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Muscle-based gene therapy and tissue engineering for the musculoskeletal system

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The recent expansion of molecular biology techniques has opened the gates for a rapid advancement in our knowledge of disease mechanisms. These techniques, in addition to advances in cell biology and polymer chemistry, are resulting in novel approaches to treating musculoskeletal disorders. Surgeons, who have traditionally used the tools of excision and reconstruction to treat patients, might now serve as surgical 'gardeners', who create microenvironments that are conducive for tissue regeneration. This review will update readers on the principles and current advances in muscle-based gene therapy and tissue engineering for the musculoskeletal system.

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▼ Tissue engineering refers to the science of creating living tissue to either replace, repair or augment diseased tissue. The engineered tissue can either be created *in vitro* and subsequently implanted into the patient, or the tissue can be created entirely *in vivo*. Regardless of the technique, tissue engineering requires at least three components: a growth-inducing stimulus, a scaffold to support tissue formation and responsive cells (Fig. 1).

Growth factors and gene therapy

Growth factors are soluble proteins that promote cell division, maturation and differentiation. Recombinant DNA techniques have increased our understanding of the function of these proteins, some of which influence bone and cartilage formation *in vivo*. These techniques have also enabled the production of large quantities of growth factors for use in muscle, bone and cartilage research. Much interest is currently being placed on identifying the optimal growth factor for a particular application. In addition, once identified, the optimal method for delivering the growth factor to the surgical microenvironment must be determined.

The most obvious means of delivering growth factors *in situ* is direct application of the recombinant protein. In the past 36 years, since the discovery of the bone morphogenetic proteins (BMPs), researchers have demonstrated that one of the most important factors for *in vivo* healing is the presence of high and sustained levels of growth factor. Most growth factors are rapidly cleared and have half-lives of minutes[1]. Even when the growth factor is bound to a collagen scaffold, its half-life is limited to several days. Additionally, although microgram doses are sufficient for the *in vitro* manipulation of cells, doses required for *in vivo* regional regeneration are typically in milligram quantities. Researchers have, therefore, turned to regional gene therapy for the sustained delivery of growth factor to a local site *in vivo*.

One gene therapy approach is the transfer of a gene encoding the desired growth factor into cells using viral or non-viral vectors; the transduced cells subsequently secrete the desired protein into the microenvironment. Gene therapy can take two forms: *in vivo* (direct) and *ex vivo* (Fig. 2). The *in vivo* approach consists of directly injecting or implanting the gene–vector construct into the patient; this approach is attractive because of its technical simplicity. However, it is limited by the inability to perform *in vitro* safety testing on the transduced cells. The alternative *ex vivo* approach consists of isolating cells from a tissue biopsy. The cells are then grown in culture and transfected or transduced *in vitro*.

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The genetically altered cells can then be tested *in vitro* both for successful gene transfer and abnormal behavior before their introduction into the patient.

To facilitate clinically applicable gene therapy, it is necessary not only to delineate the protein needed for proper tissue function and identify its gene, but also to be able to reproducibly deliver it in a durable manner to the diseased or injured tissue. Muscle cells have emerged as a promising vehicle for gene therapy and tissue engineering for the musculoskeletal system (Fig. 3).

Muscle-derived stem cells

Several attributes make muscle an ideal tissue for tissue engineering:

(1) A muscle biopsy or tissue harvest during early surgical procedures or during primary care visits is relatively easy and can be repeated without compromising the patient's health.

(2) Muscle-derived cells tolerate *ex vivo* manipulation well, and millions of muscle-derived cells can be cultured quickly (within 1 week)[2,3].

(3) Upon re-injection *in vivo*, muscle-derived cells naturally fuse to form multinucleated, post-mitotic myotubes that do not undergo cellular turnover[4]. Most bone defects are surrounded by muscle, so the injected muscle-derived cells have a natural myogenic milieu on which to fuse. These myotubes can potentially express the protein of interest in overabundance by their fusion into larger, multinucleated myotubes and myofibers.

(4) Muscle tissue contains inducible osteoprogenitor cells[2,4].

(5) Muscle cells are easily transducible by a variety of viral vectors[5–9].

Because of these characteristics, muscle cells have been used extensively as a vehicle for gene therapy in multiple muscle-related diseases, such as Duchenne muscular dystrophy[9–13]. They have also been used in many non-muscle-related gene therapy applications: transfer and expression of factor IX for hemophilia B (Ref. 14), systemic delivery of human growth hormone for growth retardation[15], gene delivery of human adenosine deaminase for the adenosine deaminase deficiency syndrome[16]; gene transfer of human proinsulin for diabetes mellitus[17]; expression of tyrosine hydroxylase for Parkinson's disease[18]; expression of FasL to prevent immunorejection of pancreatic islet cell transplants[19]; and the injection of muscle cells into the joint, including the meniscus, synovium and ligament, for the treatment for arthritis and improvement of intra-articular tissue healing[20–22].

It is known, however, that whole muscle is composed of a heterogeneous population of cell types. Skeletal muscle contains satellite cells – the myogenic precursor cells. These cells are resting, mononucleated precursor cells that are capable of fusing to form post-mitotic, multinucleated myotubes and myofibers. Skeletal muscle can also contain stem cells.

Stem cells are unique in their characteristic capacity for protracted renewal, production of daughter cells (which can proceed towards lineage commitment and terminal

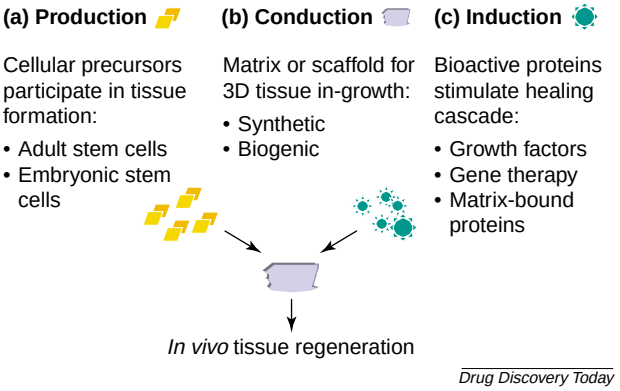

Figure 1. Components of the tissue engineering model include **(a)** cellular precursors, which participate in tissue production, **(b)** a matrix or scaffold to provide structural support for tissue in-growth, and **(c)** bioactive proteins or growth factors, which induce the tissue regenerative cascade.

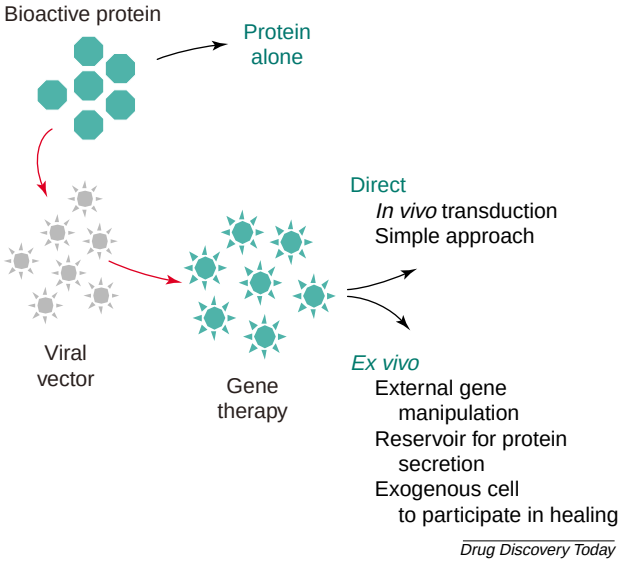

Figure 2. Bioactive proteins can be delivered to tissues via gene therapy, using either a direct *in vivo* approach or an *ex vivo* approach. The direct method places the gene–vector construct directly into the patient. In the *ex vivo* approach, allogenic or autologous cells are first isolated in culture and then transduced *in vitro* before their reintroduction into the patient.

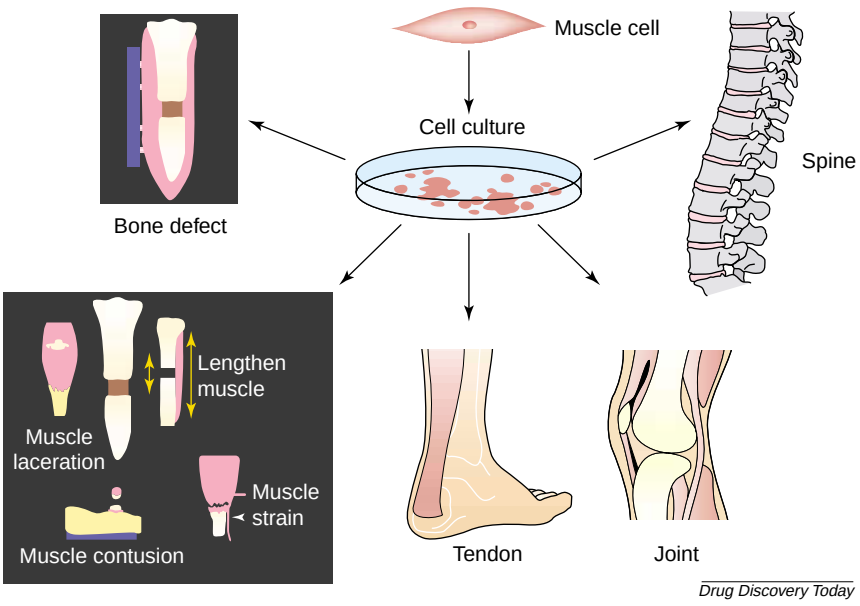

Figure 3. Muscle cells have been used as both the cellular precursors and cellular vehicles for bioactive protein delivery in many musculoskeletal research applications, including muscle regeneration and repair, tendon, meniscus and fracture healing, and spinal fusion.

differentiation) and multipotency, as well as the ability to persist in these activities throughout life[4,23–26]. Accordingly, muscle-derived stem cells produce populations of daughter cells that are capable of terminal differentiation into muscle cells, which in turn can differentiate into myofibers, while a subset remains undifferentiated[23]. These stem cells do not express markers of mature muscle tissue, are capable of prolonged production of progeny, and are multipotent[4,23–25]. There are relatively few muscle-derived stem cells in comparison with the amount of committed satellite cells within a given muscle[4].

A specific population of highly purified muscle-derived stem cells (MC13 cells) has recently been identified that express both early myogenic markers, such as desmin, c-met, myocyte nuclear factor (MNF) and Bcl-2, in addition to stem-cell markers, such as Sca-1, Flk-1, and CD34 (Ref. 4). *In vitro* and *in vivo*, MC13 cells were able to fuse and form multinucleated myotubes. When stimulated *in vitro* with BMP-2 and BMP-4, these cells show marked morphological changes and express osteocalcin and alkaline phosphatase, suggesting their differentiation into osteoblasts[4].

The use of muscle-based tissue engineering has potential for healing in both the soft tissues and bony structures of the musculoskeletal system. This review will focus on the application of muscle-derived cells for bone healing. In addition, the limited use of muscle-derived cells for skeletal muscle disease and articular disorders will also be discussed.

Bone healing

The problem of bone healing

Fracture healing remains a fundamental problem in orthopaedic surgery. Although the majority of fractures heal well, difficulty in the form of delayed healing or non-union of fracture can be devastating. Fractures in anatomically compromised locations (e.g. talar neck or scaphoid), those with inadequate fixation or infection, those resulting from high-energy-type injuries with soft tissue stripping or segmental bone loss, or those occurring in poor bone-stock (i.e. osteoporosis) often result in delayed healing. In a recent review, 5–10% of the 5.6 million annual fractures in the USA proceeded to delayed or impaired healing[27]. Treatment options for the orthopedic surgeon confronting these complex fractures, especially those that involve segmental bone loss, include bone autograft, vascularized bone-grafting, allograft supplemented with osteogenic proteins, bone transport or amputation[27–30]. Unfortunately, patients generally endure a lengthy recovery with numerous procedures, potential donor-site morbidity and often an unsatisfactory end result[30]. Similar difficulties occur with closure of the residual bony defects in craniofacial reconstruction, and oncology and trauma surgery. Consequently, research directed towards improving fracture treatment and the development of optimal bone-substitutes remains an area of intense interest.

Current approaches

A variety of bone grafts and implants has been used in orthopedic surgery; however, present bone substitutes do not behave physiologically or mechanically like true bone. For optimal bone healing, the milieu must contain osteoconductive, osteoinductive and osteogenic elements[4]. Scaffolds provide the platform for bone in-growth and satisfy the osteoconductive requirement. Osteoinductive factors trigger osteoprecursors, which are stem cells in the microenvironment, to initiate the healing cascade of chemotaxis, mitosis and differentiation[31]. Growth factors and other bioactive proteins usually play this role[31]. Finally, osteogenic elements, such as the native osteoprecursors or muscle-derived stem cells[3], directly participate in bone formation after stimulation by the osteoinductive factors, by manufacturing osteoid in the osteoconductive scaffold.

To date, the most successful bone-grafting material is autogenous cancellous bone. This substitute has osteogenic, osteoconductive and osteoinductive properties, because it contains surviving osteogenic cells, bone collagen, bone mineral, BMPs and growth factors. However, the use of autogenous bone grafts has many shortcomings: patients often experience significant pain, infection and blood loss at the harvest site and a maximum failure rate of 15% is usually expected[32,33]. Other approaches with synthetic materials, including metal prostheses, calcium-phosphate-based ceramics and pastes, methyl methacrylate constructs, and polymers, have been useful in a limited manner. The overwhelming shortcomings of these materials are their collective inability to integrate into surrounding tissues and their inability to become vascularized. Integration is important for long-term functional outcomes, as the structure of a patient's tissue is dynamic and changes with time.

Muscle-derived stem cells in bone healing

As discussed in previous sections, one of the three requirements for bone healing is an osteoconductive agent or scaffold. Normally, a given tissue is composed of several different cell types organized in a hierarchical structure. If dissociated, cells can reorganize on small levels but need a supportive scaffold to form larger, organized structures. Such scaffolds provide the platform for bone in-growth and incremental graft replacement. We have documented the ability of injected muscle-derived stem cells to fuse into myotubes and myofibers *in vivo* after injection into muscles.

Osteoinductive factors trigger osteoprecursors or pluripotent stem cells in the microenvironment to initiate the healing cascade of chemotaxis, mitosis and differentiation. Numerous studies have documented the ability of muscle-derived stem cells not only to be easily transduced by a variety of viral vectors expressing BMPs, but also to efficiently deliver biologically active doses of these proteins to the fracture site[2–4,34]. Indeed, it has been shown that muscle-derived cells were capable of producing between 444 and 2557 ng of BMP over a three-day period after stable transduction[34]. These cells, therefore, act in an osteoinductive manner as triggers of pluripotent cells in the microenvironment. In addition, and perhaps more interestingly, they can participate in the bone-healing process by differentiating into osteoblasts themselves[4]. Therefore, once transduced with a BMP-expressing vector, this cytoarchitecture serves as a reservoir for the secretion of bone growth-factors, the osteoprogenitor targets for the secreted protein, and as a matrix for bone formation.

Day and colleagues found that muscle-derived stem cells carrying a reporter gene were capable of mediating gene transfer to bone defects[35]. Muscle cell populations were then isolated from mice using the preplate technique[5], which has been previously used to purify myogenic cells[36]. This capitalizes on the different abilities of muscle-derived cells to adhere to type I collagen-coated flasks. In preplate 6, the cells' ability to express alkaline phosphatase after exposure to BMP-2 protein increased in a dose-dependent manner, whereas the percentage of desmin-positive cells decreased[2,4,36]. Thus, muscle consists of a heterogeneous collection of cells, including osteoprogenitor cells[2]. Bosch and coworkers then examined the ability of these muscle-derived cells to produce bone: after transduction with adenovirus-BMP-2 (AdBMP-2), cells were injected into the triceps surae of immunocompromised (severe combined immunodeficient, SCID) mice, where they induced heterotopic bone formation within two weeks. It was estimated that transduced cells *in vitro* could produce nanogram quantities of BMP-2 (Ref. 37). The muscle-cell fate was followed by cotransduction of a *lac*Z retroviral vector, and preplate 6 cells were found both at the osteoid edge and within lacunae, suggesting that the cells had become both osteoblastic and osteocytic in nature[2,38]. When co-localized using *lac*Z and osteocalcin immunohistochemistry, they were found to be capable of expressing bone protein[2,38].

Musgrave and colleagues continued this line of research and found the following: that AdBMP-2 produced bone when injected directly into skeletal muscle[39]; that human muscle cells, isolated by the preplate technique, could also deliver BMP-2 *ex vivo* and stimulate heterotopic bone formation[34]; and that although many tissue-derived cells were capable of responding to BMP-2 by producing bone, the optimal cell populations for production of ectopic bone were a bone-marrow-derived cell line (OIMC) and muscle-derived cells[3,37].

As mentioned previously, a subclone of the muscle-derived cells found in preplate 6 has been shown to express stem-cell markers, including Sca-1, FLK and CD34 (Ref. 4). When transduced with AdBMP-2 and placed into a crucial-sized calvarial defect in immunodeficient mice, they achieved >85% closure of the defect within two weeks and 95–100% closure by four weeks[4].

To apply this technology to clinical situations, however, the muscle stem cell–vector–growth-factor construct must induce bone formation and healing in immunocompetent animals. Recently, muscle-derived stem cells transduced with a retroviral vector encoding BMP-4 have been used to produce ectopic bone formation and stimulate bone healing in normal immunocompetent animals (Wright *et al.*, pers. commun.).

Skeletal muscle

Both direct and *ex vivo* gene therapy to skeletal muscle for inherited diseases, such as Duchenne muscular dystrophy,

have long been investigated[6–12]. Clinical trials using autologous myoblast transplantation for Duchenne muscular dystrophy have previously proven safe[13], although myoblast survival is often sub-optimal for inherited muscle diseases[5]. However, myoblast transplantation combined with *ex vivo* gene therapy is an attractive approach to deliver appropriate growth factors and responsive cells for acquired traumatic muscle injuries. The ongoing discovery of novel growth factors could facilitate gene therapy approaches to improve acquired muscle-injury healing. Insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) have been demonstrated to improve muscle healing and strength (fast-twitch or tetanic strength) in various rodent models of injury including contusion, laceration and strain[40–42].

Articular disorders

Multiple articular disorders are candidates for gene therapy and tissue engineering applications. Novel approaches to treating arthritis, chondral and osteochondral defects, miniscal tears and ligaments are being investigated.

Cartilage

Cartilage defects and progressive osteoarthritis are among the most frequent, yet challenging conditions in orthopedics. In contrast to the healing capacity of bone, articular cartilage heals poorly. Current repair techniques include cartilage debridement and resurfacing[43,44], subchondral drilling[45], arthroscopic abrasion[46,47] and microfracture[48]. Common to these techniques is the disruption of subchondral bone to allow osteochondral progenitors from the marrow to gain access to, and participate in, repair of the cartilage defect. The repaired cartilage, however, is structurally inferior to native cartilage and contains significantly less proteoglycan[49]. More recent strategies for cartilage repair seek to provide an alternative source of cells to stimulate healing. These have included the use of autologous chondrocyte transplantation[50] and transplantation of cartilage plugs[51]. Although adult and embryonic chondrocytes can be used for transplantation, the limiting factor is chondrocyte supply. Other researchers have tried to overcome this limitation by generating chondrocytes from mesenchymal stem cells[52]. These cells can be induced to become osteochondral progenitor cells in culture before reimplantation.

Finally, muscle-derived cells are easily accessible, and their potential for repairing articular defects has been explored. When transplanted into full-thickness articular defects in rabbits, muscle-derived cells improved the healing of the defects with an efficiency equivalent to that of chondrocyte cartilage transplantation (Adachi *et al.*, unpublished).

Meniscus

Meniscal injuries represent an attractive application for gene therapy and tissue engineering. The possibility of creating a custom replacement meniscus *in vitro* using scaffolds, cells and gene therapy for subsequent *in vivo* implantation is intriguing. Alternatively, meniscal cells could be modulated *in vivo* using gene therapy to promote healing of certain injuries. Meniscal cells are amenable to the gene transfer of both marker genes and various growth-factor genes, using either direct or *ex vivo* gene therapy, with gene expression persisting for up to six weeks[53]. Successful *ex vivo* gene transfer has been accomplished using either muscle-derived stem cells[21] or meniscal cells[53]. Research is ongoing into the preferred growth factors to promote meniscal healing, techniques to improve long-term gene expression, and the optimal scaffold needed to create *de novo* menisci.

Ligaments

Gene therapy techniques are also being applied to ligaments. *Lac*Z gene transfer to ligament has proven feasible in animal models using either the direct or the *ex vivo* approach, using both adenovirus and retrovirus. *Ex vivo* gene transfer to ligaments has been successfully achieved using either ligament fibroblasts or skeletal muscle-derived cells[22]. Many growth factors, such as bFGF, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), IGF-1 and -2, transforming growth factor-β (TGF-β) and BMP-12, might play roles in ligament healing. Data suggest that PDGF stimulates cell division and migration, whereas TGF-β and the IGFs promote extracellular matrix synthesis[22]. Direct gene-transfer of PDGF-β, using a viral–liposome conjugate vector, into rat patellar ligament resulted in initial improved angiogenesis and subsequent enhanced extracellular matrix synthesis[54].

Summary

In this review, we have summarized one segment of tissue engineering for the musculoskeletal system. To bring tissue engineering technology to clinical fruition, however, it is necessary to collaborate in a multidisciplinary manner to identify and optimize the adequate cell, growth factor and scaffold construct for each application. Future work should focus on the use of stem cells, of both adult and embryonic origins, in autologous, allogeneic and perhaps xenogeneic settings. Details of the cells' proliferative potential, genetic engineering and immunogenic potential should be explored. In addition, researchers must learn to combine stem cells with growth factors in a viable spatiotemporal relationship in order to recreate the *in situ* microenvironment. The potential for regeneration of tissue

for healing the musculoskeletal system is vast, with much work still to be accomplished.

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