

BIOACTIVE SCAFFOLDS IN SKELETAL MUSCLE REGENERATION AND TISSUE ENGINEERING

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Introduction

Skeletal muscle has an impressive capacity to regenerate following damage. This is mediated by muscle precursor (stem) cells (called satellite cells), which lie between the plasma membrane of a muscle fibre and the surrounding basal lamina (1,2). Once activated, satellite cells proliferate and migrate to the damaged area of the muscle, differentiate and fuse to form new muscle fibres (3). However, when a traumatic injury results in a significant loss of muscle, the natural repair processes are unable to bridge the gap between the remaining tissue, leading to the accumulation of scar tissue, cosmetic deformity and a loss of muscle function. Current treatment options, such as autologous muscle grafts, involve significant donor site morbidity and may not be applicable to the repair of load-bearing muscles. A promising alternative approach is the use of regenerative medicine and tissue engineering to enhance the repair and replacement of damaged muscle (4).

Skeletal Muscle Tissue Engineering

Tissue engineering aims to use progenitor cells in combination with three-dimensional (3D) scaffolds to repair, replace and restore the essential functions of lost or damaged tissue (5). In broad terms, tissue engineering can be separated into *in vitro* and *in vivo* approaches (Table 1). *In vitro* skeletal muscle tissue engineering involves using patient stem cells (e.g., autologous satellite cells) to

construct or grow a muscle graft in the laboratory, which is then transplanted into the patient's muscle. *In vivo* tissue engineering refers to when the new muscle tissue is grown *in situ*, and can be further subdivided into 'cells plus scaffold' and 'scaffold only' approaches. The first method involves seeding myogenic cells onto a scaffold *in vitro* and transplanting the cell-laden scaffold into the patient's damaged tissue. Alternatively, the scaffold-only approach involves transplanting cell-free material into the tissue and allowing the patient's endogenous myogenic cells to migrate, proliferate and differentiate using the bioscaffold as a template to produce new muscle.

In vitro production and transplantation of muscle tissue has a number of technical challenges, the most obvious being how to achieve appropriate vascularisation and innervation of the transplanted tissue construct. Whether cultured myogenic cells attached to a scaffold will be subjected to the same rapid and massive death that occurs when cultured myoblasts are transplanted *in vivo* also needs to be addressed. Furthermore, it has proven difficult to grow muscle in the laboratory that is capable of generating significant levels of tension. As the principal function of skeletal muscle is to generate active force, this is an important characteristic of any engineered muscle (6).

For these reasons, attention in recent years has turned to *in vivo* tissue engineering and the development of biocompatible scaffolds which, once inserted, guide the growth and differentiation of cells to repair damaged

muscle tissue. These scaffolds provide support to the developing muscle and regeneration occurs entirely *in vivo*, so vascularisation and innervation develop as directed by the tissue itself. This approach also avoids potential problems associated with survival of transplanted cultured cells.

The overall aim of a skeletal-muscle-specific scaffold is to create an artificial niche, which allows the natural processes of stem cell activation, proliferation, differentiation and self-renewal to occur. In their normal biological

Table 1. *In vitro* versus *in vivo* skeletal muscle tissue engineering.

Tissue Engineering		
<i>In vitro</i>	<i>In vivo</i>	
Graft (cells and scaffold)	Cells and scaffold	Scaffold only
Process 1. Develop appropriate functionalised scaffold 2. Harvest stem cells from muscle biopsy 3. Seed cells on scaffold 4. Generate a muscle graft 5. Implant graft in damaged muscle 6. Grafted muscle assists resident cells in repair	Process 1. Develop appropriate functionalised scaffold 2. Harvest stem cells from muscle biopsy 3. Seed cells on scaffold 4. Implant scaffold in damaged muscle 5. Implanted cells and tissue resident cells regenerate muscle	Process 1. Develop appropriate functionalised scaffold to stimulate optimal myogenic response 2. Implant scaffold in damaged muscle 3. Tissue resident muscle cells are stimulated to regenerate muscle

environment, satellite cells are influenced by a combination of signals – including those from the host muscle fibre, the circulatory system and the extracellular matrix (ECM) (7). Optimal skeletal muscle tissue engineering will only be achieved when it is possible to develop artificial, 3D microenvironments that recapitulate the natural environment, at least to some extent.

Scaffolds in Skeletal Muscle Tissue Engineering

Finding a suitable matrix to support skeletal muscle tissue engineering remains one of the main obstacles to success. An ideal skeletal muscle scaffold should be biocompatible, biodegradable (producing non-toxic degradation products), porous and have the correct balance of stability and elasticity (8,9). Scaffolds should also mimic the ECM of skeletal muscle, thereby increasing the probability that surrounding cells will be stimulated to proliferate, migrate and differentiate to produce new, functional tissue (5).

Currently, there are limited materials available that meet the criteria above and are also easy to manufacture into a suitable construct. A range of biodegradable and non-biodegradable materials have been developed and tested in skeletal muscle tissue engineering applications. These include natural materials such as collagen, fibronectin, elastin, fibrin, hyaluronin and laminin (5). In addition to these purified natural products, heterogeneous natural ECM such as that derived from de-cellularised porcine small intestinal submucosa has been used to repair muscle defects in abdominal wall and load-bearing skeletal muscle with some success (10,11). Synthetic materials that have been investigated in skeletal muscle applications include silicones and polymers (e.g., polylactic acid, polyglycolic acid and polycaprolactone) and their copolymers (e.g., poly(lactide-co-glycolide)) (5,7).

Natural scaffolds have a number of advantages over their synthetic counterparts, such as their excellent physiological activities, mechanical properties similar to natural tissues and biodegradability. In the case of small intestinal submucosa scaffolds, these also retain a proportion of their naturally occurring bioactive molecules, such as growth factors and cytokines. However, disadvantages include the risk of infection and antigenicity from using animal-derived products, a potentially unstable material supply and the cost of production. Synthetic polymers, on the other hand, can be manufactured reproducibly and cheaply. However, they lack cell-recognition signals and do not support optimal cell adhesion, migration and proliferation without some form of chemical modification. Thus, the best approach is likely to be the use of synthetic materials modified to contain cell recognition sites and binding sites for appropriate growth factors and other bioactive molecules.

Investigating Cell-Scaffold Interactions

Although the ultimate success of a skeletal muscle scaffold lies in its ability to support growth and repair *in vivo*, much work remains to be done *in vitro* to develop the optimal artificial niche. Thus, recent methodological

advances in skeletal muscle tissue engineering (in addition to increasingly sophisticated scaffold design) include *in vitro* techniques such as the development of serum-free culture, sensitive methods to measure cell proliferation, differentiation and force generation, and the ability to observe cell-scaffold interactions at a molecular level.

Serum-free culture

Established tissue culture techniques use foetal bovine and horse sera to promote proliferation and differentiation of myoblasts, respectively. However, these sera provide an undefined mixture of growth factors. By growing cells in a completely defined, serum-free system, and supplementing with known quantities of specific growth factors, it is possible to analyse the effect of specific ECM molecules, cytokines and growth factors on myoblast growth and differentiation.

Quantitative Real-time Polymerase Chain Reaction

Cell proliferation is routinely assessed using a variety of metabolic and DNA-based assays. Similarly, myoblast differentiation can be assessed via measuring levels of creatine kinase, or by counting differentiated myotubes versus undifferentiated myoblasts, which can be distinguished by immunostaining (differentiation index). Recently, these approaches have been complemented by qRT-PCR of gene markers for myoblast proliferation and differentiation, such as insulin-like growth factor 1, skeletal muscle alpha 1 actin, myogenin and embryonic skeletal muscle myosin heavy chain 3 (12). The early measurement of differentiation via these PCR markers increases the sensitivity with which we can assess the efficacy of novel scaffolds and cell growth conditions.

Atomic Force Microscopy

Visualisation of 3D cell-scaffold interactions presents a challenge to the standard light and confocal scanning microscopy methods most often used by cell biologists. In fact, to better understand the role of scaffolds in muscle repair and regeneration, it is important to elucidate the 3D ultrastructure of these materials, as well as map cell-scaffold interactions at nanometre levels. For decades, electron microscopy (EM) has been used to visualise such ultrastructure, but routine EM methods irreversibly denature biomolecules during both specimen preparation (harsh chemical fixation procedures) and imaging (electron beam interactions) (13).

Atomic force microscopy, an alternative to EM, was invented in the mid-1980s. Atomic force microscopes (AFMs) belong to a class of scanning probe microscopes that record interactions between a probe and a surface. A typical AFM consists of a silicon or silicon nitride cantilever with a sharp tip (probe) at the end to scan a specimen surface. When the tip scans the surface, molecular interactions between the tip and the surface cause a deflection in the cantilever which is measured by an array of photodiodes. Since AFMs operate in physiologically appropriate solution conditions and perform quantitative, non-destructive, high resolution imaging (14), they have obvious advantages over EM.

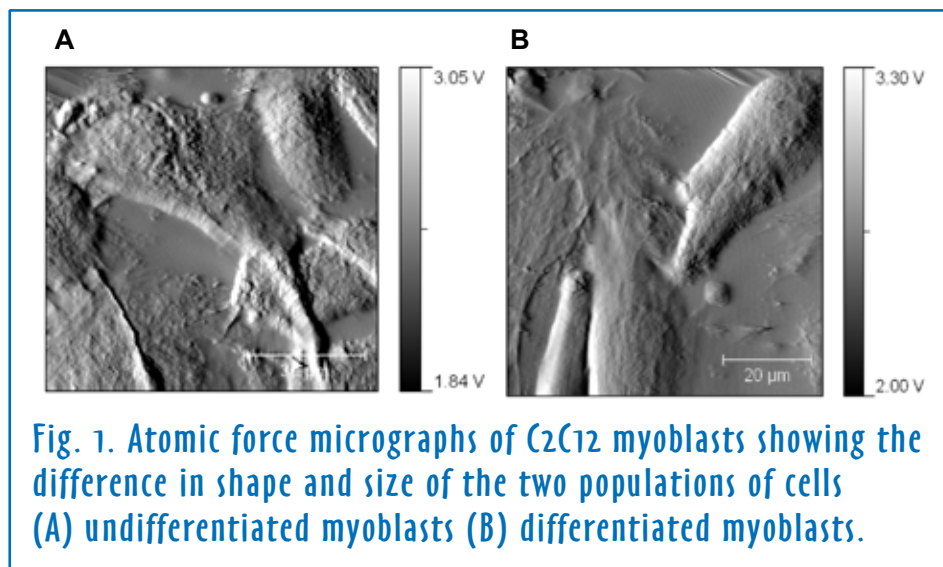


Fig. 1. Atomic force micrographs of C2C12 myoblasts showing the difference in shape and size of the two populations of cells (A) undifferentiated myoblasts (B) differentiated myoblasts.

processes, have generated scaffolds that contain nanofibrous polymer fibres that physically resemble the fibres and filaments of a natural ECM. These developments, coupled with improvements in defined cell culture media and imaging methods like atomic force microscopy, have positioned the field of regenerative medicine for big advances in the near future. Much of the *in vitro* work necessary to assess scaffolds produced by the chemists and chemical engineers for muscle regenerative medicine applications has been constrained by difficulties with imaging and with the lack of definition of cell culture media.

Atomic force microscopy can be used to characterise the topography of both synthetic and natural scaffold materials, and the interaction of cells with these scaffolds (15) (Fig. 1). Graham *et al.* (16) recently developed a new method using cryopreservation and cryosectioning of tissue samples to preserve and visualise bio-molecular structures (in particular ECM) *in situ*. Using this technique, it was possible to visualise the macromolecular structures of fibrillar collagens (skin, cartilage and intervertebral disc), elastic fibres (aorta and lung), desmosomes and mitochondria. In the last five years, it has also become possible to combine the high-resolution topographical imaging capabilities of an atomic force microscope with the optical imaging abilities of a laser scanning confocal microscope. Kassies *et al.* (17) coined the term AFFM (combined AFM and fluorescence microscope) for these instruments, which can produce single cell fluorescent images combined with atomic resolution and enable researchers to relate functional properties to morphological features of imaged cells.

However, the latest advance in atomic force microscopy, which is arguably even more impressive than AFFM, is known as simultaneous topography and receptor imaging (TREC). Whereas normal AFM images yield information only about the shape and volume of the molecule being imaged, TREC generates single molecule maps of specific types of molecules in a complex sample while simultaneously carrying out high-resolution topography imaging (18). This approach can be used to investigate receptor binding sites and their spatial distribution on natural biological surfaces. In this mode, an antibody is bound to the AFM tip, which then functions as a molecular sensor. A highly developed electronic feedback loop then allows sample topography measurement with simultaneous mapping of antibody recognition sites on the surface of the sample. We envisage this method being particularly useful for analyses of both natural muscle ECM as well as synthetic scaffolds that have been modified, with ECM proteins for example, to enhance their functional attributes.

Conclusion

Recent technological advances in the materials used for scaffolds, as well as scaffold preparation by electrospinning

Advances in adapting scaffolds to handle the force required for supporting the repair of damaged muscle tissue and in functionalising scaffolds with molecular motifs that facilitate cell adhesion, cell growth and differentiation, will change the way major muscle injuries are managed.

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