffo, 2013; Lee et al., 2010). Exogenous soluble factors and animal-derived products can pose problems, particularly relating to contamination, for cell transplantation in clinical applications (Villa-Diaz et al., 2013). Biophysical cues could be the key to solving these issues (Kingham and Oreffo, 2013). The development of next-generation cell culture protocols combining physical and chemical stimulation will allow more vigorous study of stem cell behaviour. However, the main priority of future research

should be to clarify the influence of biophysical stimulation on stem cell self-renewal, phenotype and directed differentiation, and establish reliable methods for its use in stem cell culture and cell therapy.

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Table 1. Biophysical stimulation and its effects on stem cell behaviour or cell phenotype

STEM CELL	LINEAGE	BIOPHYSICAL STIMULATION	EFFECT	REF.
hiPSC	cardiomyocytes	Electrical stimulation (2 Hz for 1 week, then 1.5 Hz after)	Cell alignment. Elongated cell morphology. Greater contractile force.	(Hirt et al., 2014)
hiPSC	cardiomyocytes	4 μm /10 μm (w/d) microgrooves	Organised cross-striation. Cell alignment. Elongated cell morphology. Organised cross-striation. Organised sarcoplasmic reticulum.	(Rao et al., 2013)
hiPSC	neural stem cells	Low-intensity ultrasound (500 mW cm^{-2})	Proliferation. Differentiation.	(Lv et al., 2013)
hESC	neural cells	Nanogrooves (350 nm/500 nm w/d)	Differentiation.	(Lee et al., 2010)
hESC	neural cells	Nanofibrils	Differentiation.	(Sridharan et al., 2009)
hESC	-	Cyclic stretching (10% strain, 10 or 30 cycles/min)	Stem cell phenotype (proliferation, pluripotency).	(Saha et al., 2006)
hESC	neural stem cell	Electrical field (16 mV/mm)	Migration.	(Feng et al., 2012)
hESC	-	Shear stress < 8 dynes/ cm^2	Stem cell phenotype (proliferation, pluripotency).	(Geuss and Suggs, 2013)
hiPSC, hESC	vascular smooth muscle cells	Uniaxial tensile strain (7% elongation), 24 hours	Cell alignment perpendicular to direction of strain. Increased ECM gene expression.	(Wanjare et al., 2015)
hiPSC, hESC	vascular smooth muscle cells	Circumferential tensile strain (7% elongation), 6 days	Increased collagen III and elastin expression.	(Wanjare et al., 2015)
hMSC	osteoblasts, fibroblasts	Cyclic uniaxial tension (10% strain, 0.1 Hz frequency)	Increased gene expression.	(Haudenschild et al., 2009)
hMSC	chondrocytes	Cyclic compression (5% strain, 0.1 Hz frequency)	Increased gene expression.	(Haudenschild et al., 2009)
hMSC	osteoblasts, adipocytes	Oscillatory shear strain (0.5 ± 4 dyn/ cm^2), up to 24 hours	Differentiation.	(Kuo et al., 2015)
hMSC	osteoblasts	Nanotopography (120 nm pits, 300 nm square lattice spacing, 50 nm offset)	Differentiation.	(Dalby et al., 2007)
hMSC	osteoblasts	Low frequency electromagnetic field (30–45 Hz, 1 mT)	Differentiation.	(Kang et al., 2013b)
hMSC	osteoblasts	Low frequency electromagnetic field (7.5 Hz, 1 mT)	Multipotency.	(Kang et al., 2013b)
hMSC	osteoblasts	1.5 MHz ultrasound	Differentiation.	(Kang et al., 2013a)
hMSC	neurons	Low frequency electromagnetic field (sinusoidal, 1 mT, 50 Hz)	Differentiation. Decreased proliferation.	(Cho et al., 2012)

hMSC	neurons	Electrical field (100 mV/min), 10 min/day	Neuron-like morphology. Increased gene expression.	(Thrivikraman et al., 2014)
hMSC	-	Blue light (455 nm or 470 nm)	Decreased proliferation.	(Wilner, 2010)
hMSC	-	Microscale ordered BCC monolayers	Multipotency.	(Wang et al., 2015d)
hMSC	-	Ordered nanotopography (120 nm pits, 300 nm square lattice spacing)	Multipotency.	(McMurray et al., 2011)
human neural stem cells	neurons	Reduced graphene oxide nano-mesh substrate; near-infrared laser light (808 nm)	Differentiation.	(Akhavan et al., 2015)
human neural stem cells	neurons	Electrical field (1 V/cm)	Elongated morphology. Differentiation.	(Pires et al., 2015)
human epithelial stem cells	-	Helium-neon laser light (632.8 nm)	Proliferation. Cell migration.	(Liao et al., 2014)

Abbreviations: hESC, human embryonic stem cells; hiPSC, human induced pluripotent stem cells; hPSC, human pluripotent stem cells; hMSC, human mesenchymal stem cells.

Table 2. Key components for biochemical induction of directed stem cell differentiation.

STEM CELL	LINEAGE	KEY COMPONENTS OF INDUCTION MEDIA	REF.
hPSC	Cardiomyocytes	activin-A, BMP-4, Dkk-1, VEGF, bFGF	(Hartman et al., 2015; Mummery et al., 2012)
hPSC	Cardiomyocytes	CHIR99021, DMH1	(Aguilar et al., 2015)
hPSC	Neural cells	LDN, CHIR99021, Noggin, SB431542	(Chambers et al., 2016)
hPSC	Astrocytes	retinoic acid, FGF-8	(Krencik et al., 2011)
hPSC	Pancreatic cells	Activin A, FGFs, retinoic acid, LDN, SANT	(Pagliuca et al., 2014; Rezanian et al., 2014)
hiPSC	Hepatocytes	DEX, activin-A, bFGF, BMP-4, HGF, OSM	(Mallanna and Duncan, 2013; Schwartz et al., 2014)
hESC	Endothelial cells	hydrocortisone, human EGF, bFGF, heparin	(Kane et al., 2010)
hESC	Pancreatic cells	activin, Wnt, FGF-10, CYC, retinoic acid, DAPT, Ex4, IGF-1, HGF	(D'Amour et al., 2006)
hESC	Pancreatic cells	activin A, Wnt, KGF, CYC	(Kroon et al., 2008)
hESC	Neural stem cells	retinoic acid	(Wesselschmidt and McDonald, 2006)
hESC	Astrocytes	retinoic acid, SHH, EGF, bFGF, CNTF, 10% FBS	(Krencik et al., 2011)
hESC	Oligodendrocytes	retinoic acid, SHH, bFGF	(Hu et al., 2009)
hESC	Neurons	retinoic acid, bFGF, EGF (and SHH for motor neurons)	(Robertson et al., 2008)
hESC	Dopaminergic neurons	SHH, FGF-8, ascorbic acid	(Robertson et al., 2008)
hESC	Retinal cells	leftyA, activin, Dkk-1, serum	(Robertson et al., 2008)
hMSC	Osteocytes	DEX, b-glycerophosphate, ascorbic acid, 10% FBS	(Pittenger et al., 1999)
hMSC	Adipocytes	DEX, indomethacin, insulin, 3-isobutyl-L-methylxanthine, 10% FBS	(Narvaez et al., 2013)

hMSC	Chondrocytes	DEX, TGF β , BMP-2, glucose, FBS	(Hadjizadeh and Doillon, 2010)
hMSC	Epithelial cells	all-trans retinoic acid, 10% FBS, DMSO	(Brzoska et al., 2005)
hMSC	Neurons	DMEM, 2% DMSO, BHA, potassium chloride, forskolin, hydrocortisone, insulin, valporic acid	(Qian and Saltzman, 2004)
hMSC	Endoderm	activin-A	(Li et al., 2013)
hMSC	Pancreatic cells	retinoic acid, activin, GLPI-1, EGF, FGF, platelet lysate, beta-cellulin, nicotinamide	(Zanini et al., 2011)

Abbreviations: BHA, butylated hydroxyanisole; BMP, bone morphogenetic protein; CHIR99021, small molecule; CNTF, ciliary neurotrophic factor; CYC, KAAD-cyclopamine; DAPT, γ -secretase inhibitor; DEX, dexamethasone; Dkk-1, Dickkopf-1; DMH1, bone morphogenetic protein signalling inhibitor; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; Ex4, exendin-4; FBS, foetal bovine serum; FGF, fibroblast growth factor; bFGF (aka FGF2), basic fibroblast growth factor; GLPI-1, glucagon-like peptide 1; hESC, human embryonic stem cells; HGF, hepatocyte growth factor; hiPSC, human induced pluripotent stem cells; hMSC, human mesenchymal stem cells; hPSC, human pluripotent stem cells; IGF, insulin-like growth factor; KGF, keratinocyte growth factor; LDN, selective BMP inhibitor LDN-193189; mESC, mouse embryonic stem cells; SB431542, Lefty/Activin/TGF β pathway inhibitor; OSM, oncostatin M; SHH, sonic hedgehog; VEGF, vascular endothelial growth factor.

Table 3. Effects of combined biochemical and biophysical stimulation on stem cells.

STEM CELLS	LINEAGE	CHEMICAL STIMULATION	PHYSICAL STIMULATION	EFFECT	REF.
hESC	cardiomyocytes	IWP4, Gsk3 inhibitor	Substrate stiffness (50kPa)	Differentiation	(Hazeltnine et al., 2014)
hESC	cardiomyocytes	p38-MAPK inhibitor, transferrin, sodium selenite, 2-mercaptoethanol	Electrical stimulation	Cell alignment. Elongated cell morphology. Increased cardiac-specific gene expression. Organised cytoskeleton. Improved Ca ²⁺ cycling.	(Chan et al., 2013)
hMSC	myocytes	Myogenic induction media	Muscle-like matrix stiffness	Differentiation	(Engler et al., 2006)
hMSC	cardiomyocytes	BMP-4, lithium chloride, IWR-1, microRNA-206 inhibitor	Aggregation, 3D culture	Differentiation	(Tchao et al., 2014)
hMSC	neural cells	Neurogenic induction media	Brain-like (soft) matrix stiffness	Differentiation	(Engler et al., 2006)
hMSC	osteoblasts	Osteogenic induction media	Collagenous bone-like (rigid) matrix stiffness	Differentiation	(Engler et al., 2006)
hMSC	osteoblasts	Osteogenic induction media	100 nm or 200 nm particle features, hexagonal arrangement	Increased alkaline phosphatase activity and osteogenic ECM deposition. Inhibited cell spreading and focal adhesion formation.	(Wang et al., 2015b)
hMSC	osteoblasts	Fibronectin; mixed osteogenic/adipogenic induction media.	Sharp-edged star patterning	Differentiation	(Kilian et al., 2010)
hMSC	osteoblasts	10% FBS, DEX, ascorbic acid 2-phosphate, β -glycerophosphate	Blue light (455 nm or 470 nm)	Multipotency	(Wilner, 2010)

hMSC	osteoblasts	10% FBS, DEX, ascorbic acid 2-phosphate, β -glycerophosphate	Red light (656 nm)	Differentiation	(Wilner, 2010)
hMSC	chondrocytes	Chondrogenic induction media with TGF β 3	5 MHz ultrasound	Differentiation	(Guha Thakurta et al., 2015)
hMSC	adipocytes	10% FBS, DEX, insulin, indomethacin, isobutylmethyl xanthine	Native-like (adipose tissue-like) matrix stiffness	Differentiation	(Young et al., 2013)
hMSC	adipocytes	Fibronectin; mixed osteogenic/adipogenic induction media.	Soft-edged star patterning	Differentiation	(Kilian et al., 2010)
hMSC	osteoblasts, endothelium	RGD-grafted oxidised sodium alginate/N-succinyl chitosan hydrogel	Low-intensity pulsed ultrasound	Differentiation	(Wang et al., 2014)

Abbreviations: BMP, bone morphogenetic protein; DEX, dexamethasone; FBS, foetal bovine serum; GSK, glycogen synthase kinase; hESC, human embryonic stem cells; hiPSC, human induced pluripotent stem cells; hMSC, human mesenchymal stem cells; hPSC, human pluripotent stem cells; IWP, inhibitor of Wnt pathway; IWR, inhibitor of Wnt response; p38-MAPK, p38 mitogen-activated protein kinase.

Figure 1 .

Figure 2 .

Figure 3 .

Figure 4 .

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Figure 5 .

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