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CELL-SCAFFOLD INTERACTIONS IN THE BONE TISSUE ENGINEERING TRIAD

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Abstract

Bone tissue engineering has emerged as one of the leading fields in tissue engineering and regenerative medicine. The success of bone tissue engineering relies on understanding the interplay between progenitor cells, regulatory signals, and the biomaterials/scaffolds used to deliver them – otherwise known as the tissue engineering triad. This review will discuss the roles of these fundamental components with a specific focus on the interaction between cell behaviour and scaffold structural properties. In terms of scaffold architecture, recent work has shown that pore size can affect both cell attachment and cellular invasion. Moreover, different materials can exert different biomechanical forces, which can profoundly affect cellular differentiation and migration in a cell type specific manner. Understanding these interactions will be critical for enhancing the progress of bone tissue engineering towards clinical applications.

Keywords: Tissue engineering; bone; regeneration; scaffold; cell-scaffold interactions.

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Introduction

The human body's natural reaction to trauma or injury is to initiate a cascade of biological processes leading to tissue repair. Bone tissue has excellent regeneration ability whereby it can repair itself in response to trauma or injury. However, if injury exceeds a critical size, bone formation is impaired and surgical intervention is required. There are a number of therapeutic strategies for promoting bone tissue regeneration. One prevalent approach is to transplant healthy autogenous tissue or tissue allograft. While significant technical advances continue to be made in transplantation treatments, the idea of tissue replacement dates back to the 16th century. Gasparo Tagliacozzi (1546-99) published '*De Curtorum Chirurgia per Insitionem*' (The Surgery of Defects by Implantation) in 1597, in which he described a nose replacement that he had constructed from an autogenous forearm flap (Murphy and O'Brien, 2010; O'Brien, 2011). Today, autografts are still widely utilised for bone grafting while allografts tend to be applied more for whole organ restoration such as the liver, kidney and heart. Despite the life-saving capacity of tissue grafting, major problems still exist. For autogenous grafts, tissue and donor site morbidity can be problematic. For allografts, immune complications and donor availability remain challenging (Damien and Parsons, 1991; Arrington *et al.*, 1996; Giannoudis *et al.*, 2005; Khan *et al.*, 2005). In light of these obstacles, there is a strong need for the development of novel synthetic or bioengineered bone graft substitutes.

Recent scientific progress in biomaterials and cell based therapeutics has created exceptional advances in the development of engineered tissues. There are three main components in the field of tissue engineering:

1. A scaffold that provides structure and substrate for tissue growth and development
2. A source of cells to facilitate required tissue formation
3. Growth factors or biophysical stimuli to direct the growth and differentiation of cells within the scaffold.

Taken together, these components make up what is known as the tissue engineering triad (Fig. 1).

Despite early successes achieved with tissue engineering, many challenges are still faced in tissue regeneration (Rose and Oreffo, 2002). Clearly, it is critical to tailor the components of the tissue engineering triad for specific tissue applications. However, not only are these components individually important, understanding their interactions is key for successful tissue engineering. It is the purpose of this review to focus on the interplay between the three components of the tissue engineering

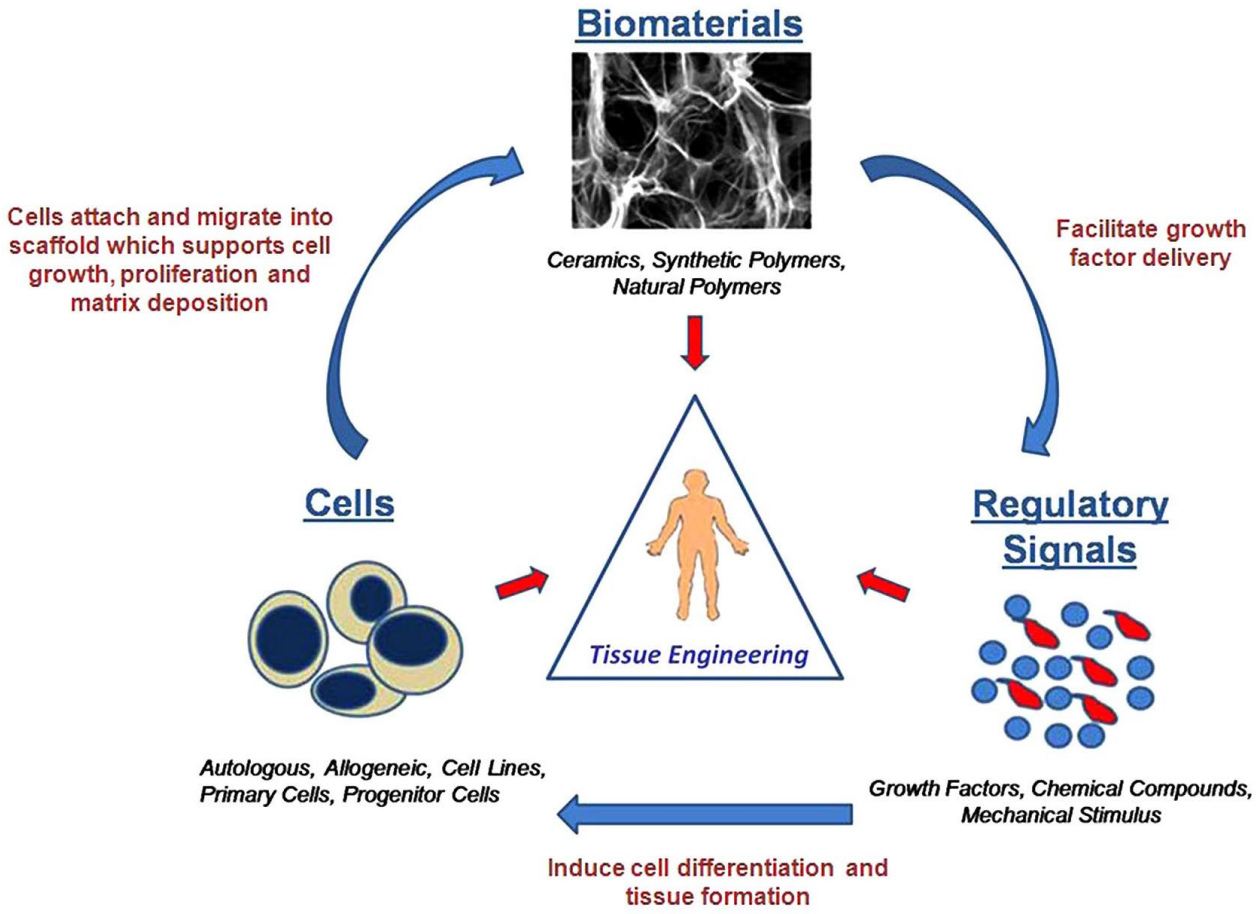

Fig. 1. The three essential components that make up the tissue-engineering triad.

triad in the context of bone tissue engineering, with particular emphasis on the importance of scaffold design on cell attraction, migration, differentiation, and their subsequent tissue generation.

Biomaterials

Over the past several decades there has been an explosion in the range of available scaffolds in terms of composition and architecture to promote osteoconduction (bone mineral and collagen deposition) and osteoinduction (osteogenic differentiation) (Albrektsson and Johansson, 2001). In the field of scaffold design, crucial considerations include the biocompatibility and biodegradability of the biomaterial used (Hutmacher, 2000; Yang *et al.*, 2001). The term biocompatible refers to the ability of a scaffold to support cell growth and tissue regeneration *in vivo,* without eliciting an inflammatory or immunogenic response that may result in its rejection. Limited local inflammation can promote healing and neovascularisation, however chronic inflammation and/or an adverse immune response can compromise both the implant and the patient (Babensee *et al.*, 1998; Hutmacher, 2000). Ideally, biodegradation should occur over a period of time that allows the scaffold to disappear in concurrence with tissue formation, leaving behind repaired or regenerated tissue. This negates the need for a second surgery to remove the implant (Murphy *et al.*, 2000; Haugh *et al.*, 2011). In the case of drug delivery, degradation needs to be controlled at a rate that can facilitate optimal drug release. The choice of material and the micro- and macro-structure of that material will influence the biocompatibility and degradability. There are three major classifications of materials that have been used for scaffolds in bone tissue engineering: (1) ceramics, (2) synthetic polymers and (3) natural polymers.

Ceramics are non-metallic compounds with a crystalline structure. Typically, they have a high level of stiffness and as a result are commonly used in bone tissue engineering. The most frequently used ceramics are calcium phosphate (CP), tricalcium phosphate (β-TCP), and hydroxyapatite (HA), and these materials are biocompatible and osteoconductive. Their biocompatibility is commonly attributed to their structural similarities to the mineral phase of bone. Nevertheless, ceramics are brittle materials and generally have a slow rate of degradation and can persist for months or years (Pilliar *et al.*, 2001; Rizzi *et al.*, 2001; Giannoudis *et al.*, 2005).

A number of synthetic, degradable polymers have been employed for bone tissue engineering. Poly(alpha-hydroxy

acid) polymers such as polylactic acid (PLA), polyglycolic acid (PGA), and the co-polymer poly-DL-lactic-co-glycolic acid (PLGA) have shown promising pre-clinical and clinical findings (Amanat *et al.*, 2007). For PLGA, the physical properties and degradation rates can be controlled by altering the monomer ratios in lactide/glycolide copolymers. These polymers also have FDA approval for specific clinical applications (Yang *et al.*, 2001; Jiang *et al.*, 2006). An attraction of synthetic polymers is an ability to manufacture scaffolds with characteristics tailored to match that of new tissue formation. However, a major disadvantage with many synthetic polymers is the release of acidic degradation by-products that can alter the pH in surrounding tissue. In turn, this can cause adverse tissue and inflammatory reactions (Yang *et al.*, 2001; Ravindran *et al.*, 2010).

Natural polymers commonly used in tissue engineering include collagens, glycosaminoglycan, chitosan, hyaluronic acid, fibrin and elastin (Berglund *et al.*, 2004; Ma *et al.*, 2004; Glowacki and Mizuno, 2008; Murphy *et al.*, 2010), isolated directly from the extracellular matrix (ECM). Cell adhesion and subsequent cell activity is mediated by specific integrin-ligand interactions between the cells and their surrounding ECM. Some of these natural polymers contain surface ligands required for cell adhesion and proliferation (Heino, 2000). Those that lack cell specificity are modified to incorporate ligands that facilitate cell-ECM interaction (Vepari and Kaplan, 2007; Kim *et al.*, 2008). Natural polymers present a more native surface relative to synthetic polymers; as a result, they are biocompatible and typically degradable with non-toxic degradation products (Hubbell, 1995). Limitations of natural polymers include their poor mechanical properties that are unsuitable for high strength applications as well as the relatively high costs associated with purification or *de novo* synthesis. Nonetheless, for orthopaedic applications natural polymers remain a popular choice of material. Collagen has been frequently explored, as it is one of the main constituents of the natural ECM. Outside of orthopaedics, collagen has achieved success in the areas of skin (Yannas *et al.*, 1989), bladder (Atala *et al.*, 2006), and airways (Macchiarini *et al.*, 2008). However, to overcome the limitations associated with natural polymers, recent advances in scaffold design and fabrication have led to a paradigm shift towards the development of biomimetic scaffolds. Biomimetic scaffolds imitate the native ECM and are often utilised *in vitro* as analogues of the natural ECM to facilitate investigations of cell-ECM interactions and processes (Shin *et al.*, 2002; Ravindran *et al.*, 2010). More importantly, they provide a compromise between the mechanical and biological prerequisites needed to rapidly promote bone healing.

An elaboration of this strategy has been to incorporate an additional phase into collagen scaffolds to improve the osteoconductivity and/or biomechanics. One example is the introduction of a ceramic phase, such as hydroxyapatite (HA) to improve the mechanical properties. Collagen-hydroxyapatite (CHA) scaffolds have demonstrated improved healing of bone defects in comparison to collagen alone (Gleeson *et al.*, 2010). However, other biomimetic scaffolds have recently emerged and are drawing new interest in the tissue engineering field (Shin *et al.*, 2003; Radisic *et al.*, 2006; Moutos *et al.*, 2007; Potter *et al.*, 2008).

Cells in Tissue Engineering

In the context of bone tissue engineering, regeneration is biologically driven by progenitor cells that are able to form new osteoblasts. These progenitors can arise from within the damaged tissue or surrounding native tissues, or they can be exogenously supplied as part of the tissue engineering solution.

An important consideration for the delivery of *ex vivo* cells is whether they are autologous, allogenic or xenogenic. Autologous cells are harvested directly from the individual undergoing repair, whereas allogenic cells are from a donor individual (of the same species). Xenogenic cells are transplanted from a different species and are less common in tissue engineering than in the field of whole organ transplantation.

There are three principal cell therapeutic strategies for treating diseased or injured tissues in patients: (1) implanting isolated cells, (2) implanting a construct assembled from cells and scaffolds, or (3) *in situ* tissue regeneration by native cells.

Implantation of isolated cells

With this strategy, whole cell populations can be directly transplanted or isolated cells can be cultured and expanded *ex vivo* prior to re-implantation. Raw bone marrow transplants are an example of the former and have been used in the treatment of a variety of malignant and non-malignant haematological diseases. Cells within the marrow have the ability to reconstitute the haematopoietic system (Takahashi and Yamanaka, 2006) and the stromal progenitors are multipotent and can differentiate into a variety of cell lineages (Pittenger *et al.*, 1999). Consequently, transplanted marrow cells have been used in the regeneration of a range of complex organs including the liver and heart (Caplan, 2007; Timmins *et al.*, 2007). Recently, Gao *et al.* (2012) demonstrated the use of implanted isolated mesenchymal stem cells (MSCs) for bone tissue engineering. MSCs isolated from healthy donor mice were implanted into mutant littermates that exhibited skeletal defects similar to those seen in ageing bone and enhanced implant osteointegration (Gao *et al.*, 2012).

Implanting a construct assembled from cells and scaffolds

In the context of tissue engineering and regenerative medicine, the use of isolated cells is not limited to direct replacement of damaged cells. Another method is to deliver a combination of whole cell isolates or *ex vivo* cultured cells seeded onto a substrate template (Lee *et al.*, 2003; Levenberg *et al.*, 2003; Kim *et al.*, 2010). Aside from implantation outcomes, implant designs are still commonly assessed *in vitro* using 3D culture systems. These 3D culture systems can utilise primary cells from

the tissue to be regenerated, isolated stem/progenitor cells, or immortalised cell lines. These systems have been used to gauge the performance of engineered constructs and achieve incremental improvements by modification of scaffold (porosity, architecture, and biomaterials), drug delivery, or cell source.

Established cell lines capable of extensive or indefinite proliferation are frequently used for *in vitro* investigations. While cell lines can demonstrate biocompatibility, these lines are by definition abnormal and may poorly reflect the *in vivo* behaviour of a tissue implant. Primary cells derived from tissue explants (whether mechanically or enzymatically isolated) can provide a pool of cells more comparable to endogenous progenitors. Nevertheless, 3D culture models with primary cells allow optimisation without complications such as post-surgical infection and host *versus* graft disease (HVGD). For some tissues, primary cells can be challenging or impossible to isolate and expand (such as pancreatic islet cells), but this is rarely a problem for bone.

For bone tissue engineering purposes, bone marrow-derived MSCs remain popular due to their multipotency. They are capable of differentiation down multiple lineages including bone, cartilage, and adipose tissue (Pittenger *et al.*, 1999; Krampera *et al.*, 2006; Uccelli *et al.*, 2008). Bone marrow is also considered to be the most accessible and enriched source of adult stem cells. Pittenger *et al.* (1999) demonstrated that cells isolated from human marrow aspirates were capable of remaining in a stable undifferentiated state in long-term cultures and could be induced towards osteochondral lineages when provided with the appropriate cues (Pittenger *et al.*, 1999). Since then, extensive investigations have been carried out in terms of MSC characterisation and clinical potential. However, there are disadvantages associated with the use of bone marrow derived MSCs. The harvest of these cells is an extremely invasive procedure with donor variability being a prevalent issue. Furthermore, the number, differentiation potential and life span of these cells can decrease with increasing age (Nishida *et al.*, 1999; Mueller and Glowacki, 2001; Stenderup *et al.*, 2003). Adipose tissue has also been shown to contain a population of multipotent cells, genetically similar to MSCs known as adipose derived stem cells (ADSCs). ADSCs are easily extracted from adipose tissue after surgical procedures such as liposuction (Fraser *et al.*, 2008) and similarly to MSCs, can differentiate towards an osteogenic lineage when treated in osteogenic medium (Zuk *et al.*, 2002) or genetically modified to over-express BMP-2 (Dragoo *et al.*, 2005). An alternative, albeit heterologous, source is umbilical cord blood which can be harvested by a non-invasive approach. Similar to bone marrow and adipose tissue, umbilical cord blood has a population of multipotent MSCs that can be differentiated down an osteogenic lineage (Kern *et al.*, 2006). However, the number of MSCs in the umbilical cord blood is extremely low in comparison with their population in the bone marrow, adipose tissue and more difficult to isolate (Musina *et al.*, 2007).

Human ES cells are a broader utility as they maintain pluripotency, can be induced into multiple mature somatic cell types (Rippon and Bishop, 2004) and can be cultured indefinitely in an undifferentiated state due to their ability to self-renew (Bishop *et al.*, 2002; Hipp and Atala, 2008). However, the use of these cells is often coupled with complex ethical, religious, and political issues (Rippon and Bishop, 2004), leading to the search for alternate sources of stem cells that could be derived without creating or destroying embryos (Hipp and Atala, 2008). In 2006, Takahashi and Yamanaka attempted to circumvent the ethical issues associated with ES cells. They reported that enforced expression of 4 key transcription factors (OCT3/4, Sox2, Klf4 and c-Myc) could re-program somatic cells to pluripotency with similar developmental potential as ES cells (Takahashi and Yamanaka, 2006). These cells were termed induced pluripotent stem (iPS) cells. However, the vast differential potential of ES and iPS cells is assessed by the ability of these cells to form teratomas (Thomson *et al.*, 1998; Yu *et al.*, 2007; Zhang *et al.*, 2008). Consequently, the use of these cells in clinical practice is thus far hindered.

In situ tissue regeneration by native cells

The isolation and expansion of autologous stem cells was once considered to be the future of tissue engineering and regenerative medicine. However, challenges associated with cost and culture times as well as limited demonstrations of efficacy have led to a re-focusing of efforts to recruit native cells to sites of tissue damage. In order to modulate the migration and tissue-appropriate differentiation of endogenous progenitors, drugs capable of affecting regulatory signals or proteins involved with the regulatory signalling cascade can be locally delivered. As such, the field of controlled drug delivery *via* tailored biomaterials is currently driving many innovations in biomaterials (Numata and Kaplan, 2010; Yu *et al.*, 2010; Hoffman, 2012; Panyam and Labhasetwar, 2012;).

Another method for modulating progenitor recruitment and differentiation is the application of gene therapy technologies. Gene therapy is a powerful tool for the manipulation of existing progenitors as well as the delivery of paracrine signals (Godbey and Atala, 2002). For example, cells can be engineered to produce VEGF to stimulate angiogenesis or rhBMP-2 to promote osteoblastogenesis. Gene therapy with viral and non-viral vectors can allow the transient or sustained release of a range of therapeutic factors, albeit often at high cost and some concerns for malignant transformation (Levenberg *et al.*, 2003). Recently, there has been a surge in research to combine gene therapy with scaffold-based templates to produce gene-activated matrices (GAMs), enhancing the capacity for repair. The scaffold acts as a depot for the gene while simultaneously providing both structural support and a matrix for new tissue deposition (Storrie and Mooney, 2006; O'Rorke *et al.*, 2010; Curtin *et al.*, 2012; Tierney *et al.*, 2012a).

Regulatory Signals

Cellular behaviour is strongly influenced by biological, biochemical, and biophysical cues from the ECM. Consequently the use of regulatory signals is the third component of the tissue engineering triad. These include

biochemical and or biophysical stimuli to induce and regulate tissue formation both *in vitro* and *in vivo*.

Biochemical stimuli modulate cell-signalling processes that regulate cellular migration, adhesion, proliferation, differentiation, and survival. Growth factors and cytokines can function locally or systemically to change patterns of gene expression in target cells. They can also be involved with up-regulating and down-regulating the synthesis of other growth factors and receptors. These proteins bind to transmembrane receptors that transduce extracellular signals to changes in gene expression (Griffith and Naughton, 2002; Rose and Oreffo, 2002; O'Brien, 2011). Growth factors and drugs that modulate growth factor signalling are broadly used within the tissue engineering field, although the use of recombinant factors can significantly add to the cost of an implant.

For bone tissue engineering, rhBMPs are approved for clinical intervention and can create new bone *de novo* (Geiger *et al*., 2003). Other growth factors used to promote bone include the Insulin-like Growth Factors (IGFs) and Transforming Growth Factor-β proteins (TGF-βs). IGF-I and IGF-II have essential roles in bone growth, development, remodeling and repair. TGF-β, initially purified from platelets, stimulates matrix protein synthesis, has dramatic effects on osteoblast and osteoclast activity (Bonewald *et al*., 1990). Blood platelets are also a rich source of platelet-derived growth factor (PDGF), a potent stimulant of mesenchymal cell proliferation and migration such as fibroblasts and vascular smooth muscle cells (Kilian *et al.*, 2004).

Calcium phosphate ceramics can themselves be a regulatory signal in terms of bone tissue engineering. CP ceramics have a compositional resemblance to bone mineral, as such, the degradation of such biomaterials can elicit a biological response similar to the one generated during bone remodelling (Barrere *et al.*, 2006). The calcium and phosphate ions released during degradation can induce bone cell activity (Zaidi *et al.*, 1989; Kanatani *et al.*, 1991) and may also be used as raw materials for new bone formation (Metsger *et al.*, 1993; Bohner *et al.*, 2012).

In addition to biochemical signals, cellular behaviour is strongly influenced by biomechanical stimuli by a process known as mechano-transduction. Multiple systems have been developed to induce different types of physical stimulation to cells in culture including spinner flask bioreactors, flow perfusion bioreactors, dynamic compression bioreactors, and hydrostatic pressure bioreactors (Darling and Athanasiou, 2003). Provision of appropriate biomechanical stimulation *ex vivo* can positively influence cell differentiation and the production of ECM and ultimately the outcome following implantation (Plunkett and O'Brien, 2011). Optimised biomechanics can also be used to improve the cellular spatial distribution (Khan *et al.*, 2005). Heterogeneous cell distribution can be a major obstacle to developing any three-dimensional (3D) tissue or organ *ex vivo*.

A greater understanding of the role of the ECM in coding molecular/ biochemical as well as physical information is enabling the development of a new generation of biomaterials. For example, stem cell fate has been shown to be influenced by the stiffness of the substrate on which they reside (Harley *et al.*, 2007; Murphy *et al.*, 2012). As a result, scaffolds themselves have become incorporated as regulatory signals in the development of tissue engineered constructs, whereby the physical and chemical structure of the biomaterial is tailored for optimal cell behaviour.

Cell-Scaffold Interactions for Bone Tissue Engineering

Optimising the design of bioactive scaffolds is guided by an understanding of the behaviour and responses of cells cultured on these scaffolds. In the past decade, there have been many significant advances in the development of synthetic and naturally occurring biomaterials. However, for bone tissue engineering two major challenges exist. Firstly, it remains difficult to fabricate cell-permissive internal architectures for some biocompatible materials. Insufficient pore structure can lead to cellular aggregations around the periphery of the construct. This in turn can lead to premature core degradation of the construct (Kelly and Prendergast, 2004). Secondly, many scaffolds, particularly those based on first generation designs, have suboptimal mechanical properties, limiting their use in load bearing orthopaedic applications. It is necessary to overcome these challenges in order to facilitate the migration and proliferation of osteoprogenitors, and to support their subsequent differentiation and matrix deposition. As such, the interaction between scaffold architecture, scaffold biomechanical properties, and the cellular response remain areas of emerging importance in the tissue-engineering field.

Cell-ECM interaction

The chemical composition and physical properties of the natural ECM have been shown to prominently influence cell morphology, motility, and migration (Friedl *et al.*, 1998; Harley *et al.*, 2008; Kanungo and Gibson, 2010). Similar to natural ECM, tissue engineering scaffolds can influence the cellular response in terms of cell proliferation and differentiation (osteoinduction), and the biomaterial and microarchitecture can influence chemo-attraction, adhesion, and migration which in turn will affect matrix deposition and mineralisation (osteoconduction) (Zeltinger *et al.*, 2001; Tierney *et al.*, 2009b;Keogh *et al.*, 2010; Ravindran *et al.*, 2010).

Cell motility and migration play an important role in many biological processes and requires a dynamic interaction between the cell, its substrate and the cytoskeleton (Huttenlocher *et al.*, 1995; Lauffenburger and Horwitz, 1996; Ridley *et al.*, 2003). This occurs by means of specific ligand-integrin interactions. Cell-ECM interactions are accompanied by cytoskeletal action, matrix remodelling, and contraction, which modulate cell fate (Friedl *et al.*, 1998). Whilst cell mediated contraction is a natural phenomenon that is essential for wound healing (Yannas, 2001), it can negatively affect tissue engineered constructs. In mechanically weak scaffolds, such as many natural polymers, it can lead to reduced scaffold volume.

This can lead to improved biomechanics, but the reduced scaffold size and porosity can result in difficulties fitting specific implant sites and subsequent osteointegration, as well as affecting the cellular response (Lee *et al.*, 2001; Govender *et al.*, 2002).

The discovery of integrins in the mid 1980s altered our view of the natural ECM in terms of its role in the physical linkage and subsequent behaviour of cells (Hynes, 1987). Several parameters contribute to the strength of integrin-ligand mediated cell adhesion. These include concentrations of adhesive ligands or substrate, number of receptors, and the receptor-ligand affinity. A shift in these parameters can have a dramatic effect on cell migration (Huttenlocher *et al.*, 1995; Gumbiner, 1996; Huttenlocher *et al.*, 1996; Lauffenburger and Horwitz, 1996). Altering ligand density has been observed to affect the strength of the cell-substrate interactions *via* differential integrin binding to adhesion ligands. In recent years, these effects have been translated in terms of 3D scaffold design. Changing the composition of biomaterials used in scaffold fabrication can lead to a difference in ligand availability and subsequent integrin binding. Tierney *et al*. (2009) demonstrated that altering the concentration of collagen and glycosaminoglycans (GAGs) in a collagen-GAG scaffold had a significant influence on osteoblast activity, indicating an effect of differing ligand availability (Tierney *et al.*, 2009a; Tierney *et al.*, 2012a). Furthermore, altering the composition of biodegradable scaffolds developed for cartilage repair has led to improved *in vivo* stability and increased matrix deposition (Moutos *et al*., 2007). Consequently, the composition of scaffolds in terms of ligand density and availability is an important consideration in scaffold design.

Scaffold architecture

Pore size, pore interconnectivity, and total porosity are essential considerations in scaffold development. Interconnection is essential for healthy cellular invasion, growth, and nutrient flow. Since the 1970s, scaffold and biomaterial pore size has been recognised as an essential consideration in tissue development (Hulbert *et al.*, 1970). If the pores are too small, cell migration is limited – resulting in the formation of a cellular capsule around the edges of the scaffold. This in turn can limit diffusion of nutrients and removal of waste resulting in necrotic regions within the construct. Conversely, if the pores are too large there is a decrease in surface area which in turn limits cell adhesion. Pore size has been observed to influence significantly cell adhesion *in vitro* (O'Brien *et al.*, 2005). Additionally, scaffold mean pore size significantly affects cell morphology and phenotypic expression (Nehrer *et al.*, 1997).

However, over the years it became evident that the optimal pore size varies depending on the biomaterials used and application of the construct. For example, in porous silicon nitride scaffolds pore sizes in the range of 30-80 μm were optimal for endothelial cell adhesion, but fibroblasts preferentially bound to larger pores (< 90 μm) (Salem *et al.*, 2002). In contrast, for PLLA scaffolds pore sizes of 63-150 μm represented the optimal range for vascular smooth muscle cells, while fibroblasts could bind a wider range of pore sizes including smaller pores (38-150 μm) (Zeltinger *et al.*, 2001). However, over the years pores sizes in the range of 20-1500 μm have been reported favourably within the literature (Hulbert *et al.*, 1970; Nehrer *et al.*, 1997; Lee *et al.*, 2004; Williams *et al.*, 2005).

In an effort to reconcile the conflicting reports, O'Brien *et al*. (2005) developed a method for identifying the specific surface area available for cell adhesion within a collagen-GAG scaffold as it related to pore-size. It was determined that both specific surface area and cell attachment decreased with increasing pore size within a range of 96-150 μm (O'Brien *et al.*, 2005). A more recent study expanded the range to 85-325 μm and demonstrated a bimodal effect of pore size on cell attachment (Murphy *et al.*, 2010). Within the lower range of pore sizes (85-190 μm), a significant peak in cell number was observed in pore size of 120 μm – similar to the findings of O'Brien *et al*. (2005). However, when this pore range was expanded the largest pore size facilitated the highest cell attachment even though specific surface area decreased (Murphy *et al*., 2012). One interpretation of these findings is that the lower peak results from optimising the specific surface area and attachment, but that a second peak emerges from an improved potential for cell migration and proliferation. More recent studies have examined the effect of pore size on long term tissue development. Siccheri *et al*. (2012) investigated tissue interaction *in vivo* within PLGA-CaP scaffolds with pores in the range of 470-1200 μm. Increased bone formation and vessel number was observed within pores in the range of 470-590 μm. Although this paper does not identify the lower limits of the optimal pore range for tissue formation, it is one of the first to identify an optimal pore range for tissue formation (Sicchieri *et al.*, 2012).These papers reflect the first important step towards fully understanding the relationship between scaffold pore size and resultant cell and tissue behaviour.

Different cell types exhibit a preference for adhesion to scaffolds with different mean pore sizes, due to the characteristic size of the cell. When migrating through a porous network, cells use a bridging mechanism whereby they use neighbouring cells as support to bridge across pores larger than the individual cell (Salem *et al.*, 2002). However, if pore size greatly exceeds the dimensions of a cell, the cell can only spread along the struts and this can influence cell migration and migration speed (Reilly and Engler, 2010). Lowery *et al*. (2010) highlighted these changes in cell conformation with changing pore size in woven polycaprolactone (PCL) scaffolds. As pore size increased, cells began to align along single fibres instead of attaching to multiple fibres (Lowery *et al.*, 2010). Pore size within a scaffold determines the number of struts and the density of ligands available for cell adhesion. Thus, as pore size decreases there is more specific surface area for cell to adhere to (O'Brien *et al.*, 2005). However, this denser network of struts limits choice in cells' direction of their movement and creates a greater resistance to scaffold penetration (Harley *et al.*, 2008).

Speed is often the metric of choice to quantify the ability of cells to migrate (Lauffenburger and Horwitz, 1996; Ridley *et al.*, 2003). However, it does not provide

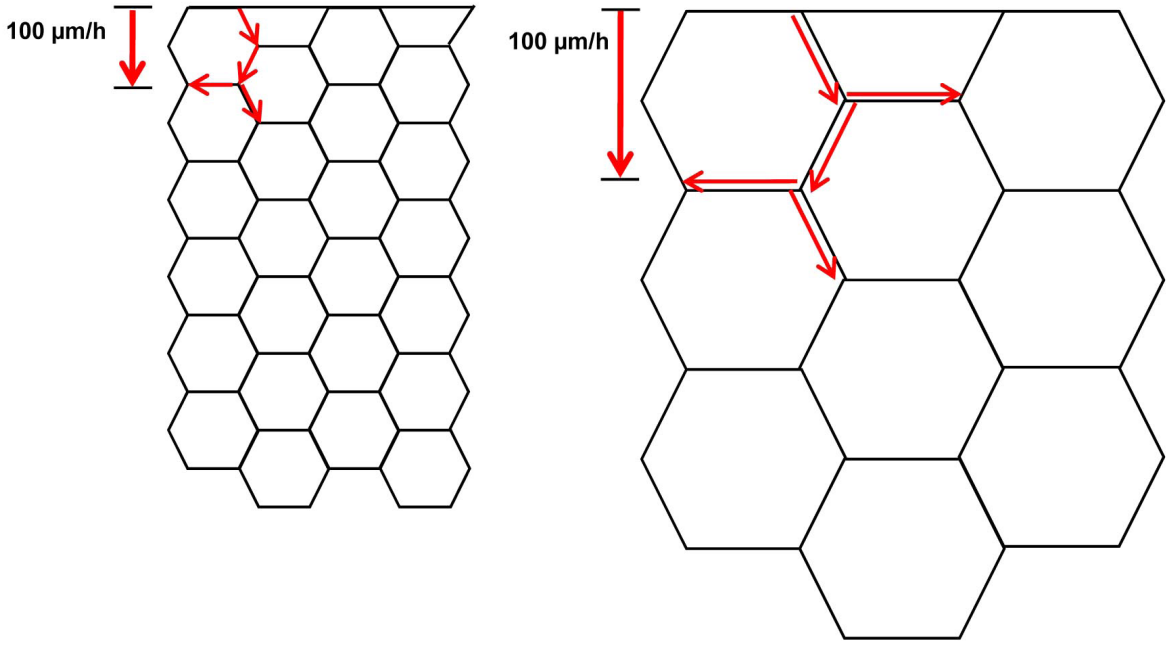

Fig. 2. Illustration on the effect of specific surface area in small pores (**A**) and large pores (**B**) on cell migration. Arrows indicate the direction of migration of cell and the distance travelled assuming the rate of cell migration is constant.

information about the direction or distribution of this motion (Zaman *et al.*, 2007). Cells travelling through larger pores may migrate slower, but their directional movement allow them to travel further into the scaffold (Fig. 2). In summary, it has been shown that larger pore size can overcome the advantages of specific surface area by increased cell migration and scaffold infiltration (Murphy *et al*., 2010).

Scaffold porosity, the percentage void space within a solid (Karageorgiou and Kaplan, 2005), and pore size are intrinsically coupled. Whilst pore size is essential for cell attachment, proliferation and migration, porosity influences nutrient delivery and waste removal through a construct. It is generally accepted that the higher the porosity the better for construct development. Depending on the biomaterial used, an increase in porosity can lead to a decrease in mechanical properties. This is generally a greater concern in natural biomaterials such as collagen, alginate-based substrates and chitosan, as they are mechanically weaker materials. However, there are a number of crosslinking techniques that can be utilised to strengthen without affecting porosity (Haugh *et al.*, 2009).

Mechanical properties

A scaffold's mechanical properties are derived from its composition and architecture. Beyond the previously discussed effects of scaffold architecture on cell migration, the capacity of a scaffold to respond to mechanical force can modulate the cellular response. These mechano-regulatory signals remain poorly understood, but may be an important consideration for future tissue engineering scaffold design.

Mechano-transduction is a process whereby cells transduce or convert physical force-induced signals into biochemical signals that are integrated into appropriate cellular responses (Ko and McCulloch, 2001; Huang *et al.*, 2004). Numerous molecules, cellular components and extracellular structures have been shown to be involved in mechanochemical transduction. These transduction elements include cell-ECM and cell-cell adhesions, membrane components, cytoskeletal filaments and nuclear structures. A current challenge is trying to understand how cells orchestrate all these transduction mechanisms to produce specific responses to mechanical signals. Not all anchorage-dependent cells respond to substrate stiffness in the same way. However, MSCs, fibroblasts and endothelial cells have demonstrated increased cell adhesion, spreading and proliferation on stiffer substrates (Discher *et al.*, 2005; Yeung *et al.*, 2005). Cell migration has also shown to be influenced by stiffness gradients. Integrins provide a mechanical link between the ECM and the actomyosin cytoskeleton of cells. Integrins can trigger signalling transduction cascades and induce focal adhesion formations as a result of ECM ligand binding and associated changes in receptor conformation. Force application to bound integrins promotes focal adhesion assembly, which in turn promotes actin filament polymerisation and induces cytoskeletal contraction. Contractile forces are generated by the ubiquitous cross-bridging interactions of actin and myosin-II filaments in stress fibres (Fig 3A). These forces are transmitted to the substrate as traction forces. It is through these forces that cells are able sense their surrounding matrix and can distinguish subtle changes in matrix elasticity (Zajac and Discher, 2008). Although living cells might sense and respond to force locally through individual mechanosensitive molecules, they integrate physical and chemical signals at the whole cell level before they respond. As such, mechanical

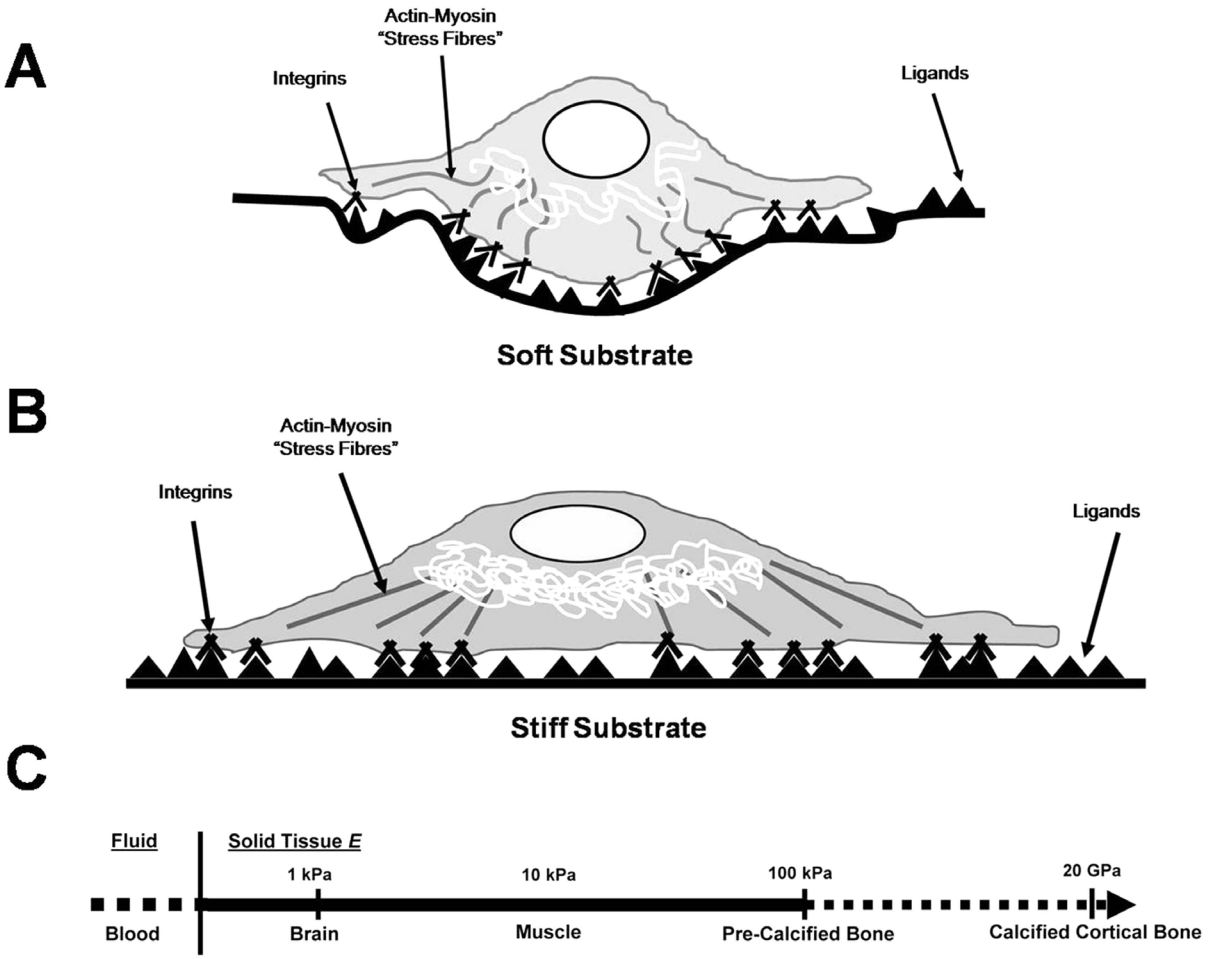

Fig. 3. Illustrations depicting the attachment of a cell to a soft (**A**) and stiff (**B**) substrate and the resultant cytoskeletal formations, adapted from Zajac *et al*. (2008).

forces generated with cell-ECM or cell-cell adhesions can influence embryogenesis and tissue formation (Evans *et al.*, 2009). The mechanical properties of native tissues are very diverse and can vary, for example, from soft brain tissue (0.1 kPa) to pre-calcified bone (100 kPa) to rigid compact bone (20 GPa) (Fig. 3B) (Zysset *et al.*, 1999; Flanagan *et al.*, 2002). As such, naïve stem cells are exposed to a range of matrix elasticity *in vivo*.

In 2006, a study by Engler *et al.* indicated a key relationship between substrate stiffness and MSC differentiation whereby MSCs sense their surrounding matrix elasticity and transduce that information into morphological changes and lineage specification. Matrix elasticity was mimicked with inert polyacrylamide (PAAm) gels. Elasticity was altered *via* crosslinking with different concentrations of bis-acrylamide and adhesion was provided by collagen type I coating of the gels. Utilising this elastically tuneable system it was shown that matrix can specify the lineage of naïve stem cells towards neurons, myoblasts and osteoblasts respectively as stiffness increased. In addition, non-muscle myosin II (NM II) was identified as an integral component of the cell's ability to couple matrix stiffness to lineage specification (Engler *et al.*, 2006). This study was the first to highlight importance of elasticity-directed differentiation as a novel and surprisingly sensitive cell regulator. Although once considered a cornerstone in literature for the effect substrate stiffness on stem cell fate, recent studies have demonstrated that there are other stem-cell-niche interactions guiding cell-substrate stiffness driven differentiation that must be taken into consideration. Trappmann *et al.* (2012) demonstrated that, similarly to the findings of Engler *et al*. (2006), MSC differentiation was regulated by the elastic modulus of PAAm gels. However, MSC differentiation was unaffected by polydimethylsiloxane (PDMS) gel stiffness. Furthermore, when PMDS gels were cultured with human epidermal stem cells, differentiation was unaffected. On PAAm gels, epidermal stem cell differentiation was only affected on gels of low elasticity (Trappmann *et al.*, 2012). Similar to Engler *et al*. (2006), these gels were coated with collagen type I and changing the collagen crosslinking concentration altered stem cell differentiation on both gel types. Contradictory to previous findings, Trappmann *et al*. (2012) concluded that the feedback mechanism that drives cell-fate decisions is directed by the mechanical force exerted by the stem cells on the collagen fibres as opposed to the gels themselves. These studies demonstrate that stem cells-ECM mechano-forces and subsequent cell responses vary with differing cell types and substrates, highlighting the complicated nature of these interactions.

Many of these studies have been carried out on polymer gels that do not recapitulate the normal 3D environment encountered by cells in porous engineered scaffolds. Recently, focus has shifted to changes sensed by cells within the elastic modulus of porous scaffolds (Murphy *et al*., 2012). Micro forces created by cellular contraction receive different mechanical-regulatory feedback, dependent on a scaffold's capacity for deformation. Previously, it has been shown that collagen-GAG scaffold architecture influences the fate of MSCs whereby the stiffest collagen-GAG scaffolds (1.5 kPa) directed naïve MSCs towards on osteogenic lineage and the most compliant collagen-GAG scaffolds (0.5 kPa) directed the MSCs towards a chondrogenic lineage. It is interesting to note that the stiffness range utilised in this study is significantly limited in comparison to the studies carried out on 2-D substrates and gels. Yet, a significant influence on stem cell fate was observed (Murphy *et al.*, 2012). This highlights the intricate relationship between cells and 3D porous scaffolds. What is determined as the elastic modulus of a whole scaffold, may not appropriately reflect the forces placed on a single cell on an individual struts (Harley *et al.*, 2007). This area of research is still in its infancy and these mechano-biological relationships and the cell pathways they modulate need to be better understood, both in 2D and 3D environments, in order to manipulate stem cell differentiation for *in vitro* and clinical applications.

Conclusions

Tissue engineering is aimed at different applications, including implantation and drug delivery. Although individually important, success depends on the convergence of the three components of the tissue engineering triad. The utility of each component ultimately relies upon the criteria required for the specific application.

Subtle changes in scaffold architecture have significant effects on cellular activity. Interestingly, there is a dual relationship between scaffold pore size and cell behaviour. Increased surface area, provided by small pores, may have a beneficial effect on cell adhesion. However, improved cellular infiltration and migration, facilitated by larger pores, outweighs this effect. Recent advances in the cell biology of the ECM and ECM receptors have provided new and important ways of thinking about the interplay between cells and scaffolds, whereby scaffold mechanical properties and composition have been shown to influence integrin-ligand interactions, thus affecting cell morphology and differentiation. These fundamental studies have demonstrated the importance of tailoring scaffold micro-architecture, cell type and regulatory signals for tissue-specific applications.

In summary, continuing efforts are being made towards developing a clinically functioning tissue-engineered scaffold. Advancing our way of understanding and thinking about the interplay between cells and scaffolds will enhance our progression towards clinical applications.

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Editor's note: All questions from the reviewers were answered by text changes. There is hence no "Discussion with Reviewers" section.