Title

Biophysics rules the cell culture, but they are yet to reach the clinic. What are we doing wrong?

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Musculoskeletal injuries represent the leading cause of physical disability worldwide with associated annual direct and indirect healthcare expenditure in excess of US$ 874 billion in the US alone [1]. Current treatments are predominantly based on tissue grafts (autografts are preferred) [2-7] and biomaterials [8-11]. Given that the former are associated with scarce availability, insufficient remodelling and adverse immune reactions [12-14] and the latter with substandard stability, poor biological response and foreign body response [15-17], their clinical suitability has been questioned and gave rise to the field of cell-based therapies [18-23]. Cell-based therapies advocate that optimal repair and regeneration can be achieved through the utilisation of the intrinsic capacity of cells to build native supramolecular assemblies; cells are the natural born extracellular matrix (ECM) builders after all. Unfortunately, cell-based therapies require *in vitro* cell expansion in artificial tissue culture media and plastics. Removed from their optimal tissue niche, cells lose their phenotype, function and therapeutic potency [24-28]. Thus, contemporary tissue engineering incorporates high levels of biomimicry in the design of functional and physiologically relevant *in vitro* microenvironments to recapitulate *ex vivo* (to a certain extent) the complexity of the *in vivo* tissue context from where the cells came from. Herein, we briefly discuss recent advancements in biophysical aspects of cell culture systems and whether these developments have influenced clinical translation and commercialisation of cell-based therapies in the musculoskeletal space.

Biophysics and dynamics (in the form of architectural, geometrical, dimensional and topographical features; biomechanical properties, such as elastic modulus and shear forces and cyclic strains; and localised density) are ubiquitous in nature and determine cell / tissue specificity and function [29-33]. For example, tendons are composed of highly ordered, bidirectionally aligned collagen fibrils (up to 100 nm – 1,000 nm in diameter), which they bundle together to form collagen fibres (1 μm to 20 μm in diameter) and collagen fibre bundles (20 μm to 500 μm in diameter) [34]. Bone exhibits a radial gradient porous structure from the outside: the cortical bone has outer porosity ~ 5 %, whilst the inner part can reach porosity up to ~ 10 %; the cancellous bone's porosity starts at ~ 50 % in the outer layer and can reach ~ 90 % in the inner layer [35]. Articular cartilage has a zonal architecture

and the organisation and alignment of the collagen fibrils / fibres is different in every zone (e.g. parallel, perpendicular, diagonal, radial) [36, 37]. Advancements in engineering have made available numerous nano- and micro- fabrication technologies (e.g. electro-spinning, imprinting) that have enabled control over permanently differentiated and stem cells' fate [38, 39]. For example, electro-spun and/or imprinted substrates have been shown to not only maintain tenocyte [40-42], chondrocyte [43, 44] and osteoblast [45, 46] phenotype, but to also direct stem cells towards tenogenic [47, 48], chondrogenic [49, 50] and osteogenic [51-53] lineages. The term 'durotaxis' is used to describe the ability of cells to migrate directionally towards areas of high ECM rigidity. ECM elasticity / mechanical compliance governs numerous *in vivo* biological processes, including cellular spreading, migration and differentiation; morphogenesis; wound healing; and disease progression [54, 55]. In the last decade, numerous *in vitro* studies have demonstrated the positive influence of substrate rigidity in tendon- [56], cartilage- [57] and bone- [58] derived cell phenotype maintenance and in stem cell differentiation towards tenogenic [59], chondrogenic [60] and osteogenic [61] lineages. Static or dynamic uniaxial or multiaxial tensile, compressive or shear mechanical loads are also crucial for musculoskeletal tissues development, function and healing [62-67]. It is not a coincidence, after all, that exercise is an integral element of any orthopaedic rehabilitation regime [68-72]. Several bioreactor systems, of variable complexity, have been used as means to control tenocyte [73, 74], chondrocyte [75, 76] and osteoblast [77, 78] phenotype *in vitro* and to direct stem cells towards tenogenic [79, 80], chondrogenic [81, 82] and osteogenic [83, 84] lineages. Musculoskeletal tissues, like any other tissue, are highly dense ECM assemblies. Yet again, traditional cultures are conducted in dilute culture media that barely imitate the density of body fluids, let alone compact tissues. To emulate this dense ECM microenvironment *in vitro*, macromolecular crowding, also known as localised density or excluding volume effect, has been proposed and has been shown to substantially modulate nuclear processes, such as gene transcription, RNA splicing and DNA replication, and protein properties, such as diffusion coefficients, folding kinetics and thermodynamic activities, both intracellularly and extracellularly

[85-89]. *In vitro* data have shown macromolecular crowding to maintain tenocyte and osteoblast phenotype [90] and to enhance chondrogenesis in stem cell culture [91].

Despite the significant volume of work in *in vitro* setting, only a handful of studies have assessed in preclinical models the influence of mechanical preconditioning in tissue regeneration. However, in all cases, the cells were seeded into / onto a scaffold, the cell / scaffold system was subjected to mechanical loading *in vitro* for a period of time and then the cell / scaffold system was implanted [92-94]. To-date, no study has ever assessed in preclinical models or in clinical setting the influence of surface topography, substrate rigidity, mechanical stimulation or macromolecular crowding preconditioning in permanently differentiated or stem cell-alone implantation. What has hampered preclinical / clinical translation and commercialisation of these game-changing technologies? Financial issues may be the first reason. There are only a few companies that manufacture bioreactor systems with the capacity to apply loads and the systems available not only are far too expensive, but also have limited capacity for cell expansion. Reproducibility issues may be the second reason. Although electro-spinning is widely available in laboratory setting, only a handful of companies have industrialised the process and it is still challenging to precisely control the dimensionality of electro-spun mats. Scalability issues may be the third reason. Although imprinting has solved the problem of reproducible scaffold dimensionality, we are still far away from making in economic fashion the, most likely, trillions of imprinted cell culture substrates required per year to expand cells for educations, research, development and clinical purposes. Lack of sufficient evidence may be the forth reason. Although macromolecular crowding has been available since the 1980's, only a handful of studies have assessed its potential in cell culture context. Standardisation may be the fifth reason. Rarely one will find a paper that authors extracted the cells in the same fashion, used the same media, applied the same preconditioning conditions and conducted the same analysis. Regulatory issues may be the sixth reason. Most of the scaffold based surface topography / substrate rigidity experiments are carried out using non-FDA approved polymers.

It is undeniable that cell culture market grows exponentially; it is expected to worth US$ 18.63 billion by 2020 [95] and US$ 37 billion by 2022 [96]. Unless a disruptive innovation comes along, it is likely that functional reparative therapies will involve the delivery of a relevant cell population that has been expanded *in vitro*. It is therefore imperative to direct our efforts towards the creation of physiologically / clinically relevant, industrially scalable and regulatory compliable *in vitro* microenvironments in order to develop in the years to come remedial patient bedside cell-based therapies.

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