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Review

Advances in Skeletal Muscle Tissue Engineering

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Abstract. *Skeletal muscle tissue engineering is a promising interdisciplinary specialty which aims at the reconstruction of skeletal muscle loss caused by traumatic injury congenital defects or tumor ablations. Due to the difficulty in procuring donor tissue, the possibilities for alternative treatment like autologous grafting (e.g. muscle flaps) are limited. This process also presents consistent problems with donor-site morbidity. Skeletal muscle tissue engineering tries to overcome this problem by generating new, functional muscle tissue from autologous precursor cells (stem cells). Multiple stem cells from different sources can be utilized for restoration of differentiated skeletal muscle tissue using tissue engineering principles. After 15 years of intensive research in this emerging field, for the first time, solutions using different strategies (e.g. embryonic stem cells, arterio-venous (AV) loop models, etc.) are being presented to resolve problems like vascularisation of tissue engineered constructs. This article reviews recent findings in skeletal muscle tissue engineering and outlines its relevance to clinical applications in reconstructive surgery.*

Skeletal muscle tissue engineering represents an interdisciplinary approach, using cell biology and engineering principles to generate functional muscle tissue by imitating neo-organogenesis from mononucleated stem cells (*e.g.* myoblasts) to differentiated myofibers (1). It applies specific characteristics of precursor cells, scaffolds and bioactive factors in order to form, manipulate and restore skeletal tissue phenotype and function. Skeletal muscle tissue, a highly specialised tissue, is often lost due to traumatic injury, extensive surgical tumor ablation or muscle fiber atrophy caused by prolonged denervation.

Until now only few alternatives for functional restoration of muscle tissue existed. The most commonly used, free tissue transfer (autologous grafting), relocates muscle tissue from autologous sites to the site of defect. However, this technique results in donor-site morbidity, causing functional loss and volume deficiency. The field of skeletal muscle tissue engineering promises alternative approaches for both functional and aesthetic treatments.

In recent years, different research groups have presented techniques of engineering three-dimensional (3D) skeletal muscle tissue that was able to produce force through contractions of muscle fibers. This *in vitro* engineered muscle cannot be compared to native muscle tissue, but it represents a major step forward. These important findings were followed by other reports that introduced methods of vascularisation of engineered constructs. Even if some tissue engineering applications (*e.g.* skin and bone) can presently be used in clinical settings, vascularisation still constitutes a major hurdle for tissue engineering operations in which constructs exceed the limits of diffusion. Most approaches were dependent on the host for generation of a blood vessel system that could provide enough oxygen and nutrients for survival. However, in capacious tissues with a high oxygen demand like skeletal muscle, vascularisation is a fundamental concern (2). Thus, reports of successful vascularisation of muscle constructs represent another major progression towards the clinical practise of skeletal muscle tissue engineering. This review summarizes these recent findings in skeletal muscle tissue engineering science and outline their importance for possible subsequent clinical usage.

Skeletal Muscle Tissue

Skeletal muscle tissue is a highly adapted tissue. The primary function is generating longitudinal force. This is established by an intricate morphological assembly which consists of uniaxially directed bundles of densely packed myofibers

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which generate force by contraction of multiple myofibrils. Myofibers are well-vascularized, multinucleated and derived from mesodermal myoblasts. Mature muscle fibers are not capable of self-renewal due to terminal differentiation. The ability of self-renewal of skeletal muscle tissue arises from a subpopulation of cells called satellite cells or myoblasts which reside beneath the basal lamina and constitute up to 1-5% of total skeletal muscle nuclei, depending on age and muscle fiber composition (3). These myoblasts, first described by Mauro in 1961, remain – in healthy muscle fibers – in the mitotic quiescent state G0 and express CD 34, M-cadherin, PAX 7, syndecan 3, c-met (4). Along the site of a muscle injury, myoblasts become activated by external local stimuli, re-enter the mitotic cell cycle and regenerate muscle tissue to a certain extent, either by forming new myofibers or fusing with damaged myofibers (3). A certain number of cells re-enter the G0 state and therefore are capable of maintaining the regenerative potential of injured muscle tissue. Migration into the injured area and proliferation of cells are often associated with the creation of a connective tissue network (scar tissue formation) which leads to a loss of functionality (5, 6). After prolonged denervation, satellite cells are not capable of re-entering the cell cycle; therefore, the potential for regeneration is lost or very limited (7). When satellite cells enter the G1-phase, Pax 7, a paired-type homeobox gene, which has previously been shown to play an important role in skeletal muscle formation, is upregulated (4).

Differentiation of mononucleated myoblasts into differentiated multinucleated myotubes can be analysed by investigating the expression of marker genes that play distinct roles during myogenesis. Among these genes, myogenic regulator factors (MRF) (8) play essential roles. These transcription factors contain a basic helix-loophelix DNA-binding domain and function as promoters for several muscle specific genes. Two important transcription factors of myogenesis are MyoD and myogenin, both members of the MRF family (9). MyoD, acting as a myogenic differentiation factor, is upregulated in the early stages of myogenesis and induces fusion of myoblasts and reduction of proliferation. It accomplishes this by up regulating cellcycle inhibitors (p21) and downregulating cell-cycle activators, such as cyclins and cyclin-dependent kinases (10). Myogenin arises in later stages of differentiation and acts more specifically in the formation of multinucleated muscle tissue, as seen in myogenin-deficient knock-out mice which terminate muscle formation due to insufficient myoblast fusion (11). Both transcription factors are upregulated during the process of skeletal muscle formation and can function as markers of differentiation. Mature myotubes can be identified by CD 56, alpha-sarcomeric actin, myogenin, MRF 4, Myo D or Myf 5 stainings (Table I) (3).

Muscle satellite cells are considered multipotential stem cells that are able not only to give rise to myogenic tissue but also to osteogenic and adipogenic tissue after treatment with biochemical signals (12). Asakura *et al.* have demonstrated that satellite cells isolated from adult mice and treated with bone morphogenetic proteins (BMPs) or adipogenic inducers differentiated into osteocytes or adipocytes and therefore illustrate multipotential mesenchymal stem cell plasticity (12).

Table I. *Markers of skeletal muscle differentiation.*

Expression markers	
Muscle derived cell	CD 34
Satellite cell	CD 34, CD 56, Myf 5, desmin
Myotubes	CD 56, alpha-sarcomeric actin, myosin heavy chains, myogenin, MRF 4, desmin

Tissue Engineering Principles and Requirements

Skeletal muscle tissue engineering employs the regenerative potential of stem cells and their potential for proliferation and maturation. The preferred source of cells for skeletal muscle tissue engineering applications are primary, nonimmunogenic satellite cells since they can easily be obtained by muscle biopsies and be cultivated *in vitro* (13). Satellite cells from different species and various aged muscles sources (fetal, neonatal, adult) can be isolated and studied in several animal models (*e.g.* rat, mouse, chicken) (14-16). Human satellite cells can also be successfully extracted and expanded *in vitro* (17, 18) meeting the requirements for future clinical applications. The human muscle phenotype is stable and cells can be stored in liquid nitrogen without loss of proliferation or differentiation capacity (17). A biopsy of 0.1 cm^3 can give rise to $5x10^3$ proliferating cells (17). Satellite cell differentiation is more comparable to myogenesis than differentiation of immortal myogenic cell lines like C2C12 (mouse) or L6 (rat) and experimental results from primary stem cells of different species can be more accurately assigned to the tissue engineering concept (13).

In order to create sufficient volumes of neo-tissue, cells must be expanded *in vitro*, facing the antagonism between proliferation and differentiation (19). Therefore, tissue engineering research should always try to utilize expanded cells and characterize the influence of cell passages on conducted experiments (20). After multiple passages, satellite cells show a decreased proliferation rate; the number of desmin positive cells decreases and induction of differentiation becomes more sophisticated (21). For tissue engineering purposes, these induction methods should succeed in a 3D environment and be feasible for later

clinical applications (20). Other cell types that have been shown to differentiate into the myogenic cell line are *e.g.* mesenchymal stem cells (MSC), hematopoeitc stem cells (HSC) (22) and embryonic stem cells (14). MSC, first described by Caplan *et al.* (23), are defined as clonogenic cells, with a lifelong self-renewal capacity and the ability of differentiating into a variety of cell types (24). Based on the source of stem cells, for regeneration purposes, one can divide them into embryonic (ESC)- or adult-derived cells, which have been well investigated and characterized over the years (24). Adult MSC, isolated from various sources (*e.g.* skin, skeletal muscle, umbilicord, blood, and most commonly, bone marrow), have been shown to differentiate into the myogenic lineage (12, 22, 25).

Sherwood *et al.* have investigated the incorporation of unfractioned bone marrow cells and HSC in damaged and uninjured skeletal muscle and demonstrated that bone marrow cells or HSC engrafted in damaged muscle tissue contributed to the restoration. The number of cells that were able to adhere seems to vary by muscle group and on the severity of muscle injury (22). Ferrari *et al.* (26) and Corti *et al.* (27) were also able to obtain limited muscle regeneration by the injection of bone marrow cells. Gang and co-workers (25) have demonstrated that MSC isolated from umbilical cord blood differentiate into skeletal muscle cells *in vitro*. Bossolasso *et al.* (28) have shown that MSC isolated from bone marrow can differentiate into muscle cells *in vitro* and can give rise to myogenic cells *in vivo* after muscle injury.

ESC, isolated from the inner cell mass of a blastocyst, can give rise to cell-lineages of all three different germinal sheets and the germ lane and are therefore considered pluripotent (29). ESC have recently entered the field of skeletal muscle tissue engineering with the reports by Levenberg and co-workers (2) who cultivated embryonic endothelian cells together with myoblasts.

In Vitro Tissue Engineering

A prerequisite to treating acquired and inherited skeletal muscle myopathies is the ability to either transplant constructs of differentiated muscle tissue or implant or inject muscle-precursor cells into the site of dysfunction for subsequent formation of functional muscle tissue (30). There are two different approaches in achieving these ends in skeletal muscle tissue engineering: *in vitro* and *in vivo* tissue engineering (Figures 1 and 2). The first concept attempts to create 3D differentiated, functional muscle tissue with or without the use of an extracellular matrix (ECM) *in vitro* by extracting stem cells from, for *e.g.,* muscle biopsies followed by their expansion and differentiation in a controlled environment (*e.g.* culture flasks, bioreactor) (Figure 1). To facilitate the formation of 3D tissue engineered muscle, the use of an ECM for providing a solid framework for cell growth and differentiation is a very effective approach but not a necessity. Dennis *et al.* (31, 32) and Kosnik *et al.* (33) have introduced a technique of creating contracting 3D muscle tissue by co-culturing fibroblasts and myoblasts. Due to the delamination of the monolayer co-culture and an endpoint fixation, a 3D contracting muscle-like construct was created which was called "myooid". The myooids had a significantly higher ECM content compared to native skeletal muscle due to the fibroblasts. Using electrical stimulation, excitability and contractibility were measured, which showed, for example, that rat "myooids" developed a specific force of approximately 1% of adult control muscles (30).

The ECM plays an important role in the process of attachment, alignment and differentiation of muscle precursor cells. An ideal matrix should provide an optimal surface for cell proliferation and differentiation in order to enhance tissue neogenesis. It also should be biocompatible and bioresorbable and nonimmunogenic with a high affinity to biological surfaces (34). In order to create 3D muscle constructs, specific combinations with scaffolds and cell types have been studied. Kamelger *et al.* (34) have compared different ECMs and shown that rat myoblasts seeded on polyglycolic acid (PGA) meshes, in alginate or in hyaluronic acid-hydrogels, can fuse *in vivo* into vascularized, multinucleated myotubes. Saxena *et al.* (35, 36) have demonstrated that myoblasts cultivated *in vitro* on an ECM also differentiate into myotubes in non-muscle environments *in vivo*.

Other studies have cultivated and successfully differentiated myoblasts on Matrigel®, an extract from the Engelbreth-Holm-Swarm mouse sarcoma, which contains various extracellular matrix proteins and growth factors in undefined concentrations (37-39). Due to its strong differentiation induction potential, Matrigel® has been used frequently, also in combination with other matrices like collagen (40). However, because of its origin and undefined content, it is only suitable for experimental models and cannot be applied in clinical settings (30).

Another possible matrix for muscle tissue engineering purposes is acellularized skeletal muscle tissue. Borschel *et al.* (41) have demonstrated that myoblasts seeded on acellularized mouse extensor digitorum muscles could produce contractile force on electrical stimulation after two weeks of *in vitro* cell culture.

Another scaffold that has already been used successfully in clinical tissue engineering application for skin and bone is fibrin. It combines multiple advantages such as biocompatibility, biodegradability and the binding of growth factors, such as FGF-2 and VEGF (42). It allows diffusion of growth and nutrient factors and provides a solid framework in which myoblasts can proliferate, migrate, and differentiate into 3D skeletal muscle constructs. The high surface area of fibrin encourages cell-matrix interactions,

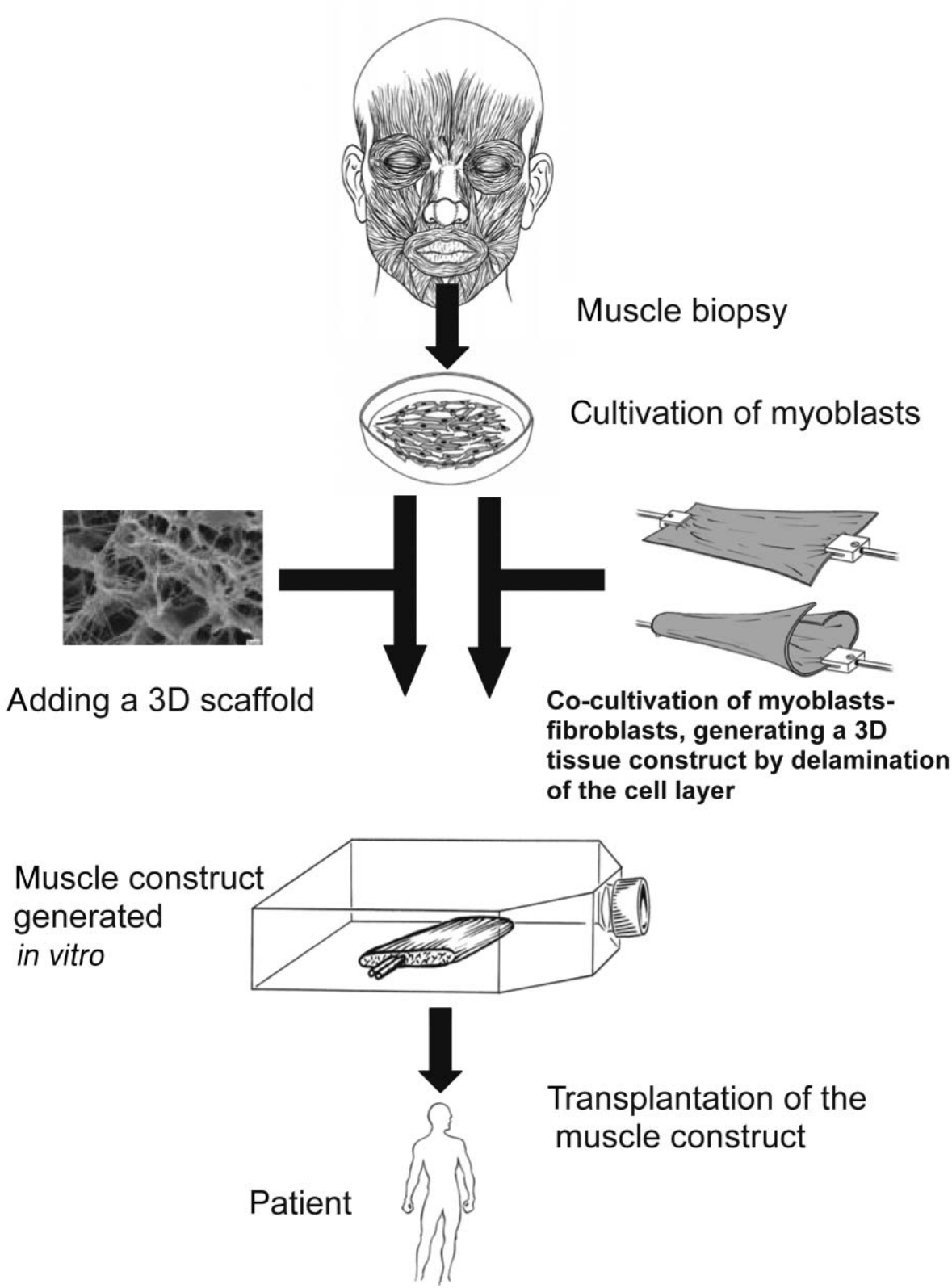

Figure 1. *The in vitro tissue engineering concept. The in vitro approach to skeletal muscle tissue engineering attempts to create 3D-differentiated, functional muscle tissue with the use of an extracellular matrix (ECM) or by co-cultivation of myoblasts and fibroblasts in vitro by extracting stem cells from, for e.g., muscle biopsies followed by their expansion and differentiation in a controlled environment.*

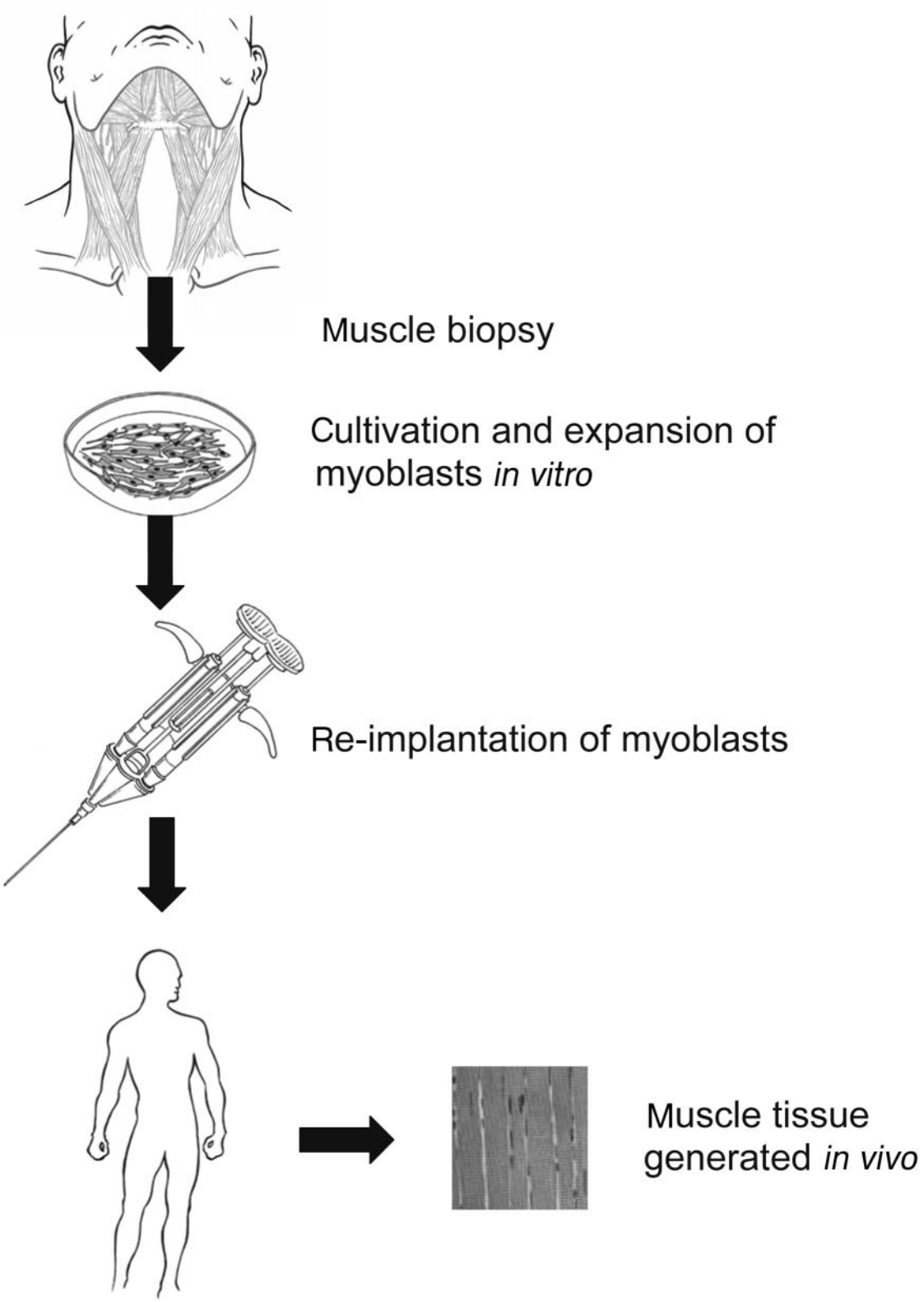

Figure 2. *The in vivo tissue engineering concept. The in vivo tissue engineering approach aims to reconstruct functional tissue through the cultivation and expansion of satellite cells in vitro followed by re-implantation using a transport matrix, which allows subsequent differentiation of cells in vivo.*

gives sufficient space for extracellular matrix generation, and is resorbable once it has served as structural guidance for muscle formation (1). The degradation rate can be adapted to the number of cells involved because the mixture includes aprotinin, a protease inhibitor. Furthermore, fibrin can be injected directly into muscle defects using a two-way syringe when the formation of the muscle construct is initiated *in situ* at the site of the defect, as demonstrated by Beier *et al.* (9). Huang *et al.* have also shown that myoblasts seeded on fibrin gels differentiated into contracting myotubes which generated a maximum twitch force of 329±26.3 μN and a tetanic force of 805.8±55 μN. The engineered muscle showed normal physiological functions like length-tension and force-frequency relationships (42).

In vitro formation of skeletal muscle has become a general model for understanding the essential steps and principles of myogenesis, including mechanisms of cell differentiation, cell-matrix interactions and morphogenesis (30, 43). For *in vitro* tissue engineering applications it is important to have stimulating factors which can initiate fusion of mono-nucleated myoblasts into multinucleated myofibers in a 3D environment. There are methods of inducing maturation which are very effective (*e.g.* the use of Matrigel® or the co-cultivation of myoblasts and neuronal tissue), but can only be applied in non-clinical research studies (20). Other techniques try to imitate *in vivo* conditions during myogenesis and therefore drive cells into the differentiation pathway. One important factor during myogenesis that can influence gene expression, protein synthesis and total RNA/DNA content is mechanical stimulation (40, 44-46). Powell *et al.* have demonstrated that mechanically stimulated 3D human skeletal muscle cells seeded on a collagen matrix showed an increase in cellular proliferation, myofiber organisation and ECM composition. Mechanical stimulation led to a increased number of myofibers which also measured greater in diameter (40). Chronic electrical stimulation also attempts to mimic neuronal stimulation during myogenesis. It has been shown that electrical stimulation of primary myoblasts can influence the expression of a myosin heavy chain (MHC) (47) and myogenic transcription factors (48), and also promotes the maturation of 2D primary muscle cultures *in vitro*, due to the expression of adult MHC isoforms (20, 49). It also increases cell proliferation (50). It has also been shown that electrical stimulation is not able to induce the fusion of myoblasts in a 3D environment and therefore can only act as a promoter of differentiation after the fusion of myoblasts into myotubes (20). Other bioactive substances that promote maturation of skeletal muscle cultures are insulin, insulin-like growth factors (IGFs) and acidic fibroblast growth factor (aFGF) (51-53). Muscle tissue developed *in vitro* using tissue engineering principles had a force generation increased by 50% using IGF-1 treatment (42).

In Vivo Tissue Engineering

The second approach to skeletal muscle tissue engineering aims to reconstruct functional tissue through the cultivation and expansion of satellite cells *in vitro* followed by re-implantation using a transport matrix, which allows subsequent differentiation of cells *in vivo* (Figure 2). This method, also called myoblast transfer therapy (MTT), was invented primarily for the treatment of inherited muscular dystrophies. MTT was developed as a cell-mediated gene transfer method which aims at the substitution of dystrophin, a fiber stabilizing protein, by transplanting myoblasts from normal donors into dystrophic host muscles (54). Donor myoblast survival presents this therapy's major problem, as many of these transplanted, allogenic cells suffer a swift death due to host immune reactions. Clinical trials with MTT had little success. However, when hosts were immunosuppressed, survival rates of transplanted myoblasts were significantly prolonged (55). Hence, in considering the use of autogenic cells in tissue engineering approaches, donor cell survival no longer constitutes an insurmountable obstacle.

In vivo tissue engineering can also be applied in the emerging field of cardiac muscle tissue engineering in which researchers attempt to rebuild functional heart tissue by injecting stem cells into diseased or infracted areas (56).

In novel *in vivo* approaches, satellite cell cultures are utilized as cell-based gene therapy for the delivery of therapeutic proteins (*e.g.* insulin, insulin-like growth factor 1, erythropoietin and human growth factor hormone (18, 57)). Transduced muscle precursor cells are capable of long-term delivery of soluble hormones and growth factors when transplanted into host muscles, due to their capacity for efficient myoblast incorporation and expression of foreign DNA (58). Powell *et al.* (18) have developed a muscle-based delivery system for therapeutic proteins using transduced bioartifical muscle for recombinant human growth factor. Advantages of this method include a higher survival rate, fusion efficiency, and the possible retrievability of transplanted muscle constructs over simple myoblast injections.

Comparing the *in vitro* and *in vivo* tissue engineering methods one can see that the differentiation processes of *in vivo* applications appear less difficult, due to optimal conditions for myoblast proliferation and maturation *in vivo*. Combining this advantage with the amenities of the fibrin matrix, our group developed a new transportation system for expanded primary myoblasts in a 3D fibrin matrix using the clinically-approved two-way syringe system (DUPLOJECT®). With this injection-system we were able to reconstitute microsurgical set muscle defects through the injection of expanded myoblasts within an *in situ* molding biocompatible matrix. Detection of transplanted cells was feasible for as long as 12 weeks after injection and showed

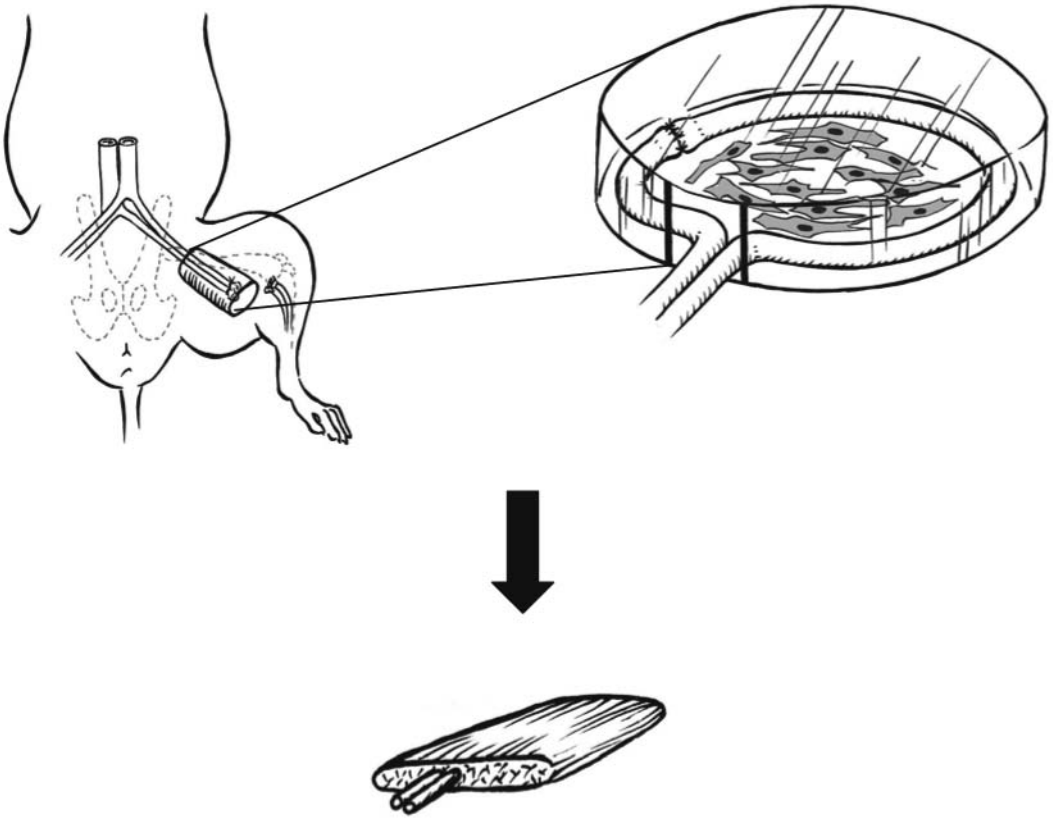

Figure 3. *The arterio-venous loop chamber model. Schematic diagram of the arterio-venous shunt loop model used to create new vascularized tissue constructs. (Adapted from (16, 60)).*

normal morphological integration into host muscle fibers. No scar formation was observed (9).

Vascularisation of Tissue-engineered Constructs

In both *in vitro* or *in vivo* approaches to muscle tissue engineering, the developed neo-muscles never outgrew the limitations of the diffusion capacity. Since skeletal muscle is a complex tissue with a high energy and oxygen demand, it appears clear that, for clinically-relevant dimensions, constructs require vascularisation. Both approaches have addressed this problem and both could represent major progress for muscle tissue engineering.

The first results of generation and vascularisation of autologous tissue constructs were presented by Tanaka and co-workers, who developed an *in vivo* model of angiogenesis by creating an encapsulated microvascular arterio-venous shunt loop in the groin of rats (59) (Figure 3). The AV shunt was set between the right femoral artery and vein with an interconnection of an autologous vein graft surrounded by a collagen matrix. The authors showed that a vascularised 3D fibrous tissue construct developed spontaneously in the immediate area surrounding the AV loop with an intrinsic capillary system, derived from the loop vessels (59). Even in the absence of an ECM, a vascularized neo-tissue grows (60).

In this AV loop chamber model, a vascular network is generated without the use of exogenous angiogenic factors, making this approach very unique. The angiogenic potential of this model appears to initiate endothelial proliferation and the migration of surrounding cells, which leads to the sprouting and formation of *de novo* vasculature. The encapsulated construct thus created can be delimited by the surrounding tissue and hence be feasible for use in later transplantations. The composed construct, supplied and nourished by the AV pedicle, can easily be connected to other nutrient vessels and therefore affiliates the intrinsic vascular system of the construct with other vessels at the site of the transplantation, raising the probability of tissue survival.

Cassell and co-workers (61, 62) have evaluated the influence of the surrounding extracellular matrix on the formation of vascularized neo-tissue in the AV loop chamber model and have shown that polylactic-coglycolic acid (PLGA) and, to a lesser extent, Matrigel® can accelerate the formation of neo-tissue and increase the vascular architecture.

Subsequently, Messina *et al.* (61) analysed the interactions and potential of the angiogenic model in the formation of neo-tissue when various different forms of skeletal muscle (preincubated, fresh, human, rat, myoblasts and nonviable freezethawed muscles) are inserted in the loop chamber as the inductive source. Interestingly, only the utilization of primary myoblasts led to the generation of skeletal muscle tissue, which certainly exceeded the diffusion capacity with a diameter up to 1 cm. The authors were able to verify that the new skeletal muscle was derived from the implanted donor cells. All other approaches involving the implantation of skeletal muscle, regardless of species, lead to formation of well vascularised adipose tissue with only small amounts or no striated muscle fibers. It appears that extracellular matix components of the implanted muscle tissue function as an adipogenic stimulus attracting host cells from the surrounding tissue to form adipose tissue. Using the femoral vessels as axial pedical, Borschel and co-workers produced vascularized functional skeletal muscle tissue in rats derived from primary myoblast cultures which generated longitudinal contractile force when electrically stimulated (41). The histological appearance and physiological behaviour of the tissue-engineered muscles resemble native skeletal muscle characteristics.

Bach *et al.* (13) also utilized expanded primary myoblasts to demonstrate that axial prevascularisation seems to promote myoblast survival and does not lead to dedifferentiation of myoblasts.

The first reports about the vascularisation of *in vitro* tissue-engineered skeletal muscle came from Levenberg and co-workers (2). They developed a 3D multiculture system in which mouse myoblasts were cultivated together with human embryonic endothelial cells (hES cell-derived endothelial cells) or with human umbilical vein endothelian cells (HUVEC) and seeded them on a poly-(L-lactic acid)/polylacticglycolic acid (PLLA/PLGA) sponge-like scaffold. After two weeks in culture, the myoblasts differentiated into multinucleated myofibers and endothelian cells formed a vessel system throughout the muscle construct *in vitro*. In order to stabilize the *de novo*- formed vessel network, the author added mouse embryonic fibroblasts to the cell culture to mimic structural support by pericytes and smooth muscle cells. This led to an enormous endorsement of vascularisation of the formed constructs which was verified by the total area of endothelian cells and surface area of lumen structures. Large *de novo*-formed vessels could only be detected in four-week-old cultures – in comparison to two-week-old cultures – and became smooth-muscle actin-positive, supporting the thesis that embryonic fibroblasts enhance stabilization of the vessels over time. The author was also able to demonstrate that the tri-cultures with embryonic fibroblasts secreted the highest levels of vascular endothelian growth factor (VEGF) in analysed cultures, which might explain the enhancement of the vascular network. To test the hypothesis that prevascularization of tissue engineered constructs leads to an improvement in terms of donor cell survival *in vivo*, Levenberg *et al.* (2) transplanted the neo-constructs subcutananeously, intra-muscularly, and intrabdominally in mice. Analysis of survival rates, integration, differentiation, and vascularisation proved that prevasularization improved both cell survival rates and blood perfusion of the implanted constructs. This verifies that prevascularization of tissue engineerred constructs is very effective. The results of Levenberg *et al.* are a major step forward in tissue engineering science, but further studies are necessary to explore whether the results in this xenogenic cell culture model can be transmitted to human models. Furthermore, the utilisation of embryonically-derived cells remains ethically delicate.

Conclusion

A loss of muscle tissue can result in the functional impairment and cosmetic deformation of patients. Tissue engineering and regenerative medicine are growing fields of interest in human life sciences due to promising results and a wide area of potential applications. By combining the principles of biology and engineering, the development of functional substitutes that might restore, maintain, or even improve human organs and tissue draw closer than ever. The study of skeletal muscle tissue engineering has also contributed to our basic understanding of cell differentiation, matrix interaction, and cell survival in *in vivo* models. First results of important issues like vascularisation were addressed and have achieved promising results. However, there are still major hurdles to overcome in skeletal muscle tissue engineering science, such as the innervation of constructs. Nevertheless, the potential applications and benefits for reconstructive surgery are growing with the development of more sophisticated techniques in tissue engineering science.

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