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Muscle-based tissue engineering for the musculoskeletal system

Review Article

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Abbreviations: DMD, Duchenne muscular dystrophy; **AAV**, adeno-associated virus; **BMPs**, bone morphogenetic proteins; **rhBMP**, recombinant human BMP; **TGF-β**, transforming growth factor-β; **ACL**, anterior cruciate ligament

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Summary

Somatic gene therapy through the transfer of genes into a particular tissue to alleviate a biochemical deficiency has emerged as a novel and exciting form of molecular medicine. Due to a number of factors, muscle tissue has emerged as a promising target for muscle based gene therapy and tissue engineering. First, many muscle groups are readily accessible and tolerate delivery by injection well. Second, muscle is composed of multinucleated, post-mitotic myofibers and may facilitate high and long term persistence of transgene expression. Third, muscle can be easily and repeatedly biopsied without compromising the health and function of human and animal subjects. Finally, muscle is very well vascularized, making systemic delivery through the bloodstream feasible. Based on these unique features of the skeletal muscle, we have described four different applications of muscle based gene therapy and tissue engineering: inherited muscle diseases, muscle injury and repair, bone healing and finally intra-articular disorders. Since the field of muscle based gene therapy and tissue engineering has expanded and matured over the last few years, we will review some hurdles facing the practical application of this technology as well as potential approaches to circumvent these limitations to eventually apply this technology to the treatment of pathologies and conditions of the musculoskeletal system.

I. Introduction

The advent of gene therapy and tissue engineering has facilitated novel approaches to the treatment of musculoskeletal disorders. The delivery of growth factors, cells, and therapeutic genes promises to revolutionize a medical field historically limited to biomechanical approaches. Significant scientific contributions have been made in the last three decades toward the understanding of skeletal muscle biology and its potential therapeutic applications. However, despite the tremendous progress, many questions currently remain unanswered. This paper reviews the current status of muscle-based tissue engineering for musculoskeletal disorders and discusses the focus of ongoing research.

As the molecular basis of an expanding number of inherited disorders has been discovered, increasing focus has been placed on gene therapy as a potential therapeutic approach. The transfer of a functional gene into a particular tissue has been explored in many disease systems using a variety of gene delivery approaches. Human inherited disorders of muscle are not uncommon diseases of childhood. Hence, skeletal muscle has been studied as a target tissue for the delivery of genes encoding proteins that may be therapeutic for inherited muscle disorders. However, since the multinucleated and post-mitotic myofibers in

skeletal muscle are capable of both long-term transgene expression and systemic delivery of proteins to the blood circulation, direct and *ex vivo* gene transfer to skeletal muscle has also been investigated as a means to create a tissue reservoir for the secretion of non-muscle proteins (Dhawan et al., 1991; Dai et al., 1992; Lynch et al., 1992; Jiao et al., 1993; Simonsen et al., 1996; Lau et al., 1996; Bosch et al., 1998; Musgrave et al., 1998).

The direct gene therapy approach, albeit technically straightforward, presents theoretical risks of *in vivo* genetic manipulation and possible reversion to pathogenicity of attenuated viral vectors. Furthermore, the direct approach does not provide for the introduction of cells capable of participating in the healing response. The *ex vivo* approach addresses these issues by limiting genetic manipulation of the cells to the culture flask thereby eliminating the potential risks of *in vivo* genetic manipulation and viral reversion. The *ex vivo* approach also allows for isolation and expansion of muscle-derived cells possibly capable of participating in the therapeutic process. Evidence exists that suggests muscle-derived cells can participate in both muscle and bone healing (Urist, 1965; Huard et al., 1992a,b; 1994a,b; Bosch et al., 1998). Finally, myoblast transplantation is a clinically feasible approach to delivering competent cells with complementary genomes to patients with inherited muscle diseases such as Duchenne muscular dystrophy (Huard et al, 1992a,b; 1994a,b). Therefore, current muscle-based tissue engineering approaches are aimed at both inherited and acquired musculoskeletal disorders.

The theory behind muscle-based tissue engineering is predicated on the unique biology of skeletal muscle derived cells. First, as discussed below, skeletal muscle contains satellite cells. These cells are resting mononucleated precursor cells capable of fusing to form post-mitotic, multinucleated myotubes and myofibers. The post-mitotic, multinucleated myofibers are stable cells theoretically capable of persistent gene expression. Therefore, by focusing tissue engineering approaches on the satellite cell, one may be capable of maximizing the degree and persistence of gene expression. Second, as alluded to earlier, skeletal muscle may contain a population of mesenchymal stem cells. Mesenchymal stem cells are resting cells capable of differentiation into several different lineages (Caplan, 1991). *In vitro* (Katagiri et al., 1994; Young et al., 1995; Warejcka et al., 1996) and *in vivo* (Bosch et al., 1998) data suggest cells residing within skeletal muscle are capable of differentiation into several different tissue lineages. Consequently, muscle-derived cells may be capable of regenerating many different tissues. Tissue engineering based on these cells not only facilitates gene delivery but may also supply the needed stem cells for healing. Finally, muscle-derived cells are clinically accessible and reliably isolated. Skeletal muscle biopsies are of low morbidity and available on an outpatient basis. Furthermore, *in vitro* isolation of muscle-derived cells has been well described (Blau and Webster, 1981; Rando and Blau, 1994; Qu et al., 1998). Based on these unique characteristics, the field of skeletal muscle-derived cells in muscle-based tissue engineering is burgeoning.

In this chapter, we summarize muscle based tissue engineering applications for the musculoskeletal system including inherited muscle disease (Duchenne Muscular Dystrophy), muscle injuries and repair, bone healing, and intraarticular disorders.

II. Muscle based gene therapy for inherited muscle diseases

The muscular dystrophies − which were the first target for gene therapy to skeletal muscle − are characterized by progressive muscle wasting and weakness. Duchenne muscular dystrophy (DMD), inherited on the X chromosome, is one of the most common and severe inherited myopathies. DMD is a devastating muscle disease characterized by a lack of dystrophin expression in the sarcolemma of muscle fibers (Hoffman et al., 1987; Arahata et al., 1988; Sugita et al., 1988; Zubryzcka-Gaarn et al., 1988).

Dystrophin, one of the largest known human genes, has a high frequency of mutation affecting 1 in 3,500 males. Dystrophin appears to function in the maintenance of muscle membrane integrity. Its absence in DMD muscle causes damage to the membrane during muscle contraction, resulting in eventual muscle fiber necrosis (Bonilla et al., 1988; Watkins et al., 1988; Menke et al., 1991). There is no treatment, and affected children die in their late teens of cardiac and respiratory failures. Because genetic testing and counseling does not dramatically lower the incidence of this disorder, it is crucial to develop therapeutic approaches to alleviate the muscle weakness in DMD patients. The ultimate goal of therapy for DMD is to provide enough dystrophin to the membrane cytoskeleton of the majority of the DMD muscle fibers to be therapeutically effective. Various approaches have been explored to transfer dystrophin into skeletal muscle, including myoblast transplantation and gene delivery based on non-viral vectors (direct DNA injection, liposome) and viral (retrovirus, adenovirus and herpes simplex virus) vectors.

A. Myoblast transplantation

Myoblast transplantation (MT) consists of the implantation of normal myoblasts into dystrophic muscles to create reservoirs of muscle cells capable of dystrophin expression (Watt et al., 1982; 1984; Morgan et al., 1988; 1990;1993; Allamedine et al., 1989; 1990; Law et al., 1988; Karpati et al., 1989; Partridge et al., 1989; 1991). MT in animal models, as well as in DMD patients, is capable of delivering dystrophin and occasionally improving muscle strength, but is hindered by immune barriers, poor dispersion

of the injected myoblasts, and rapid loss of the injected cells (Gussoni et al., 1992; Huard et al., 1992a,b; 1994a,b; Karpati et al., 1992; Tremblay et al., 1993a,b; Kinoshita et al., 1994; Vilquin et al., 1995a; Guerette et al 1997; Qu et al, 1998).

B. Gene therapy

Since the efficiency of gene therapy (GT) using naked DNA has been very limited (Acsadi et al., 1991; Danko et al., 1993; Davies et al., 1993), a virus-mediated gene delivery system may provide a promising alternative for dystrophin gene delivery. However, gene transfer via recombinant viral vectors has also been limited by numerous technical problems. Current retroviral vectors have not been found to transduce muscle fibers since they require dividing cells for integration and expression (Dunckley et al., 1992). However, an intermediate level of retroviral transduction occurs in immature and adult regenerating muscles which is likely due to myoblast mediation (Dunckley et al, 1992, 1993, van Deutekom et al, 1998a,b). Although adenoviral vectors can deliver genes to post-mitotic cells including myoblasts and newborn muscle fibers, the efficiency of gene transfer to mature muscle fibers is severely reduced (Quantin et al., 1992; Ragot et al., 1993; Vincent et al., 1993; Acsadi et al., 1994a,b, 1995; Huard et al 1995a, van Deutekom et al, 1998a,b). Moreover, gene delivery mediated by a first generation adenoviral vector induces immune responses to the vector, leading to rejection of the transduced cells (Smith et al., 1993; Engelhardt et al., 1994a,b; Yang et al., 1994a,b; Vilquin et al., 1995b).

It has been demonstrated that the replication-defective herpes simplex virus (HSV-1), which has been extensively used as a gene delivery vector to the central nervous system (Glorioso et al., 1992; 1994), can also be used as a gene delivery vector to skeletal muscle. HSV-1 efficiently infects myoblasts and myotubes *in vitro.* Furthermore, the intramuscular injection of the viral vector results in infection and transduction of a significant number of newborn mice muscle fibers and *some* adult mice muscle fibers (Huard et al., 1995b). However, limitations such as differential transducibility with HSV-1 throughout the maturation of muscle fibers, cytotoxicity, and immunological problems associated with HSV-1 (Huard et al., 1996, 1997a,b) have hindered the use of HSV-1 as a gene delivery vector to skeletal muscle.

C. Combination of myoblast transplantation and gene therapy

The idea behind this approach involves the establishment of a primary myoblast cell culture from mdx mice or DMD patients. After an adequate transfection or transduction with a dystrophin cDNA, these transduced myoblasts are reinjected into the same host to bypass immunological problems against the injected myoblasts. This approach, which permits the reintroduction of myoblasts expressing dystrophin, can be useful for DMD patients, especially for those over 10 years of age whose muscle regeneration has become inefficient due to a lack of viable satellite cells.

This method was performed using adenovirus, retrovirus, and HSV-1 carrying reporter genes (β−galactosidase or luciferase) and showed that transduced myoblasts (isogenic myoblasts) fused and reintroduced the reporter genes into the injected muscle, demonstrating the feasibility of the *ex vivo* approach (Salvatori et al, 1993; Rando et al, 1994; Huard et al., 1994c, Booth et al, 1997, van Deutekom et al, 1998a,b). We have recently observed that *ex vivo* gene transfer can deliver dystrophin in mdx (dystrophin deficient) muscle, but the immune responses against the transduced cells remain (Floyd et al., 1997, 1998).

D. Barriers to viral gene transfer of mature myofibers

Viral vectors which cannot transduce post-mitotic cells, such as retrovirus, are consequently incapable of directly infecting post-mitotic myofibers. However, adenovirus and HSV-1 can infect post-mitotic cells but still poorly transduce mature muscle fibers due to different mechanisms.

Our hypothesis is that adenoviral transduction of both immature and mature myofibers is mediated at least in part by fusion of infected myoblasts. Neonatal muscle is efficiently transduced due to continued fusion of myoblasts during muscle growth, while mature myofibers are not efficiently transduced due to a lack of myoblast fusion. Our experiments suggest that adenovirus requires transduction of myoblasts prior to fusion with myotubes or myofibers in order to transduce these differentiated muscle cells (van Deutekom et al., 1998a,b). By using pure cultures of myoblasts and myotubes, we have observed that adenovirus efficiently infects myoblasts but poorly infects myotubes. However, adenovirus transduces large numbers of mononucleated cells remaining in the differentiated muscle cell cultures. We have also shown that irradiation of newborn muscles prior to transduction inactivates myoblasts *in vivo* and significantly decreases the level of adenovirus transduction in neonatal myofibers *in vivo* (van Deutekom et al., 1998a,b). Alternatively, we have used isolated mature myofibers as a model to evaluate the efficiency of viral gene delivery *in vitro*. We have shown that the maturation-dependent loss of myofiber transducibility observed with adenovirus and HSV-1 is recapitulated in single muscle fibers *in vitro*, and thus is not solely due to host immune response (Feero et al, 1997). By using localized irradiation of muscle *in vivo* prior to isolation of myofibers, we observed that adenoviral infectivity of differentiated myofibers decreased significantly versus muscle fibers from non-irradiated muscles at the same stage of development.

These results suggest that adenoviral transduction in myofibers depends, at least in part, on myoblasts to mediate

myofiber transduction. The myoblast content of skeletal muscle decreases *in vivo* as a function of age: thus, the documented dramatic decrease in adenovirus infectivity of skeletal muscle in the post-natal period may be a consequence of reduced myoblast availability and/or fusion.

Recently, genetically modified adenoviral (ADV) vectors have been developed (Wickham et al., 1996) that express heparan sulfate directed targeting peptides at the end of fiber proteins in assembled virions (ADV PK). These viruses no longer bind cells via the native attachment receptor, yet they retain the ability to enter cells via internalization receptors (α_v β_3/β_5 integrin). The use of these new viruses will help to determine the role of the adenovirus' attachment receptor in the maturation-dependent adenoviral transduction of muscle fibers, since it has been proposed that the gradual loss of viral receptors is involved in the maturation-dependent adenoviral transduction of skeletal myofibers (Acsadi et al, 1994b). Our results, obtained in newborn and adult mouse skeletal muscle, indicate that despite the enhanced attachment characteristics, ADV PK remains hindered by both the protective extracellular matrix and diminished myoblast mediation in mature muscle (van Deutekom et al., 1998c).

On the other hand, HSV-1 is capable of infecting both myoblasts and myotubes with a similar efficiency *in vitro.* In addition, the irradiation of newborn muscle prior to HSV-1 infection does not significantly decrease HSV-1 transduction of myofibers *in vitro* and *in vivo* (van Deutekom et al., 1998a,b). Since HSV-1 is capable of transducing myotubes and newborn myofibers without myoblast transduction, we have performed experiments to characterize the cause(s) of the poor HSV-1 transduction in mature myofibers.

Preliminary data suggests that the poor level of HSV-1 transduction in mature myofibers is the consequence of the basal lamina maturation causing a physical block to virus accessibility and penetration (Huard et al., 1996, 1997a,b). In order to support this hypothesis, we have shown increased transduction efficiencies in adult myofibers from dy/dy mice (Huard et al, 1996). Dy/dy mice have defective basal lamina due to merosin deficiency. Moreover, isolated myofibers from adult dy/dy muscles (Soleus and EDL) were also found less refractory to HSV-1 transduction in contrast to that observed with age-matched control (dy/+) normal adult myofibers *in vitro* (Feero et al., 1997).

E. Approaches to circumvent the maturation-dependent viral transduction of muscle fibers

1. Artificial induction of muscle regeneration

Based on these results, it appears logical that artificial induction of muscle regeneration using agents which release satellite cells and promote myoblast proliferation and fusion may result in a higher level of transduction with adenovirus, retrovirus, and HSV-1 in mature myofibers. In support of this hypothesis, a higher level of viral transduction (adenovirus, retrovirus, HSV-1) has been observed in mature regenerating mdx muscle (Acsadi et al., 1994a, van Deutekom et al., 1998a,b). Different myonecrotic agents have been tested for their ability to specifically induce muscle regeneration and allow efficient viral transduction in mature muscle. We have observed that cardiotoxin treatment prior to adenoviral and retroviral transduction improves the efficiency of gene transfer in mature muscles (van Deutekom et al., 1998a,b). In order to determine whether the improved adenoviral transduction levels obtained in regenerating mature muscle were due to myoblast mediation, the presence of immature myofibers, or a combination of both, we irradiated regenerating muscle prior to adenoviral injection to inactivate the myoblasts. The irradiated muscles of the mice treated with cardiotoxin 2 and 3 days prior to adenoviral injection displayed a significantly decreased viral transduction in comparison to the non-irradiated muscles of the same mice (van Deutekom et al., 1998a,b). These low transduction levels suggested that the adenoviral transduction observed in the non-irradiated muscles of these mice was mainly due to myoblast-mediation. In contrast, the irradiated muscles of the mice treated with cardiotoxin 4 and 5 days prior to adenoviral injection did not show reduced transduction efficiencies, suggesting that the high adenoviral transduction levels were most likely due to the presence of immature myofibers.

2. Permeability of the basal lamina

Based on the hypothesis that the basal lamina acts as a physical barrier to viral injection in adult myofibers, we used agents that permeate the basal lamina prior to HSV-1 infection in an effort to achieve efficient transduction of adult myofibers. Different fenestrating agents, such as plasminogen activators and neutral proteases (streptokinase, urokinase), were tested to permeate the basal lamina and allow for HSV-1 to penetrate and transduce mature muscle fibers. This approach was first tested *in vitro* on mature muscle fibers isolated from adult mice, since *in vitro* muscle fibers represent a good model system for viral gene delivery to skeletal muscle (Feero et al., 1997). We observed that pre-treatment with streptokinase (5 units) and urokinase/plasminogen activator (10 units each) for 60 min. prior to HSV-1 infection in isolated myofibers (2-month-old) enhanced the level of HSV-1 transduction in mature isolated myofibers (van Deutekom et al., 1998a,b).

3. Common approaches to improve adenovirus and HSV-1 transduction in mature muscle

The maturation-dependent viral transduction with adenovirus and HSV-1 may be related at least in part by common mechanisms. Since we have observed that adenovirus displays a higher level of gene transfer in adult

dy/dy mice, it is likely that adenoviral penetration and transduction in mature myofibers is hindered at least partially by the basal lamina which acts as a physical barrier. As mentioned above, approaches to fenestrate the basal lamina may consequently allow for a better adenoviral transduction in mature myofibers. Using new mutant HSV-1 vectors which display a reduction in cytotoxicity to muscle cells, we have observed that an intermediate level of HSV-1 transduction occurs in regenerating muscle (van Deutekom et al., 1998a,b). This observation suggests that approaches which artificially release myoblasts in mature muscle may help achieve efficient transduction of HSV-1 in mature muscle. In fact, artificial induction of muscle regeneration with cardiotoxin improves HSV-1 transduction in mature muscle (van Deutekom et al, 1998a,b).

4. The myoblast-mediated ex vivo gene transfer approach

The *ex vivo* gene transfer may circumvent the inability of viral vector to transduce mature myofibers. The ability of adenovirus, retrovirus, and HSV-1 to efficiently transduce myoblasts can be used in an *ex vivo* approach. This approach consists of first transducing myoblasts *in vitro*, then transplanting them intramuscularly *in vivo*. We have achieved an efficient level of adenovirus, retrovirus, and HSV-1 transduction in mature muscle fibers using the *ex vivo* approach (van Deutekom et al., 1998a,b). In fact, a higher level of gene transfer was observed using the *ex vivo* approach than with the direct gene transfer using the same amount of viral particles (Booth et al., 1997). Although the poor survival of the injected myoblasts limits the efficiency of the myoblast-mediated *ex vivo* gene transfer of viral vectors in mature muscle, it has recently been found that the poor survival of the injected myoblasts is related at least in part to inflammatory reactions (Guerette et al., 1997; Qu et al., 1998). In an effort to bypass this limitation, myoblasts engineered to express molecules capable of expressing anti-inflammatory substances were used. Engineered myoblasts expressing interleukin 1 receptor antagonist protein (IRAP) were capable of improving the survival of the injected myoblast post-implantation (Qu et al., 1998). Furthermore, the use of specific populations of muscle-derived cells improves the cell survival after transplantation and consequently enhances the success of myoblast transplantation (Qu et al., 1998).

5. The use of new viral vectors

More recently, recombinant adeno-associated viral vectors (rAAV) have been used as gene delivery vehicles for skeletal muscle cells. Although a high efficiency of gene transfer occurs in mature muscle and a long term transgene expression of up to 18 months has been observed in mouse skeletal muscle (Kessler et al., 1996; Xiao et al., 1996; Reed Clark et al., 1997), the use of this viral vector will be limited by its restricted gene insert capacity (<5Kb). This is especially true in the field of DMD in which the dystrophin cDNA is 14 Kb. The identification of a new form of truncated dystrophin which displays a protection to the skeletal muscle fibers may eventually allow for its insertion into the AAV and consequently its delivery into dystrophic muscles.

A schematic representation of the maturation-dependent viral transduction of skeletal muscle (adenovirus, retrovirus, and HSV-1) and the aforementioned approaches to improve the viral transduction of mature skeletal muscle are presented in **Figure 1**.

III. Muscle injury and repair

Muscle injuries comprise a large percentage of recreational and competitive athletic injuries. Muscle injuries may result from both direct (contusions, lacerations) and indirect trauma (strains, ischemia and neurological injuries). Upon injury, satellite cells are released and activated in order to differentiate into myotubes and myofibers, thereby promoting muscle healing. However, this reparative process is usually incomplete and accompanied by a fibrous reaction producing scar tissue. This scar tissue limits the muscle's potential for functional recovery (Hurme et al., 1991, 1992).

Investigations in animals identified possible clinical applications for muscle-based tissue engineering to treat muscle injuries (Garrett et al., 1984, 1990). Animal models of muscle laceration, contusion, and strain currently exist (Jarvinen and Sorvari, 1975; Carlson and Faulkner, 1983; Garrett et al., 1984, 1990; Nikolaou et al., 1987; Taylor et al., 1993; Crisco et al., 1994; Hughes et al., 1995). We have developed reproducible orthopaedic muscle injuries in mice: Laceration is performed by incising 75% of the width and 50% of the thickness of the gastrocnemius muscle (Menetrey et al, 1998a,b). Contusion is created by dropping a 16 gram iron ball from a height of 100 centimeters (cm) onto the gastrocnemius muscle (Kasemkijwattana et al, 1998a,b). Strain is created by elongating the muscle-tendon unit at a rate of 1 cm/min (Kasemkijwattana et al 1998a,c). Under these conditions, muscle myofiber regeneration is found at 7 and 10 days after injury, but begins to decrease at 14 days and continues decreasing until 35 days. Concomitantly, fibrosis is observed beginning at 14 days and gradually increases until 35 days (Kasemkijwattana et al., 1998a,b,c; Menetrey et al., 1998a,b). Fibrosis appears at the time muscle regeneration diminishes and, therefore, appears to hinder the healing response.

Injured skeletal muscle releases numerous growth factors acting in autocrine and paracrine fashion to modulate muscle healing. These proteins activate satellite cells to proliferate

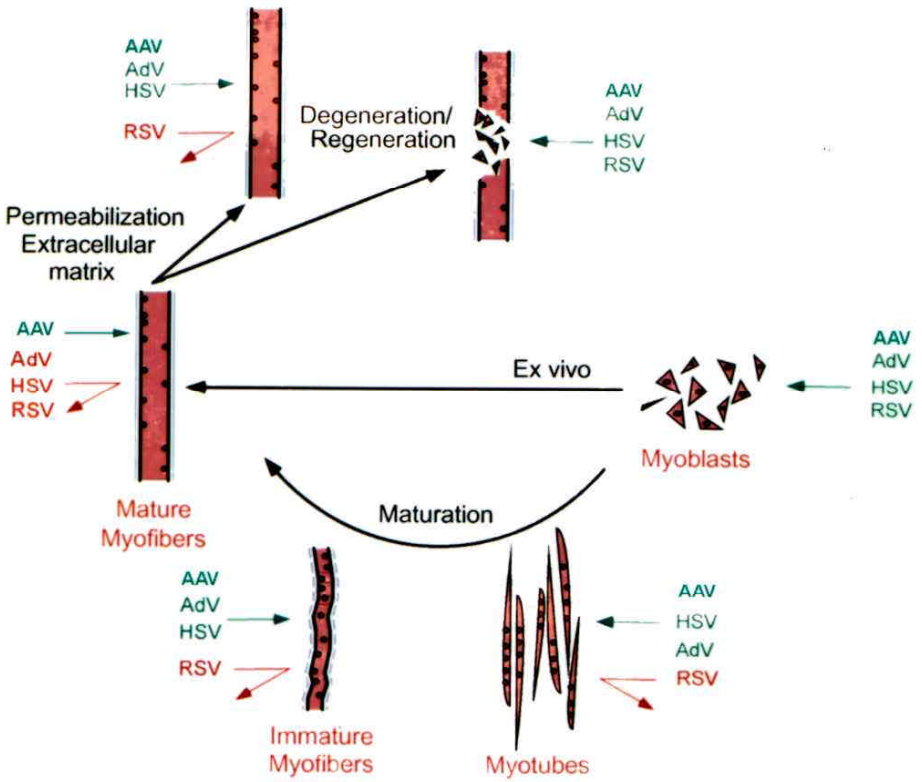

Figure 1. Schematic representation of retroviral (RSV), herpes simplex viral (HSV), adenoviral (AV), and adeno-associated virus (AAV) transduction of mature skeletal muscle, as well as approaches (the permeating of the extracellular matrix, the induction of degeneration/ regeneration, and the *ex vivo* strategy) to improve the viral transduction of mature skeletal muscle.

and differentiate into myofibers (Hurme, 1992; Bischoff, 1994; Allamedine et al., 1989; Schultz, 1985, 1989). The delivery of exogenous growth factors, specifically selected to enhance myofiber regeneration, is an intuitive therapeutic approach to muscle injuries. *In vitro* experiments have identified several growth factors capable of enhancing myogenic proliferation and differentiation (Kasemkijwattana et al., 1998a; Menetrey et al, 1998b). Satellite cell activity in cell culture was assessed at 48 and 96 hours after incubation in prospective growth factors. Basic fibroblast growth factor (b-FGF), insulin-like growth factor-1 (IGF-1), and nerve growth factor (NGF) significantly enhanced myoblast proliferation, whereas b-FGF, acidic fibroblast growth factor (a-FGF), IGF-1, and NGF increased myoblast differentiation into myotubes. Consequently, bFGF, IGF-1, and NGF are the logical candidates for therapeutic applications to enhance muscle healing (Kasemkijwattana et al., 1998a; Menetrey, et al., 1998b).

The technique chosen to deliver prospective growth factors to injured muscle is of paramount importance to optimize therapeutic benefit. Options include direct injection of growth factors, direct gene therapy, *ex vivo* gene therapy, and myoblast transplantation. Individual direct injections of b-FGF, IGF-1, and NGF into injured muscle (laceration, contusion, and strain) can increase the number of regenerating myofibers *in vivo* and increase both muscle twitch and tetanic strength 15 days after injury (Kasemkijwattana et al., 1998a,b,c; Menetrey et al., 1998b). However, secondary to rapid clearance and short half-lives, the effect of direct growth factor injections is likely transient and suboptimal. Gene therapy provides a mechanism to achieve persistent protein production and, thereby, theoretically improved muscle healing. Direct gene therapy to deliver genes to skeletal muscle is possible using naked DNA, retrovirus, adenovirus, herpes simplex virus and adeno-associated virus (see Section II). Most of these vectors transduce relatively few adult myofibers. However, adenovirus is capable of tranducing a large number of regenerating muscle fibers, a condition present in injured

muscle. Direct injection of adenovirus containing the beta-galactosidase marker gene into lacerated, contused, and strained muscle results in many transduced myofibers at 5 days (**Figure 2**). Therefore, direct injection of adenovirus carrying growth factor genes (i.e. bFGF, IGF-1, NGF) should result in sustained protein production in injured muscle. Recent data shows that direct injection of adeno-associated virus (AAV) results in a high level of adult myofiber transduction in both injured and non-injured muscle (Pruchnic et al, 1998). AAV may be the preferred vector for direct gene delivery to mature skeletal muscle, although it is capable of carrying genes of only 1-4 Kb.

Ex vivo gene therapy and myoblast transplantation are two closely related methods which require *in vitro* cell isolation and culture. *Ex vivo* techniques involve muscle biopsy and myogenic cell isolation (Rando and Blau, 1994; Qu et al, 1998). The isolated satellite cells are transduced *in vitro* with the desired gene carrying vector. The satellite cells are then reinjected into skeletal muscle, fuse to form post-mitotic myotubes and myofibers, and begin growth factor production. This technique is feasible with adenoviral (Huard et al., 1994c), retroviral (Salvatori et al., 1993), and herpes simplex viral vectors (Booth et al., 1997). *Ex vivo* delivery of the β-galactosidase marker gene to injured muscle produces many β-galactosidase-positive myofibers (**Figure 2**). The *ex vivo* muscle cell-mediated approach provides not only an efficient method of delivering selected genes, but also provides cells capable of participating in the reparative process, similar to myoblast transplantation. However, myoblast transplantation lacks *in vitro* genetic manipulations. In addition to its application toward inherited muscle diseases, myoblast transplantation is shown to improve myofiber regeneration in muscle experimentally injured with myonecrotic agents (Huard et al., 1994b). Therefore, the closely related techniques of muscle cell-mediated ex vivo gene therapy and myoblast transplantation are both applicable to muscle injuries.

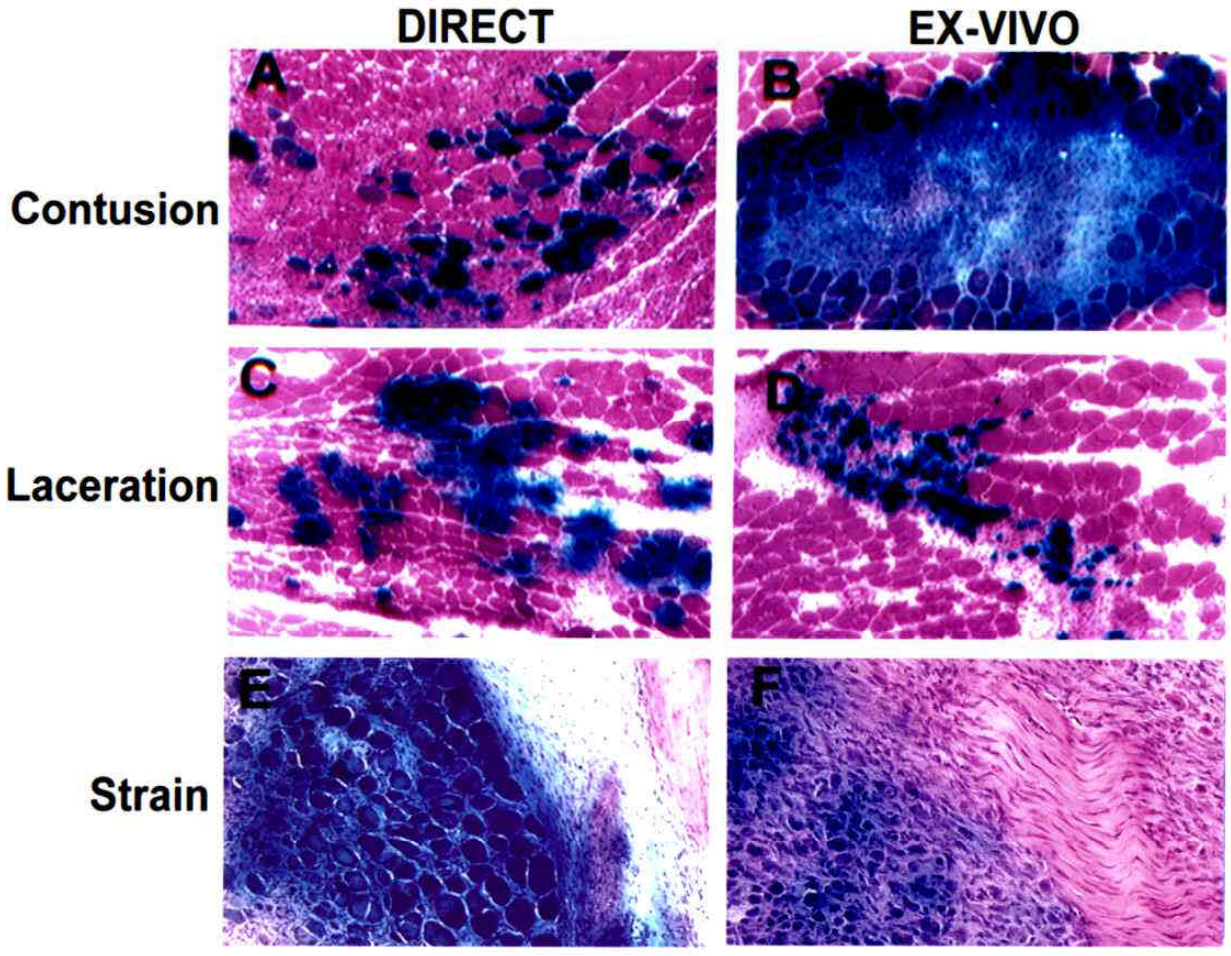

Figure 2. Adenovirus mediated direct and *ex vivo* gene transfer of β-galactosidase in lacerated, contused, and strain-injured muscle. The direct (**A, C, E**) and *ex vivo* (**B, D, F**) gene transfer into contused (**A, B**), lacerated (**C, D**), and strain-injured muscle (**E, F**) lead to successful gene delivery of β-galactosidase marker gene in the injured site at 5 days post-injection. Magnification X10 for A-F.

Muscle-based tissue engineering offers exciting potential therapies for muscle disorders. A large number of recreational and professional athletic injuries involve skeletal muscle (Garrett, 1990). Therapies to improve functional recovery and shorten rehabilitation may both optimize performance and minimize morbidity. Further research is ongoing to refine these muscle-based tissue engineering applications. The results of such investigations may provide revolutionary treatments for these common muscle injuries.

IV. Bone healing

Multiple surgical specialties, including orthopaedic, plastic, and maxillofacial, are concerned with bone healing augmentation. Physicians in these disciplines rely on bone augmentation techniques to improve healing of fracture non-unions, oncologic and traumatic bone defect reconstructions, joint and spine fusions, and artificial implant stabilizations. Unfortunately, current techniques of autograft, allograft, and electrical stimulation are often suboptimal. Therefore, tissue engineering approaches toward bone formation have immense implications.

Intramuscular bone formation is a poorly understood phenomenon. It can be present in the clinically pathologic states of heterotopic ossification, myositis ossificans, fibrodysplasia ossificans progressiva, and osteosarcoma. Radiation therapy and the anti-inflammatory drug, indomethicin, can suppress myositis ossificans. However, neither the mechanism of formation nor suppression of ectopic bone is clearly understood. The first evidence toward the existence of growth factors capable of stimulating intramuscular bone was gathered 30 years ago (Urist, 1965). Now, a growing family of bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, is recognized. Human BMP-2 in recombinant form (rhBMP-2) and BMP-2 cDNA encoding plasmids induce bone formation when injected into skeletal muscle (Wang et al., 1990; Fang et al, 1996). Current applications focus on injecting rhBMP-2 directly into non-unions and bone defects. However, muscle-based tissue engineering has enormous promise in the arena of bone healing and may shed light on the physiologic mechanism of ectopic bone formation.

Cells isolated from skeletal muscle are capable of responding to rhBMP-2 both *in vitro* and *in vivo*. Primary rodent myogenic cells in cell culture respond in a dose dependent fashion to rhBMP-2 by producing alkaline phosphatase, an osteogenic protein (Bosch et al., 1998). Furthermore, the purer the population of myogenic cells, as evidenced by desmin staining, the greater the alkaline phosphatase production (Bosch et al., 1998). Recombinant human BMP-2 inhibits myogenic differentiation as it stimulates osteoblastic differentiation of the muscle-derived cells (Yamaguchi et al., 1991; Katagiri et al., 1994; Kawasaki et al., 1998). Therefore, the *in vitro* data suggests that myogenic cells are capable of responding to rhBMP-2 and entering an osteogenic lineage.

Primary rodent muscle-derived cells are capable of being engineered to produce intramuscular bone *in vivo* (Bosch et al., 1998). The *ex vivo* approach is utilized to transduce the primary muscle-derived cells with an adenovirus carrying the BMP-2 cDNA. Intramuscular injection of as little as 300,000 transduced cells produces bone in severe combined immune deficient (SCID) mice (Bosch et al., 1998). The bone produced contains osteoid and bone marrow elements as evidenced by hematoxylin and eosin (H&E) stain and von Kossa stain for mineralization (see **Figure 3**). Not only do the transduced muscle cells produce BMP-2, but strong evidence suggests that the injected cells also respond to BMP-2 by producing bone (Bosch et al., 1998). In addition to the *ex vivo* approach, an adenovirus mediated direct gene transfer of BMP-2 produces large amounts of intramuscular bone (Musgrave et al., 1998). Consequently, both the *in vitro* and *in vivo* data support the hypothesis that muscle cells may be engineered to become osteogenic cells. The ramifications of myogenic cells' capabilities to form bone are immense.

Muscle-based tissue engineering to produce bone may be applicable to multiple skeletal abnormalities. One such scenario is large bone defects resulting from trauma or oncologic resections. Muscle-derived cells capable of bone formation may be exploited to reconstruct the bone defect and minimize the use of autograft, allograft, and bone distraction. Currently, we are investigating whether a muscle flap can be engineered to produce bone and, thereby, reconstruct an experimental bone defect. Both *ex vivo* and *in vivo* gene therapy techniques are being applied in this model. Another approach is to transform muscle, restricted to the confines of a silicone mold, into bone of desired geometry such as a proximal femur or midshaft tibia (Khouri et al., 1991). The muscle-based approach to bone defect reconstructions is especially appealing in light of the often poor vascularity of traumatic and oncologic bone defects. The combination of vascularized muscle and *de novo* bone formation offers revolutionary possibilities worthy of further investigation.

V. Intraarticular disorders

Degenerative and traumatic joint disorders are encountered frequently as our population becomes more active and lives longer. These disorders include arthritis of various etiologies, ligament disruptions, meniscal tears, and osteochondral injuries. Currently, the clinician's tools consist primarily of surgical procedures aimed at biomechanically alterating the joint (anterior cruciate ligament [ACL] reconstructions, total knee replacement, menciscal repair or excision, cartilage debridement, etc.). Tissue engineering applied to these intraarticular disease states theoretically offers a more biologic and less disruptive

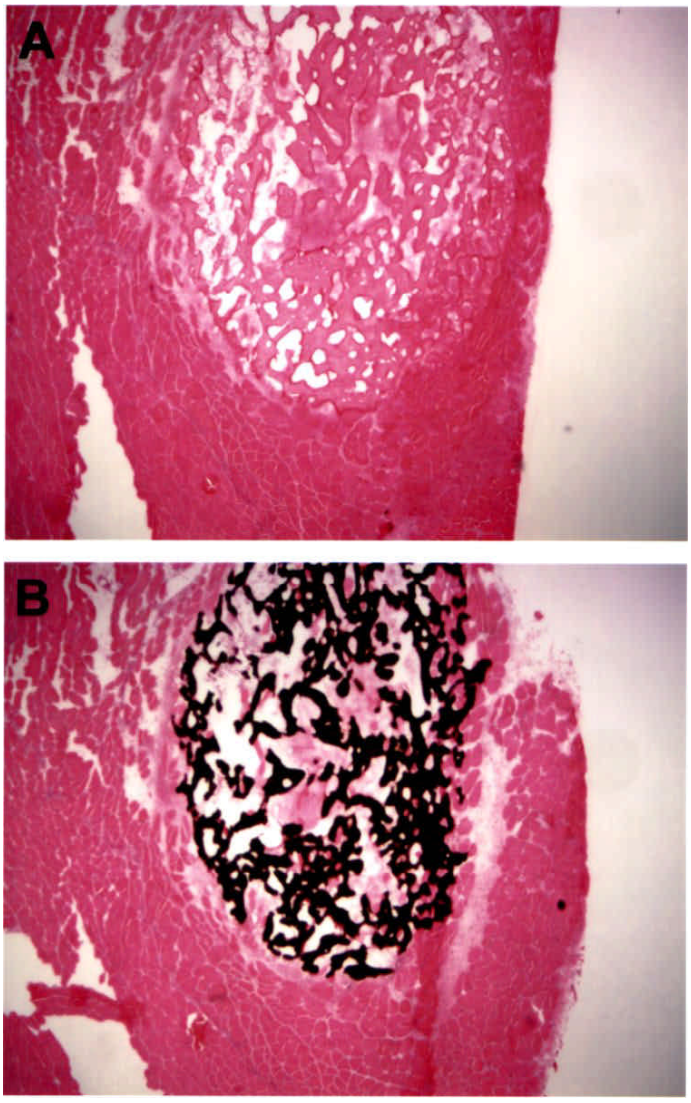

Figure 3. Myoblast mediated gene transfer of bone morphogenic protein-2 (BMP-2) leads to ectopic bone formation within skeletal muscle. The injection of adenovirally-transduced myoblasts to express BMP-2 in the gastrocnemius muscle of a scid mouse leads to ectopic bone formation, which is evidenced by H&E (**A**) and von Kossa (**B**) stains. Magnification: X10 for A, B.

reparative process. Both direct (Nita et al., 1996) and *ex vivo* (Bandara et al., 1993) gene therapy approaches to arthritis models have been reported. The synovial cell-mediated *ex vivo* approach, while offering advantages of *ex vivo* gene transfer such as the safety of *in vitro* genetic manipulation and precise cell selection, is hindered by a decline of gene expression after 5-6 weeks (Bandara et al., 1993). Due to its ability to form post-mitotic myotubes and myofibers, the skeletal muscle satellite cell offers theoretical advantages of longer term and more abundant protein production.

Muscle cell-mediated *ex vivo* gene delivery to numerous intraarticular structures is possible. Intraarticular injection of primary myoblasts, transduced by adenovirus carrying the β-galactosidase marker gene, results in gene delivery to many intraarticular structures (Day et al., 1997). Tissues expressing β-galactosidase at 5 days after injection in the rabbit knee include the synovial lining, meniscal surface, and cruciate ligament (Day et al, 1997). In contrast, injection of transduced synovial cells results in β-galactosidase expression only in the synovium (Day et al., 1997). Likewise, injection of transduced immortalized myoblasts results in gene delivery to various intraarticular structures, including the synovial lining and patellar ligament surface. However, the purified immortalized myoblasts fused more readily and resulted in more *de novo* intraarticular myofibers than the primary myoblasts. This illustrates the importance of obtaining a pure population of myogenic cells, void of fibroblast and adipocyte contamination often seen in primary

myoblasts. Muscle cell-mediated *ex vivo* approaches are predicated on myoblast fusion to form myofibers, the multinuclear protein-producing factories. Intraarticular injection of transduced immortalized myoblasts into a severe combined immune deficient (SCID) mouse results in myotubes formation and transgene expression in multiple structures at 35 days. Therefore, intraarticular gene expression (for at least 35 days) resulting from muscle cell-mediated tissue engineering is feasible in animal models. Based on this data, a muscle cell-mediated gene transfer approach may deliver genes to improve the healing of several intra-articular structures specifically to the ACL and meniscus.

The ACL is the second most frequently injured knee ligament. Unfortunately, the ACL has a low healing capacity, possibly secondary to its encompassing synovial sheath or the surrounding synovial fluid. Because complete tears of the ACL are incapable of spontaneous healing, current treatment options are limited to surgical reconstruction using autograft or allograft. The replacement graft, often either patella ligament or hamstrings tendon in origin, undergoes ligamentization with eventual collagen remodeling (Arnosczky et al., 1982). Therefore, recent research is directed at augmentation of this ligamentization process using growth factors to affect fibroblast behavior. *In vivo* data suggests that platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and epidermal growth factor (EGF) promote ligament healing (Conti, 1993). Transient, low levels of these growth factors resulting from their direct injection into the injured ligament are unlikely to produce a significant response. Therefore, an efficient delivery mechanism is essential to the development of a clinically applicable therapy. Muscle cell-mediated *ex vivo* gene therapy offers the potential to achieve persistent local gene expression and subsequent growth factor delivery to the ACL. Investigations into the effect of muscle cell-mediated *ex vivo* gene therapy to enhance the healing of torn ACLs, reconstructed ACLs, and the bone ligament interface are currently ongoing.

The knee meniscus plays a critical role in maintaining normal knee biomechanics. Primary functions of the meniscus include load transmission, shock absorption, joint lubrication, and tibiofemoral stabilization in the ACL deficient knee. The historical treatment of menisectomy for meniscal tears has been replaced by meniscal repair when tears involve the meniscus' peripheral, vascular third. Growth factors, including platelet-derived growth factor (PDGF), are capable of enhancing meniscal healing (Spindler et al., 1995). *In vitro* data currently under review details numerous growth factors' effects on fibroblast proliferation and collagen production (in preparation). Regardless of which growth factor is proven optimal for meniscal healing, the cardinal issue of protein delivery must be addressed. Direct intrameniscal growth factor injections are unlikely to produce sustained levels without the need for multiple injections, a scenario not clinically appropriate. Efficient and sustained delivery of desired growth factors may be best accomplished by gene delivery. Muscle cell-mediated *ex vivo* gene delivery offers the possibility of sustained, high level gene expression. Investigations utilizing the muscle cell-mediated *ex vivo* approach to deliver marker genes and growth factors directly to the rabbit meniscus are currently underway. Such studies may lead to novel therapies for meniscal injuries, preventing significant morbidity from these chronically disabling injuries.

VI. Future directions

Muscle-based tissue engineering is a burgeoning new discipline with unknown possibilities. Data gathered thus far proposes to challenge traditional scientific beliefs at many levels, from basic muscle cell biology to clinical medicine. In addition to the characterization of possible skeletal muscle-derived mesenchymal stem cells, investigators must aggressively pursue potential clinical applications for muscle-based tissue engineering (see schematic representation in **Figure 4**). The development of muscle-based tissue engineering approaches to inherited muscle diseases, acquired muscle injuries, bone healing, and intraarticular disorders is underway. Furthermore, investigations have been initiated into the utility of muscle-based tissue engineering to heal cartilage defects, spinal injuries, and flexor tendon lacerations. An explosion of research, from basic science to clinical medicine, is mandated to fully elucidate the potential of muscle-based tissue engineering for musculoskeletal disorders (see **Figure 4**).

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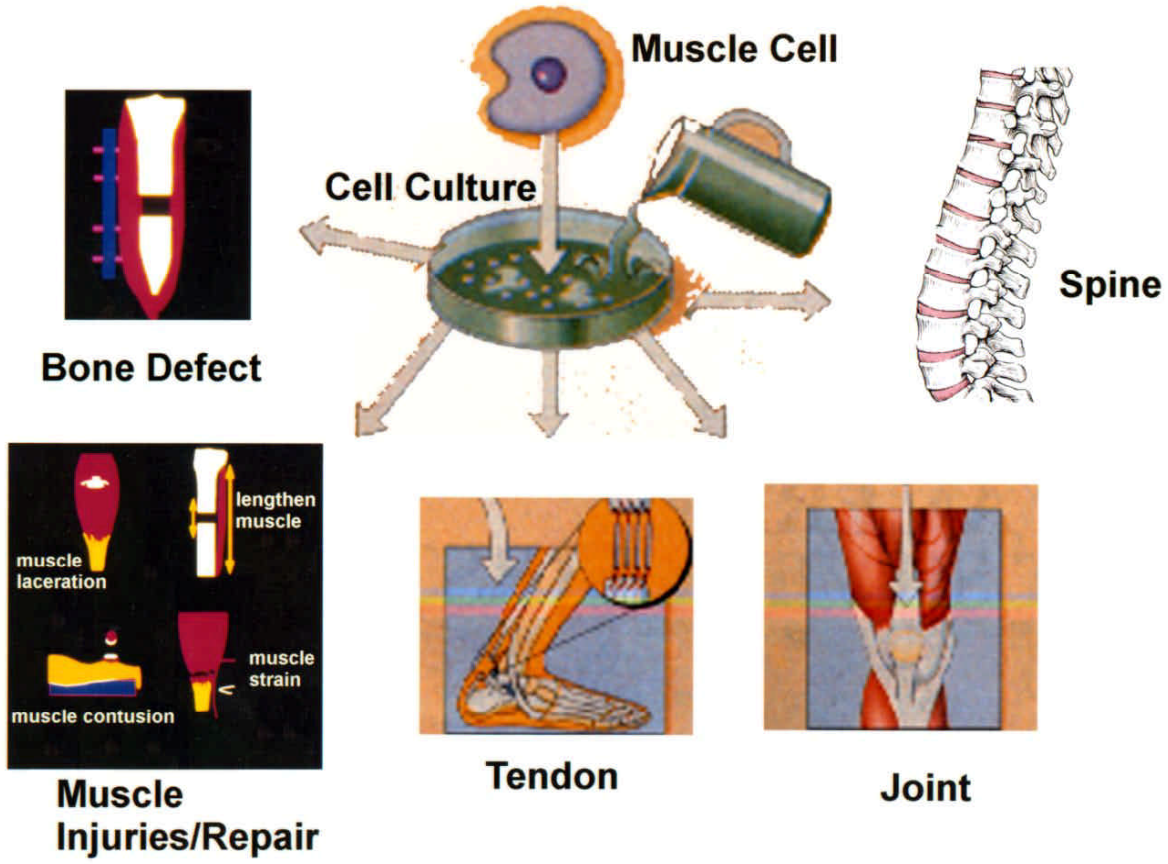

Figure 4. Schematic representation of the different applications of muscle based tissue engineering to various areas of the musculoskeletal system, including: muscle injuries and repair, bone defect, intra-articular structures, spinal injuries, and tendon repair.

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