



The extracellular microscape governs mesenchymal stem cell fate

William J. Hadden¹ and Yu Suk Choi^{2*}

Abstract

Each cell forever interacts with its extracellular matrix (ECM); a stem cell relies on this interaction to guide differentiation. The stiffness, nanotopography, protein composition, stress and strain inherent to any given ECM influences stem cell lineage commitment. This interaction is dynamic, multidimensional and reciprocally evolving through time, and from this concerted exchange the macroscopic tissues that comprise living organisms are formed. Mesenchymal stem cells can give rise to bone, cartilage, tendon and muscle; thus attempts to manipulate their differentiation must heed the physical properties of incredibly complex native microenvironments to realize regenerative goals.

Keyword: Extracellular matrix, Mechanotransduction, Adhesion, Stem cell differentiation

Background

Stem cell fate was long assumed to rely solely on biochemical messengers, yet the story of cellular differentiation is now proving much more intricate than initially theorized. As early as 1898 Councilman described cellular chemotaxis in the setting of acute interstitial nephritis and postulated that soluble substances in certain locations were responsible for the development of lymphoid foci [1]. Later studies in the 1970's identified biochemical factors responsible for cellular migration in cancer models [2], and further research in the following decade described the molecular signals necessary for the induction of differentiation in T cells and glial progenitors, respectively [3, 4]. Still later studies demonstrated the powerful capacity of a biologically-inspired chemical signal gradient to induce cellular migration and differentiation [5–7]. Because of this pattern of accumulated knowledge, the potential for biochemical signals to induce stem cell migration, differentiation and proliferation has been appreciated for many years and early intimations regarding mechanically-directed behaviour drew less notice [8].

Although historically less recognized, clues to the importance of physical properties in tissue development

are ubiquitous in nature. Life uses malleable muscles to lever tendons across rigid joints, it creates discreet neuromuscular junctions from tissues with markedly different physical properties and it protects an intricate, delicate neural apparatus with rigid bony structure. But perhaps most relevant to regenerative medicine, pathophysiological states are often defined by an altered mechanical microenvironment. Gliosis following a cerebrovascular insult, fibrosis after a myocardial infarction and collagen deposition after epithelial disruption all forever alter the mechanical microenvironment experienced by individual cells resident to those tissues [9]. This shifting mechanical microscape makes it all the more necessary for therapies intent on rectifying pathology to prepare for the inductive properties inherent to the pathophysiological environments they will encounter.

Specifically, the elasticity [10–13], nanotopography [14, 15], protein composition [16, 17], and mechanical strain [18] inherent to the extracellular matrix (ECM) are all independent factors guiding stem cell lineage commitment. Even more fascinating is recent work suggesting stem cells retain mechanical memory for surfaces previously encountered [19]. This has striking implications not only for the inherent validity of the conclusions drawn from studies using *in vitro* models, but on the understanding of the *in vivo* temporal changes potentially implicit in aging. Understanding and deconstructing this complex menu of signals is core among

* Correspondence: yusuk.choi@uwa.edu.au

²School of Anatomy, Physiology and Human Biology, University of Western Australia, Entrance 2, Hackett Dr, M309, Level 1, Crawley, WA 6009, Australia
Full list of author information is available at the end of the article

the current challenges faced by regenerative medicine as each ECM inflicts a specific array of stressors on its resident cells. Factors such as cell density, shape and size [20–22], as well as the degree and type of cell-cell interaction and the dimensionality experienced within each tissue further complicate attempts to recapitulate these environments *ex vivo*.

The implications for understanding the interaction between cells and their respective ECMs are critical and far-reaching. The hopeful search for panacea in many medical fields has focused significant energy on stem cell therapy; from basic science to randomized clinical trials [23–25]. While seemingly safe in their application [26], medicine is marginally closer to sound and applicable stem cell therapies in regenerative medicine [27, 28]. There are of course many reasons this large cumulative effort has progressed slowly toward clinical resolution. In part, bureaucratic engines always run slower than scientific minds and funding for clinical trials in this field is doubly wrought with financial and political mines [29]. The emergent and rapidly expanding use of adult stem cell sources has softened some of the political stigma attached to the prefix ‘embryo’. Still, our lack of understanding and our inability to control all biochemical and biomechanical inputs that direct stem cell fate is certainly rate-limiting. The global sum of all signals encountered by a differentiating cell, whether chemical or physical, past or present, act as one unique set of circumstances directing the commitment of each cell toward a specific lineage [30]. Nature has replicated these exquisite circumstances with innumerable diversity for millennia; proof that such discretion is possible.

Mesenchymal stem cells (MSCs) are easily accessible progenitors that can give rise to fat, muscle, tendon, cartilage and bone [31, 32]. The promise of a tool to combat some of the most burdensome human pathologies, including myocardial infarction, osteoporosis and osteoarthritis, makes this stem cell source particularly appealing to biomedical engineers [33]. The future of medicine for an aging population may well depend on our ability to understand and control those slow and seemingly inevitable processes that alter physical structures. This review explores the guiding role of the ECM in MSC differentiation and discusses attempts to deconstruct and translate these ECM component signals for tissue engineering in the search for regenerative therapies.

The physical world

Stiffness

The stiffness of infarcted fibrous heart tissue is much greater than that of the adjacent healthy myocardium [9, 34]. This clear physical demarcation has so far complicated the ability of introduced stem cell therapies to remodel and replace infarcted myocardium. Indeed

ECM stiffness (Young’s modulus) has been shown to influence spreading [35, 36], migration [10], proliferation [37] and differentiation [17, 38] in multiple stem cell lineages [11, 12], and is a key physical property guiding stem cell behaviour (Fig. 1a). Tissue stiffness not only dictates differentiation, but informs cytoskeletal arrangement on a macro scale. Myotubes form myosin/actin striations preferentially on those substrates that mimic the passive stiffness of normal muscle [13, 39]. This is also true of artificial environments that mimic rigid collagenous bone and soft neural tissue; where the absolute stiffness in isolation directs osteogenic and neurogenic differentiation respectively [11]. This realization has led to the pursuit of substrates whose stiffness can be modulated in both two [40] and three [41] dimensions under static or dynamic conditions.

Collateral to dimensionality, efforts to create stiffness gradients in both space (Fig. 1b) and time (Fig. 1c) have added further pieces to our most accurate models of *in vivo* stiffness conditions. Controlling stiffness gradients in spatial planes has been accomplished with a variety of methods, including repeated freeze-thaw cycles with liquid nitrogen and cylindrical polyvinyl alcohol columns [42], heat gradients within polymerizing polydimethylsiloxane [37], polyelectrolyte monolayers with patterned cross-linker [43], inclusion of rigid particles in a soft hydrogel [44], and microfluidic mixing of different polyacrylamide (PA) solutions. Still other methods have employed photo-initiators and a patterned [40, 45, 46] or moving [47–49] photomask overlying a polymerization chamber to create stiffness gradients. However, tissues are not static in time. Temporal control over synthetic substrate stiffness is required to fully appreciate and simulate *in vivo* environments [50]. Early successful attempts at dynamic control over substrate stiffness came using photo-responsive elements embedded within those substrates [51–53], or chemicals whose disparate molecular weights stiffened hyaluronic acid hydrogels [54]. This approach allowed external control over substrate stiffness and gave researchers license to guide chondrogenic differentiation with specific timing or mimic the development of mature cardiomyocytes from mesodermal precursors. Building from this approach, near-infrared photons used to excite embedded gold nanorods have allowed researchers to write custom stiffness patterns into synthetic hydrogels [55], and still others have developed hydrogels that respond to their biological environment by releasing metalloproteinase (MMP) inhibitors in response to the activity of that enzyme group [56].

Just as tissues are not static in time, nor are they two-dimensional. As methods to build three-dimensional models of the ECM become more sophisticated, gains that moved this field from static to dynamic to bio-responsive are being incorporated into plant-inspired, 3D-printed

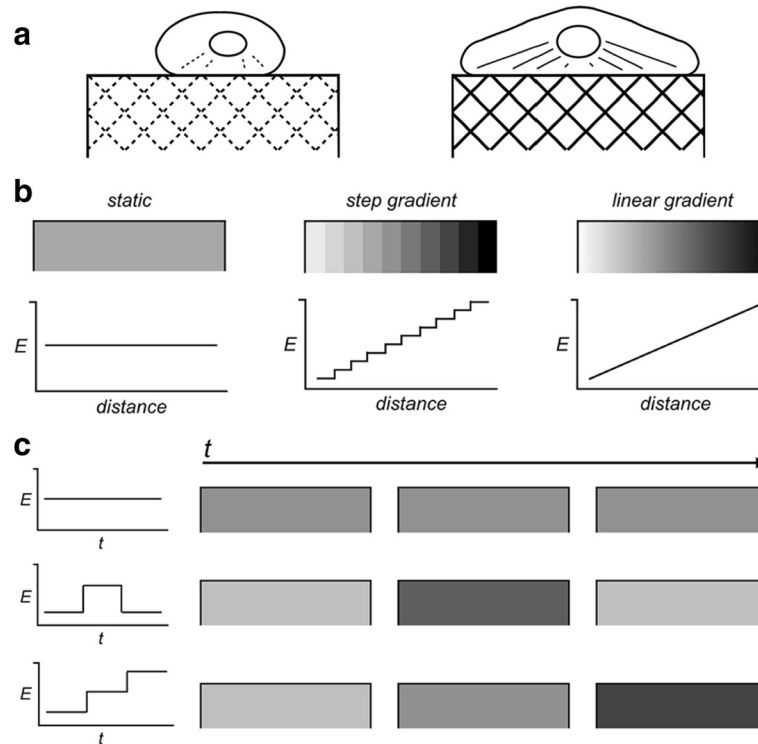


Fig. 1 Schematic representation of static and dynamic ECM stiffness. **a** stiffness influencing stem cell behaviour (soft on the left and stiff on the right); **(b)** spatial dynamic; and **(c)** time dynamic changes of ECM stiffness (E : Young's Modulus)

hydrogels that not only respond to the chemical signals of *in vivo* environments (i.e. MMPs) but to the very physical properties resident cells are feeling [57].

Stress & strain

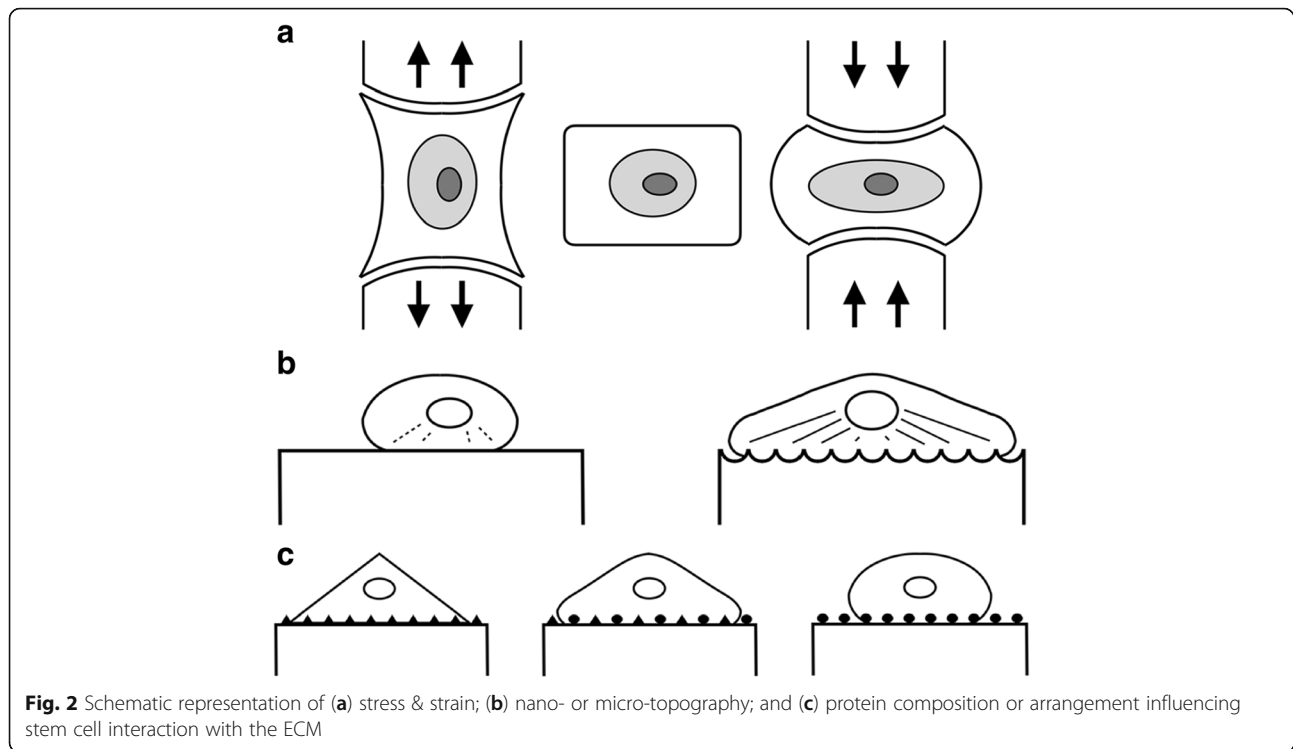
Lungs expand due to relative compartmental pressure changes and recoil due to the innate elasticity of their interstitium; hearts beat in rhythmic contraction-relaxation cycles; tendons and cartilage bear the longitudinal and compressive forces generated by ambulation. Thus the strain and stress applied to culture environments are important considerations for ECM models (Fig. 2a). Early studies with MSCs showed that uniaxial strain will induce transient increases in collagen I expression and alignment along that axis [58]. Further, a 3-5% tensile strain on a collagen I substrate can induce osteogenesis in MSCs [59], and biaxial stretching systems can influence alignment of MSCs via mechanosensing [60]. Following from this, the imposition of cyclical tensile strain to mimic the loading and unloading of the skeletal system was shown to promote osteogenesis and angiogenesis on three-dimensional collagen I scaffolds [61]. Conversely, applying a coordinated tensile strain coupled with electrical stimulation on a decellularized porcine myocardial ECM scaffold sped up myocardial differentiation of MSCs seven-fold [62]. MSCs co-cultured with intervertebral disc cells

will readily undergo chondrogenic differentiation under cyclical compressive force [63]. Similarly, cyclic hydrostatic pressure (mimicking compression) has also induced chondrogenesis in MSCs [64], and compression in the form of applied gravity and low-density ultrasound have restored collagen content and mineralized bone via osteogenic differentiation of MSCs [65, 66].

These fascinating observations are testament to the shear economy of differentiation; all available inputs are synthesized to trigger fate decisions. Even fluid shear stress modulated by $\beta 1$ integrin and experienced by many tissues in the body, has shown particular applicability to MSC osteogenic differentiation [67]. Further, these effects are not static or one-directional, as the ability of MSCs to deform the ECM around them differs depending on developmental stage of those MSCs. Fetal, neonatal and adult heart tissue all respond differently to ECMs that are evolving in composition through time [68]. This implies that although inanimate, there is a developmental dependency between stem cells and their ECM; a reciprocal guidance developing in concert.

Nanotopography

The spicules are thinner and the spaces between them greater in osteoporotic trabecular bone. This altered environment imposes new distance, surface area and



volume parameters on its residents (Fig. 2b), and we now know that this altered nanotopography also directs MSC fate (aligning electrospun nanofibers in a synthetic matrix will force increased mineralization of differentiating osteoblasts) [69]. Concordantly, nanofibers mimicking the pattern of cartilage ECM encourages chondrogenesis [70]. The fascinating detail with which stem cells use their nanoenvironment to acquire cues is perhaps best exemplified in by results showing that the mere addition of Si-OH groups to a matrix scaffold can induce osteogenesis of MSCs in the absence of growth signal supplementation [71]. Thus, not all cues are either biochemical or mechanical; some exist at the boundary between and the causality of their inductive property is less clear. The gross physical pattern of the ECM can also provide a constraining environment thus allowing more cytoskeleton tension within MSCs, which in turn leads to greater osteogenic differentiation [72], and the addition of a flow stimulus (albeit crossing into the realm of shear forces) through specific nanotopography modulates fibrochondrogenesis of MSCs [73]. Lastly, Yang et al. best exemplified the intertwined nature of three-dimensional microenvironment and the forces that environment imposes on resident cells with their assays of stem cell mechanobiology on substrates with built-in elastomeric microposts [15]. This last citation relays the inherent difficulty in deconstructing component physical cues of a given microenvironment.

Protein composition

Skeletal muscle relies on an ECM dominated by laminin while bone tissue is replete with collagen-containing ECM. This stark difference between two descendants of mesoderm tells us that ligand composition, density and spatial arrangement is consequential (Fig. 2c). Undoubtedly, the interaction between stem cells and the ECM is bidirectional and geography-dependent, so the potential for interaction (i.e. the availability of binding sites) impacts heavily on the ultimate fate of stem cells exposed to these environments [16]. Specifically, the integrin density connecting cells to the ECM will fate different lineage commitments for MSCs residing in adipose or skeletal tissue and helps in part to explain why adipogenesis proceeds despite the induction of osteogenic stress in adipose tissue [74]. The density and spacing of the cell-adhesive tripeptide arginine-glycine-aspartate (RGD) can influence MSC fate, and for some time the tethering of collagen linkage points across inherent substrate pores was thought to guide differentiation (although this latter point is becoming somewhat contentious) [62, 75, 76]. Finally, the nature of the ligand itself can factor in the outcome of MSC differentiation; hyaluronic acid (HA) has been shown to induce chondrogenesis and chondroitin sulphate (CS) has been shown to induce osteogenesis [77]. Analogous to previously discussed silicon-hydroxide group addition and perhaps most intriguing of all, is the ability of matrices to offer physical binding sites for chemical and hormonal signals

(in this case melatonin), thus acting as reservoirs that blur boundaries between biomechanical and biochemical induction of differentiation [78–80].

Rebuilding the native microenvironment

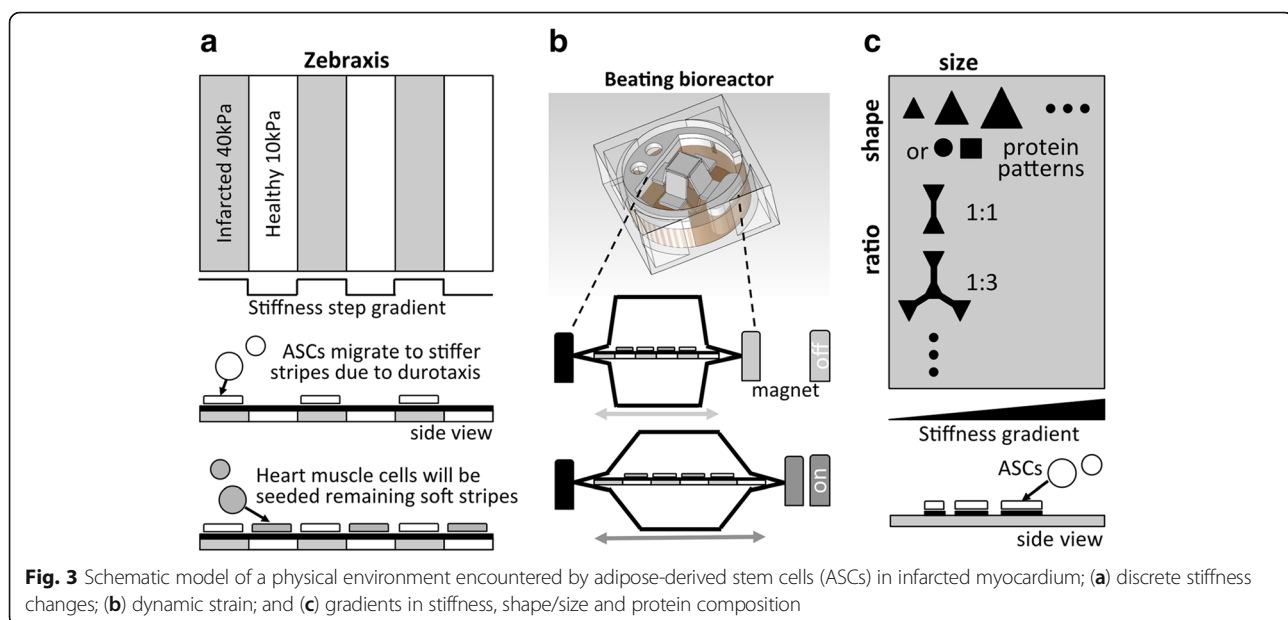
Tissue engineering is now faced with the challenge of how to most accurately and reliably reproduce and modulate each individual physical factor and then combine them into one cohesive environment. The enormity of this task is immediately realized when, for example, we attempt to faithfully reconstruct the physical changes wrought by myocardial infarction and seed those environments with adipose-derived stem cells. Discrete stiffness changes (Fig. 3a) coupled to an actively beating bioreactor (Fig. 3b) must be platformed on multiple gradients of stiffness, nanotopography and protein composition (Fig. 3c) in both two and three dimensions. The shear complexity of this deconstructive approach therefore also demands the exploration of endogenous ECMs to satisfy this same purpose. Indeed, recent studies have shown that the matrix laid down by bone marrow- or adipose-derived stem cells promotes the growth of those cell lines more so than other cell types, even after decellularization and reseeded. To this end, the induction of small, high frequency deformations in osteogenic cultures can elicit more efficient production of ECM for MSC differentiation [81]. Further, these biologically synthesized matrices promote differentiation to the lineages that originally laid them down [82]. Even artificial three-dimensionally-printed scaffolds seeded with differentiated cells of a specific tissue (which are subsequently removed) for the sole purpose of decorating that artificial matrix with biological ECM, create more effective

inductive matrices for MSCs [83, 84]. An artificial collagen-polyglyconate scaffold seeded with smooth muscle and urothelial cells has generated bladder tissue sufficient for transplantation [27] and decellularized trachea has been recolonized by recipient epithelial cells after implantation [28]. Additional recommended reviews in this field further explore the use of decellularized or naturally-derived ECMs [85–88]. While the collaborative regenerative power of resident cells in native environments is clear, this research affirms the idea that 3D printing with intricately designed biocompatible materials may offer promising solutions as these technologies converge upon common applications [57].

Regenerative goals

Muscle

In the wake of much promise, early studies in cardiomyocyte differentiation showed MSC osteoblastic differentiation when exposed to infarcted fibrous heart tissue [9]. Although clinically disappointing, this outcome was scientifically interesting and correlated with findings regarding the absolute stiffness differences inherent to infarcted heart tissue and healthy myocardium [34]. This work served to punctuate the importance of local tissue stiffness in cardiac stem cell therapy and heralded the need for a deconstructive approach to better understand the consequences of the individual forces experienced by stem cells in vivo. Consequent of this realization, researchers have recreated functional myotubes on striped surfaces of alternating stiffness [12, 89], and shown that myoblast differentiation is delayed in the setting of fibrosis [90]. The capability to reintegrate this knowledge with therapy is now beginning to take form. Jeffords and colleagues have



developed cardiac matrix hydrogels using a genipin cross-linker to more accurately mimic the stiffness conditions favourable for endothelial revascularization of infarcted heart tissue [54, 91, 92]. Interestingly, the application of an acellular ECM substitute composed of high stiffness hyaluronic acid provided better cardiac function after myocardial infarction (MI). In this case, simply altering a pathological ECM allowed resident cells to effect superior outcomes [93].

Tendon/ligament

MSC enhancement of potential tendon and ligament grafts has centred on cyclical tension of a collagen scaffold either artificially produced or decellularized from a donor site, then re-impregnated with MSCs to promote fibroblastic differentiation [94, 95]. This type of loading elicited uniaxial alignment of MSCs on synthetic yarns and promoted the production of collagen I [96], and a 3 % strain induced tendon-like gene expression profiles in MSCs seeded on these scaffolds [97]. Tendon regeneration also exemplifies the extraordinary impact of as yet unknown resident biomolecular cues [98]. The leap from decellularized donor tissue to wholly synthetic grafts hinges on this understanding [99], and highlights the limitations of synthesizing a tissue with incomplete knowledge of its components. Current steps in this direction employ co-electrospinning to form synthetic anterior cruciate ligament ECM with in-built mechanical gradients for better integration when implanted [100], or the application of combined mechanical loading of a 3D ECM model to regenerate periodontal ligaments [101].

Cartilage

Existing at relatively unpredictable biological junctions, cartilaginous tissue is a model for the combined impact of tensile, shear, compressive and stiffness conditions experienced by biological tissues and transmitted through their respective ECMs. These component parts have been deconstructed with some success and slowly reintegrated to form a broader understanding of this tissue's development. Mimicking the native environment experienced by cartilaginous tissue with cyclic hydrostatic pressure loading increases chondrocyte differentiation of BMSCs [102–105]. Concurrent with these results, cartilaginous differentiation is also upregulated when MSCs are mechanically compressed in a gel matrix [106], or forced to compress under low intensity pulsed ultrasound [107]. As mentioned earlier, distortional stress analogous to artificial gravity in a centrifuge system can increase proliferation and proteoglycan/collagen II production in an MSC population subjected to these conditions [108]. Finally, an amalgamation of tensile, shear and compressive forces meant to replicate joint stress and strain has been shown to guide MSCs toward

chondrogenesis [109]. Just as tendon ECM serves as an example of the importance of biophysical and biomolecular integration of signals, cartilage ECM reveals the importance of various interacting mechanical inputs required for proper differentiation.

Bone

With osteogenic potential, bone regeneration is an obvious important application for MSCs. Stiffness [11], mechanical loading [110] and three-dimensional pore size [111] all impact osteogenic differentiation. When considered, the nature of anatomical progression from cortical to cancellous bone and the repetitive load-bearing function of this tissue would hypothesize as much. To investigate these ends, intermittent shear stress and cyclic hydrostatic pressure have been shown to elicit mechanosensitive gene profiles in MSCs and further work has shown enhanced production of bone ECM using pulsatile versus fixed hydrostatic shear flow [112, 113]. Interestingly, high acoustic frequencies will also guide MSCs toward osteogenesis and spare the adipogenic induction seen at lower frequencies [114], and larger three-dimensional pore size will elicit more osteogenic differentiation [111]. Analogous to earlier lessons unveiled by Brietbach et al., endocardial valve ossification has been observed when MSCs encounter the required inductive mechanical stress [9, 115]. This further highlights the crossing and complicated differentiation pathways that evolve from MSC therapy under subtly different mechanical circumstances and emphasizes the importance of applying negative results to a broader paradigm.

Fascinatingly, MSC osteogenesis illuminates a far-reaching implication of the dynamic interaction between stem cells and their ECM: aged MSCs tend to mineralize their environment at a slower rate under the same mechanical circumstances as their younger counterparts because of a reduced ability to undergo mechanotransduction [116]. This interaction implies a critical feedback loop and cyclical regression of bone density with age. Considering that osteogenesis from an MSC derivative population is greatly enhanced after injury when titanium implants are coated with a collagen matrix [117], current joint replacement or fracture fixation techniques may benefit from bio-regenerative therapy just as they will be limited by the endogenous capacity of aging MSCs to facilitate concordant repair.

Conclusions

Clearly the physical environment experienced by a resident mesenchymal stem cell through its ECM has much to say regarding the ultimate fate of that cell. Absolute stiffness (as well as patho- or physiological gradients in ECM stiffness) guides development and dictates

response to a disease insult. Nanotopography can blur the boundary between geography and the physical forces imposed by that microenvironment, and ligand density can similarly blend biochemical signals with biomechanical ones. Stress and strain experienced cyclically (heart), sporadically (tendon) or constantly (bone) inform the modelling, alignment and economy of materials in those respective tissues. Further, all of these ECM components evolve through time and are reciprocal in their interaction with resident cells: osteoblasts can form matrix that stiffens their environment leading to further osteogenic differentiation; the formation of functional myotubes leads to concerted beating that exacerbates the cyclical stress imposed on that tissue; native alignment of tendon tissue informs future recruits to that tissue as to the appropriate function therein; and the decoration and incorporation of growth signals in developing bone ensures signals for future bone regeneration [98].

Effective regenerative medicine requires exquisite models of the ECM; models that account for the infinitesimal fractional inputs accumulated across billions of years of life interacting with its environment. The ruthless economy of evolution demands that whether biochemical or mechanical, past or present, static or dynamic, no force or dimension or bond is unnecessary. Thus tissue engineering is proving complex beyond imagination, but its stated goals are not unattainable.

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W.J.H. and Y.S.C. drafted the manuscript. Both authors read and approved the final manuscript.

Competing interests

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Ethics approval and consent to participate

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Author details

¹University of Sydney Medical School & Kolling Institute of Medical Research, Sydney, NSW, Australia. ²School of Anatomy, Physiology and Human Biology, University of Western Australia, Entrance 2, Hackett Dr, M309, Level 1, Crawley, WA 6009, Australia.

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