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Tissue Engineering of Skeletal Muscle

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1. Introduction

1.1 Has Churchill been right?

"*Fifty years hence, we shall escape the absurdity of growing a whole chicken in order to eat the breast or wing, by growing these parts separately under a suitable medium*" (Churchill 1932)

In 1932, Sir Winston Churchill predicted that it will be possible in future to grow and engineer muscle tissue *in vitro*. As a confirmation of his prediction, Vandenburgh *et al.* observed contracting muscle tissue engineered *in vitro* for the first time in 1988 (Vandenburgh, H.H. *et al.* 1988). Only one year later – in 1989 – the group showed that mechanical stimulation of embrionic myoblasts *in vitro* facilitates longitudinal growth of engineered skeletal muscle tissue (Vandenburgh, H.H. & Karlisch 1989). This rapid development raised high expectations for future clinical applications of tissue engineering (TE) of skeletal muscle. Indeed, engineered muscle tissue could be used in a wide range of clinical situations.

A frequent clinical application of skeletal muscle tissue is the microsurgical transfer of myocutaneous free flaps for the coverage of soft tissue defects. As one major disadvantage, the use of free flaps is inevitably linked with a certain morbidity at the donor site including the loss of functional muscle tissue. In this situation, engineered muscle tissue could help to reduce the donor site morbidity. Above all, the advantage of muscle TE lies in the generation of functioning muscle tissue to replace certain muscles after damage or denervation (Klumpp *et al.* 2010). For example, the treatment of facial nerve palsy is momentarily limited to the transfer of autologous muscle tissue innervated by another nerve (the trigeminus nerve, e.g.) or free transfer of distant muscle tissue (Terzis & Konofaos 2008). Though multiple techniques and modifications exist, the results yielded in those clinical situations are moderate (Kumar & Hassan 2002, Terzis & Noah 1997). Furthermore, Kim *et al* demonstrated that myoblast transplantation is a promising method for the reconstruction after partial glossectomy (Kim, J. *et al.* 2003). Herein, TE of skeletal muscle for the replacement of functional muscle tissue could offer an individual alternative.

However, a clinical application of skeletal muscle TE has not been realized to date due to certain obstacles which will be discussed in the following. Though, *in vitro* engineered tissue of skeletal muscle could already play an important role for the clinical treatment of inborn muscle diseases as well as muscle injuries. Once again, Vandenburgh and co-workers engineered dystrophic muscle tissue using it as drug screening platform for Duchenne muscular dystrophy (DMD) treatment (Vandenburgh, H. *et al.* 2009). Thus, a wide range of possible drugs can be analyzed without using time-consuming and costly *in vivo* models (Vandenburgh, H.). Vandenburgh's study demonstrates an economic approach for drug

screening in general and orphan drugs in particular. Beside the relatively rare musculoskeletal disorders, engineered skeletal muscle also enables the analysis of more frequent diseases. Kaji *et al* introduced an *in vitro* model of electrically stimulated and contracting muscle tissue to analyze the insulin- and exercise-dependant glucose uptake which plays a role in insulin resistance of type 2 diabetics (Kaji *et al.*). Thus, skeletal muscle TE already plays a role for clinical treatments, though a direct bench-to-bedside approach has yet to become reality.
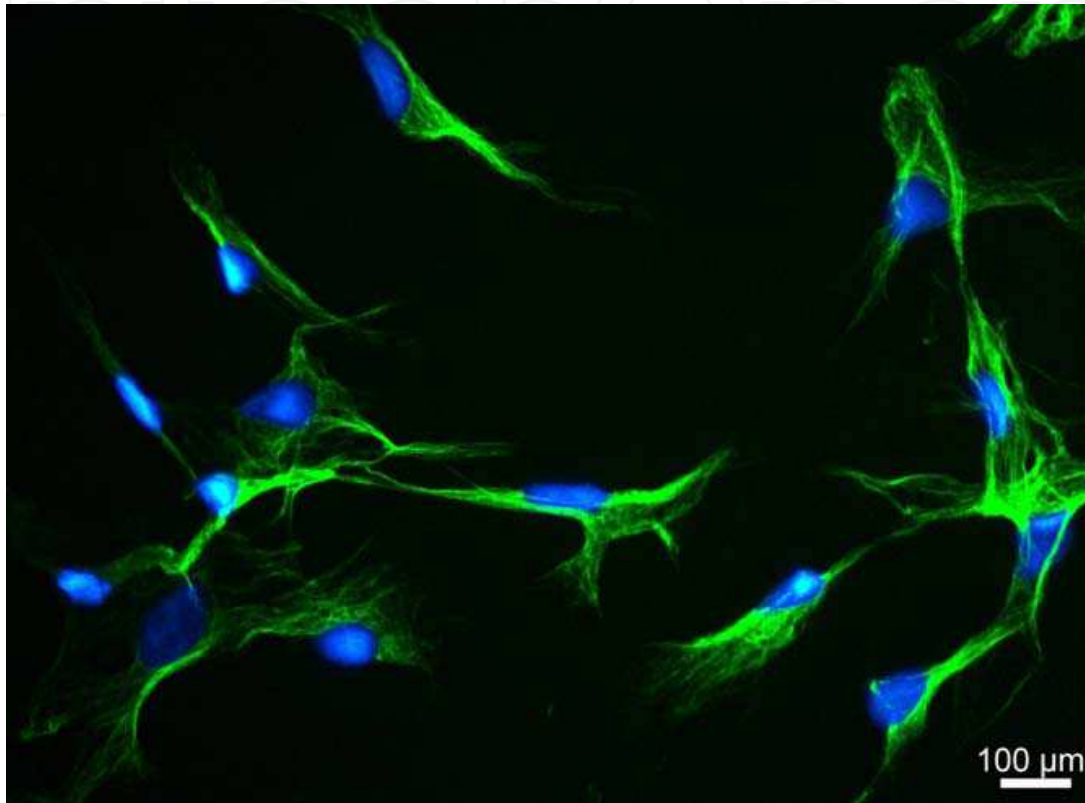

Fig. 1. Skeletal muscle precursor cells (myoblasts) *in vitro*. Immunofluorescent staining for desmin (green) an intermediate filament specifically expressed in myogenic differentiation prior to the formation of myotubes and fusion into muscle fibers. Nuclei are stained non-specifically with DAPI (Diamidine-phenylindole-dihydrochloride; blue). Magnification 400x

2. Finding the matrix for muscle TE

2.1 Materials
A variety of materials has been analyzed and reviewed regarding their suitability for skeletal muscle TE. On one side, natural materials like collagen I in the first place are preferred due to their biocompatibility and their close resemblance to the natural extracellular matrix (ECM). On the other side, synthetic materials generally show greater stability as well as cost-saving and easy handling.

Regarding collagen I - the main component of the natural ECM of mature skeletal muscle tissue - its advantage lies in high elasticity which is a pre-requisite for muscle contraction. Thus, its mechanical properties *in vivo* meet the demands for new skeletal muscle tissue perfectly. Furthermore, bovine as well as avian collagen show very low immunogenicity *in vitro* and can be safely used *in vivo* (Peng *et al.* 2010). Therefore, collagen sponges and gels

have been studied *in vitro* (Madaghiele *et al.* 2008) and *in vivo* (Kroehne *et al.* 2008). However, the rapid shrinkage of hydrogels *in vitro* as well as the low stability of collagen I are important disadvantages (Beier *et al.* 2009) and limit its use in long-term experiments. Another material frequently used for tissue engineering *in vitro* and *in vivo* is fibrin. The stability of fibrin gel is dependent on the concentration of fibrinogen and thrombin and can be augmented by addition of aprotinin which inhibits fibrinolysis (Meinhart *et al.* 1999). In addition, fibrin is known to accelerate vessel ingrowth into the matrix *in vivo* due to its binding sites for vascular endothelial growth factors (VEGF), fibroblast growth factor (FGF-2) and the cytokine interleukin-1 (IL-1) (Mosesson 2005). Still, fibrin gels show a definite loss of stability after 4 weeks *in vivo* (Arkudas *et al.* 2009). As an exception to the relative instability of natural polymers, silkworm fibroin, the structure protein of silk, shows an astonishing tensile strength of 100 –300 MPa and stability (Zhao *et al.* 2003). An *in vivo* stability of over 1 year has been reported for 17% fibroin concentration but, the cell-toxic HFIP (hexafluoro-iso-propanol) as organic solvent is necessary. Even in all-aqueous dissolution of silk fibroin with concentrations of 6 to 10% fibroin, the *in vivo* stability has been found to range between 2 and 6 months (Wang, Y. *et al.* 2008). Thus, silk fibroin provides an adequate stability for tissue engineering *in vivo*. Additionally, silk is in clinical use as suture material for a long time. However, hypersensitivity and adverse reactions have been reported and put the biocompatibility of silk into question (Soong & Kenyon 1984). The chemical and immunogenic properties of silk of *Bombyx mori* silkworms have been studied intensively since and sericin, the glue protein of silk, has been identified subsequently as the immunogenic agent (Panilaitis *et al.* 2003). The use of sericin-depleted fibroin scaffolds clearly increased the biocompatibility of silk matrices and Meinel *et al* could show that the biocompatibility of pure fibroin is comparable to collagen I and even superior to poly(d,l-lactic-co-glycolic acid) (PLGA) (Meinel *et al.* 2005). Thus, silk fibroin derived from silkworms has been used extensively as sponge-like scaffold for tissue engineering *in vitro* (Mandal & Kundu 2009) and *in vivo* (MacIntosh *et al.* 2008, Unger *et al.*). But as a drawback, the high stability and tensile strength of silk fibroin comes along with low elasticity and hydrophilicity that lead to poor cell attachment *in vitro*. In addition, the low elasticity limits the use of silk fibroin scaffolds for TE of skeletal muscle.

Biodegradable synthetic polymers have also been widely used for muscle TE. Their advantages lie in easy handling and very good stability *in vitro* and *in vivo*. For example poly(l-lactic acid) (PLLA) and the more lipophilic co-polymer PLGA have been used in different orthopaedic applications due to their non-toxic properties and long-term stability in vivo (PLLA: 24 months, PLGA: approx. 6 months) (Gunatillake & Adhikari 2003). Though, inflammatory responses (Bostman 1992) and cell toxic effects *in vitro* (Ignatius & Claes 1996) have been reported due to the acidic degradation products of PLLA and PGA. One of the most frequently used synthetic polymer in TE research is poly(ε-caprolactone) (PCL). This biodegradable synthetic polymer shows a slow degradation rate resulting in a long-term stability of approximately 1 year *in vivo* (Bolgen *et al.* 2005). Furthermore, PCL is highly biocompatible and therefore suitable for *in vivo* applications (Cao *et al.* 2009). PCL has been used as films (Sarkar *et al.* 2008) and more frequently as electrospun fibers. However, PCL is also highly hydrophobic and therefore shows poor cell attachment *in vitro* (Zhang, H. & Hollister 2009). Hence, the hydrophobicity of PCL has to be attenuated before cell seeding through plasma treatment (Martins *et al.* 2009) or by coating the scaffold or blending with other materials like collagen to enhance cell attachment (Schnell *et al.* 2007, Zhang, Y.Z. *et al.* 2005). Among the variety of biodegradable synthetic polymers materials like poly(aniline)

(PANi) (Borriello *et al.*) and oxidized polypyrrole (Gomez & Schmidt 2007) stand out due to their electrical conductivity. Though their mechanical properties are similar to PCL including poor cell attachment, electrical conductivity is an interesting feature that qualifies those materials for muscle TE, especially (Li *et al.* 2006).

Taking the properties of the most frequently used biopolymers and synthetic polymers into account, the complexity of mature skeletal muscle tissue asks for a combination of different complementary materials to engineer a matrix that meets the special demands of skeletal muscle TE. Composite scaffolds containing both, synthetic polymers for stability as well as biopolymers for enhanced cell attachment and elasticity, are therefore preferred in muscle TE research. E.g. PCL has been combined with collagen (Choi *et al.* 2008), gelatine (Kim, M.S. *et al.*), PLLA (Engelhardt *et al.*) and other materials. Also the combination of silk fibroin with collagen leads to suitable mechanical properties with good cell attachment *in vitro* (Wang, G. *et al.*, Zhou *et al.*).

2.2 Matrices

Comparable to the wide variety of materials, the methods of processing different forms of matrices are equally numerous. Thereby, mechanical properties of a certain matrix, degradation rates and cell attachment depend on the scaffold's architecture. Whereas single-cell-layers can be easily cultured on two-dimensional scaffolds (films, micropatterned scaffolds), the architecture of three-dimensional scaffolds is more complex. To ensure cell survival and proliferation *in vitro*, a three-dimensional matrix should enable diffusion of oxygen, nutrients and metabolites as well as the migration of cells inside the scaffold. Otherwise, cells will only proliferate at the periphery but not in the scaffold's centre (Ishaug-Riley *et al.* 1998). Therefore, the most important features of matrices for three-dimensional TE are high porosity (ideally approximately 90% (Freed *et al.* 1994)), adequate pore-size (Lee *et al* have shown that a range of 50 – 200 μm pore-size are sufficient for smooth muscle cells (Lee *et al.* 2008)) and high interconnectivity of the pores (van Tienen *et al.* 2002) to enable cell migration inside the matrix.

Concerning the pore size, hydrogels usually show freely diffusion of nutrients and oxygen. Cells incorporated in hydrogels can migrate through the scaffold by degrading the gel but at the same time the stability of the gel decreases continuously. Furthermore, the architecture of hydrogels randomly spread pores (Fig. 2). However, the natural extracellular matrix within functional skeletal muscle tissue is highly orientated.

The parallel alignment of ECM and skeletal muscle tissue is the pre-requisite for effective muscle contraction and force-generation along a longitudinal axis. Curtis and Wilkinson first described the "cell guidance theory" by demonstrating that microgrooved matrices with a parallel micropattern provoke parallel aligned cell growth along the pattern of the scaffold (Curtis & Wilkinson 1997). This phenomenon is also present in myoblast culture (Choi *et al.* 2008, Huang *et al.* 2006, Huber *et al.* 2007) facilitating the generation of aligned myotubes (Gingras *et al.* 2009). Therefore, several techniques for aligned scaffold architecture have been developed including selective laser sintering or three-dimensional printing reviewed by Karande *et al* (Karande *et al.* 2004). Another method to gain spatially orientated pores in sponge-like matrices is unidirectional freeze-drying of materials like collagen (Madaghiele *et al.* 2008) or silk fibroin (Mandal & Kundu 2009, 2009). Hydrogels are gradually frozen leading to controlled formation of ice crystals which result in controlled porosity after evaporation of the aqueous part of the hydrogel. Additionally, Schoof and co-workers demonstrated that also the pore-size can be controlled by variation of the freezing-

temperature (Schoof *et al.* 2001). Though, there are certain disadvantages linked to the freeze-drying method. First of all, secondary surface modifications of the sponges like coating procedures to enhance cell attachment are difficult and sparsely controllable. Secondly, the alignment of the pores is only spatial whereas the architecture of the matrix surrounding the pores usually remains at random.
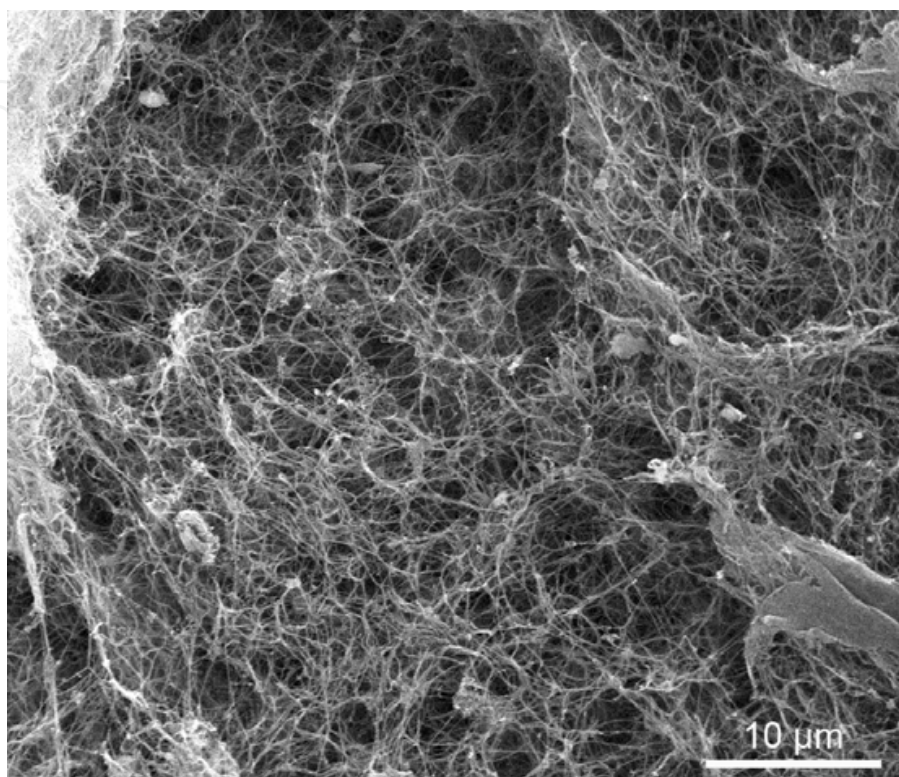

Fig. 2. Scanning Electron Microscopy (SEM) of a fibrin-collagen blend hydrogel. The aqueous part of the hydrogel is evaporated after critical point drying and fibrillar structures of fibrin and collagen is left behind. The random architecture of the hydrogel is clearly visible. 5000x magnification

On the contrary, electrospinning as an alternative method offers strict alignment of the resulting scaffolds (Ayres *et al.* 2006). The technique of electrospinning results in fibers formed by electrical voltage (Boudriot *et al.* 2006). Though, the process depends on multiple parameters, e.g. concentration and viscosity of the spinning solution, the voltage applied during the spinning process or flow rate of the spinning solution. The mechanical and chemical properties of electrospun matrices can be adjusted to the demands of the respective tissue by varying these parameters. Thus, a variety of synthetic and biopolymers can be electrospun at the micro- or nanoscale (Sell *et al.* 2009). Nanofibrous matrices electrospun from ECM proteins such as collagen I or hyaluronic acid mimic the natural ECM exactly and therefore ensure excellent cell attachment, cell viability and differentiation (Barnes *et al.* 2007). As mentioned before, biopolymers often lack the suitable stability for *in vivo* application, whereas the hydrophibicity of synthetic materials prevents rapid cell attachment. Again, the special demands of skeletal muscle TE can be met by combination of synthetic polymers and biopolymers. Different polymers can therefore be combined primarily by spinning polymer-blend solutions, core-shell spinning or co-spinning of different polymer solutions. Methods for secondary surface modification are coating

(Riboldi *et al.* 2005) or plasma treatment (Martins *et al.* 2009) of the matrix after the spinning procedure. Blending different polymers, e.g. PCL and collagen (Fig. 3), is a very simple method to generate composite matrices that combine the properties of both polymers depending on the ratio (Schnell *et al.* 2007).
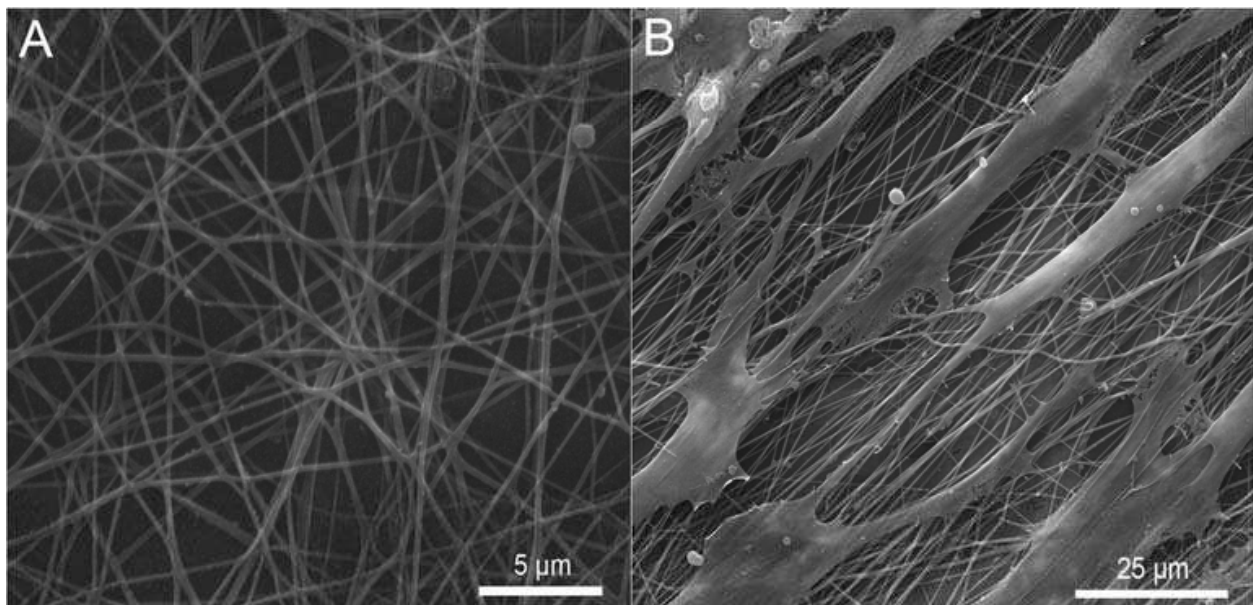

Fig. 3. Scanning Electron Microscopy (SEM) of electrospun PCL-collagen blend nanofibers. A: Randomly spun nanofibers. Magnification 10000x. B: Muscle precursor cells cultured on electrospun nanofibers with parallel alignment. The cell growth along the fibers' direction is clearly visible. Magnification 2500x

The more complex core-shell-spinning technique uses two separate polymers which are electrospun co-axially with the second polymer surrounding the first polymer at the core. Zhang *et al* used PCL as core fiber with a shell of collagen. They proved the core-shell-nanofibers to be superior to collagen-coated PCL fibers regarding cell attachment *in vitro* (Zhang, Y.Z. *et al.* 2005). Jiang and co-workers have introduced electrospun core-shell fibers as drug delivery system (DDS) (Jiang *et al.* 2005). Thereafter, the emerging field of nanofibers and nanoparticles as DDS has found its way into TE research (Sill & von Recum 2008). Controlled release of different drugs e.g. growth factors (Sahoo *et al.*) or angiogenic factors (Yang *et al.*) upgrades nanofiber matrices into "smart" matrices (Moroni *et al.* 2008).

However, electrospinning of aligned nanofibrous matrices is linked with poor control of the pore size as the main disadvantage. The generation of electrospun three-dimensional matrices, especially, results in densely packed scaffolds (fig. 3) that hinder cell infiltration (Baker & Mauck 2007, Telemeco *et al.* 2005). Therefore, co-spinning of water-soluble sacrificial fibers such as poly-(ethylene-oxide) (PEO) has been shown to overcome this problem (Baker *et al.* 2008). The sacrificial PEO fibers are interspersed inside the three-dimensional matrix and dissolve easily in water and alcohol during sterilization procedure before cell seeding. The resulting interspaces between the residual fibers then enable cells to migrate through the matrix (Baker *et al.* 2008). Though the control of pore size and interspaces in orientated nanofiber matrices is still challenging, the electrospinning technique holds great potential for TE and regenerative medicine and therefore pretends to be the most promising matrix for skeletal muscle TE at the moment.

3. Cell source

3.1 The satellite cell

Satellite cells form the major source for muscle regeneration *in vivo* after injury (Snow 1977). First described by Mauro in 1961 by electron microscopy, the term "satellite cell" was initially used for resident cells beneath the basal lamina of mature skeletal muscle fibers (Mauro 1961). Meanwhile, the "genetical footprint" of this cell population is well-known and satellite cells are specifically identified by expression of the transcription factor Paired-box 7 (Pax7) (Seale *et al.* 2000). Furthermore, satellite cells express MyoD (also known as Myf5; Fig. 4), M-cadherin, c-Met, syndecan-3 and 4 (Cornelison *et al.* 2001) and CD 34 (Beauchamp *et al.* 2000). In the past, it has been a point of discussion whether satellite cells are stem cells or myogenic progenitor cells (Zammit *et al.* 2006). Kuang *et al* proved that a small sub-population, i.e. 10% of satellite cells, shows stemness properties and repopulate the satellite cell niche *in vivo* (Kuang *et al.* 2007). These true stem cells are positive for Pax7 but negative for MyoD, whereas the majority of satellite cells are also positive for MyoD. The myogenic transcription factor MyoD marks the commitment of activated satellite cells to the myogenic line (Weintraub *et al.* 1991).
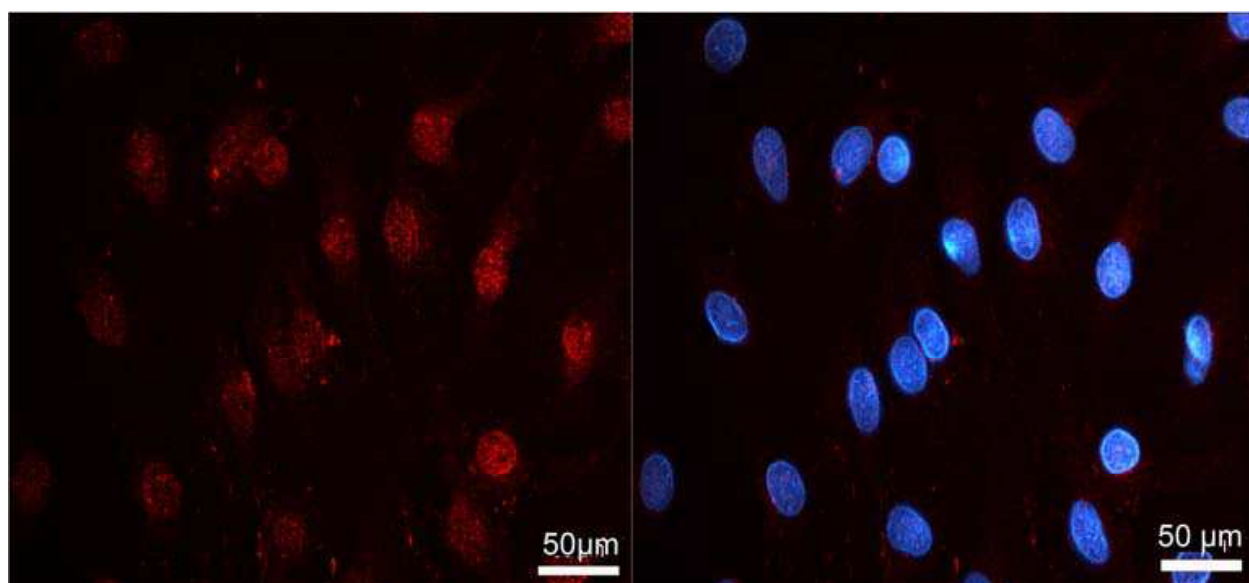

Fig. 4. Muscle precursor cells (MyoD positive cells) *in vitro*. Left side: Immnofluorescent staining for MyoD which is mainly located at the nuclei. Right side: Merge of MyoD staining and DAPI-counterstain. Magnification 400x

This myogenic imprinting renders the satellite cell to be a safe cell source for *in vivo* as well as clinical application without risking dedifferentiation and tumorigenesis. Therefore, satellite cells are the preferred cell source for clinically orientated muscle TE research (Otto *et al.* 2009). Kuang and his group have demonstrated that the Pax7+/MyoD+ cell population is renewed by the Pax7+/MyoD- cells through asymmetric self-renewal (Kuang *et al.* 2007). MyoD-positive cells in turn regenerate injured muscle tissue by differentiation into new muscle fibers. Thus, even large muscle tissue defects can be regenerated by a relatively small cell population *in vivo* (Collins *et al.* 2005, Le Grand & Rudnicki 2007). Unfortunately, this astonishing potential of self-renewal and myogenic differentiation of satellite cells *in vivo* is usually lost when satellite cells are isolated and cultured *in vitro* (Yaffe 1968). Boonen and

his group proposed the loss of the satellite cell niche at the basal lamina *in vivo* (Boonen & Post 2008). This highly specific niche preserves the stem cell status of quiescent satellite cells (Blau *et al.* 2001). Isolated satellite cells increase their expression of MyoD and differentiate after loosing contact with the basal lamina and thus loose their proliferative potential. Therefore, the generation of suitable numbers of satellite cells for muscle TE by *in vitro* culture and expansion is still challenging. Recently, Gilbert *et al* have demonstrated that the satellite cell function depends on substrate elasticity (Gilbert *et al.*). The group found the best proliferative potential of isolated satellite cells when cultured on PEG hydrogels cross-linked with laminin with an elasticity of 12 kPa which equals the elasticity of muscle tissue *in vivo*. After implantation of the cultured cells in a muscle injury model *in vivo* the engraftment rate was even comparable to freshly isolated and directly implanted satellite cells. Hence, Gilbert and co-workers showed that the satellite cell niche can be mimicked *in vitro* and thus the proliferative potential of cultured satellite cells can be preserved.

Recently, the existence of yet another cell population in adult skeletal muscle tissue has been proved: The telocyte was described by Popescu *et al* in cardiac muscle tissue first (Popescu & Faussone-Pellegrini). The typical shape of telocytes with their prolongations ("telopodes") situated in the vicinity of nerves, vessels and cardiomyocyte progenitors suggests a role in intercellular signalling as regulators in myocardial regeneration and as "nursing cells" for cardiac progenitors (Gherghiceanu & Popescu). In a recent study, Popescu and his group have identified telocytes also in skeletal muscle tissue (Popescu 2011). Beside their typical prolongations, telocytes are known to express c-kit and caveolin-1, but are Pax7 negative and thus differ from the satellite cell population. Additionally, telocytes secrete VEGF (Suciu *et al.*).

3.2 Stem cells

Stem cells of different origin offer a unique proliferation potential as the main advantage. To date, adult stem cells play the most important role in TE research, though other sources exist (embryonic or induced pluripotent stem cells (iPSC) e.g.) (Klumpp *et al.*). Since engineering of three-dimensional tissue of skeletal muscle asks for a large quantity of muscle cells, adult stem cells are a suitable cell source in TE research and regenerative medicine (Barile *et al.* 2009, Mollmann *et al.* 2009, Roche *et al.* 2009). Therefore, mesenchymal stromal cells (MSC) are a feasible alternative cell source for skeletal muscle TE due to their high proliferation rates *in vitro* and their low imunogenicity *in vivo* (Chen, L. *et al.* 2009) that even enables allogenic transplantation of MSCs (García-Castro J 2008, Rossignol *et al.* 2009). MSCs can be derived from different tissues, e.g. from bone marrow (BMSC) or adipose tissue derived (ADSC) (Deans & Elisseeff 2009). BMSCs are well-known and have been widely used for cytotherapy in regenerative medicine (Brazelton *et al.* 2003). However, in case of skeletal muscle TE, ADSCs should be preferred due to higher potential for myogenic differentiation as well as higher proliferation rate compared to BMSCs (Kern *et al.* 2006, Zhu *et al.* 2008). Still, the experiences of *in vivo* studies revealed a poor incorporation rate of transplanted MSCs into myofibers (Gussoni *et al.* 1997), ranging between 5 and 10% of the transplanted MSCs in DMD patients (Brazelton *et al.* 2003, Gussoni *et al.* 1997). Low incorporation rates are the main obstacle for cytotherapie in clinical settings. Though, Satija and co-workers proposed paracrine effects of transplanted MSCs *in vivo* as an important therapeutic effect (Satija *et al.* 2009). Therefore, transplanted MSCs secrete different cytokines resulting in anti-inflammatory, angiogenic and anti-apoptotic effects (Meirelles Lda & Nardi 2009, Sze *et al.* 2007) and thus facilitate local endogenous tissue repair (Nesselmann *et al.* 2008). Estrada and his group

explained the angiogenic effect of MSC through their secretion of Cyr61 (Estrada *et al.* 2009) a key factor for angiogensis and tissue repair *in vivo* (Mo *et al.* 2002). Estrada *et al* demonstrated that the sole addition of MSC secretome stimulates angiogenesis *in vitro* and *in vivo*.

Though their poor incorporation into myofibers and – compared to satellite cells - less effective myogenic differentiation *in vivo*, the paracrine effects of MSCs could augment the cell viability and myogenic differentiation of co-transplanted satellite cells.

4. Cell survival *in vivo* / vascularization

Whereas the generation of two-dimensional skeletal muscle tissue *in vitro* has been demonstrated by several groups before (Dennis *et al.* 2001, Strohman *et al.* 1990), engineering three-dimensional muscle tissue exceeding the size of 1 mm *in vitro* is still a challenge. Since common in vitro cultures of muscle precursor cells depend on diffusion solely, the thickness of generated tissue is limited to 500 µm to prevent apoptosis of cells in the central region of the construct (Kannan *et al.* 2005). Herein, Freed and co-workers proved the superiority of dynamic flow culturing due to enhanced diffusion capacity compared to static culture conditions (Freed *et al.* 1994). Still, the *in vitro* generation of relevant tissue sizes asks for an adequate vascularization. Levenberg and his group proved that vascularization of skeletal or cardiac muscle in vitro is possible and enhances the transport of nutrients and metabolites (Lesman *et al.* 2010, Levenberg *et al.* 2005). In their study they co-cultured muscle precursor cells with embryonic fibroblasts and endothelial cells seeded into a 3D polymer scaffold. When implanted *in vivo*, the *in vitro* generated vessels connected to vessels of the host and the tissue showed less apoptosis (Levenberg *et al.* 2005). However, even this approach does not meet the demands of a clinical setting, since the muscle tissue, engineered *in vitro*, requires an axial vascularization to enable the transplantation *in vivo* including a microsurgical anastomosis to the recipient site. In most *in vivo* experiments, matrices and muscle precursor cells are implanted subcutaneously leading to random vessel ingrowth from the constructs' periphery. In contrast to subcutaneous *in vivo* models, O. O. Erol and M. Spira introduced the arterio-venous (AV) loop model of the rat in 1980 (Erol & Sira 1980). For this *in vivo* model an AV-loop is created microsurgically between the saphenous artery and vein (Fig. 5) which can be implanted into various matrices (Polykandriotis *et al.* 2008).

Thus, vascularization in general as well as number and pattern of vessel ingrowth of different matrices can be analyzed (Arkudas *et al.* 2010, Polykandriotis *et al.* 2009) and the vascularized matrix offers a platform for tissue engineering for skeletal (Messina *et al.* 2005) or cardiac (Morritt *et al.* 2007) muscle *in vivo*. The feasibility of the AV-loop model in large animals (Beier *et al.* 2009) has been demonstrated recently and poses another step towards a more clinical setting (Beier *et al.* 2010).

Depending on the matrix architecture, a certain period of time is necessary for vascularization of the whole construct. This pre-vascularization time plays an important role for survival rates of implanted cells *in vivo*: Thus, cells implanted after this time period show significantly lower apoptosis rates (Arkudas *et al.* 2007). Vascular growth factors such as bFGF and VEGF (Yancopoulos *et al.* 2000) are frequently used to reduce the pre-vascularization time *in vivo* (Arkudas *et al.* 2007). Therefore, different approaches have been tried for controlled drug release of VEGF. Beside the use as soluble factor or immobilized in fibrin hydrogels (Arkudas *et al.* 2007), VEGF can be bound to nanoparticles (des Rieux *et al.*) or nanofibers (Vournakis *et al.* 2008) as drug delivery systems (DDS) to improve angiogenesis *in vivo* (Zisch *et al.* 2003). Kim *et al* have demonstrated the positive therapeutic

effect of nanoparticle based VEGF release in ischemic muscle tissue (Kim, J. *et al.*). Hypoxia-regulated systems enable an even more selective delivery of VEGF to ischemic sites only, e.g. in myocardial repair (Ye *et al.*). Finally, the VEGF expression of (co-) implanted MSCs could also enhance angiogenesis *in vivo*.
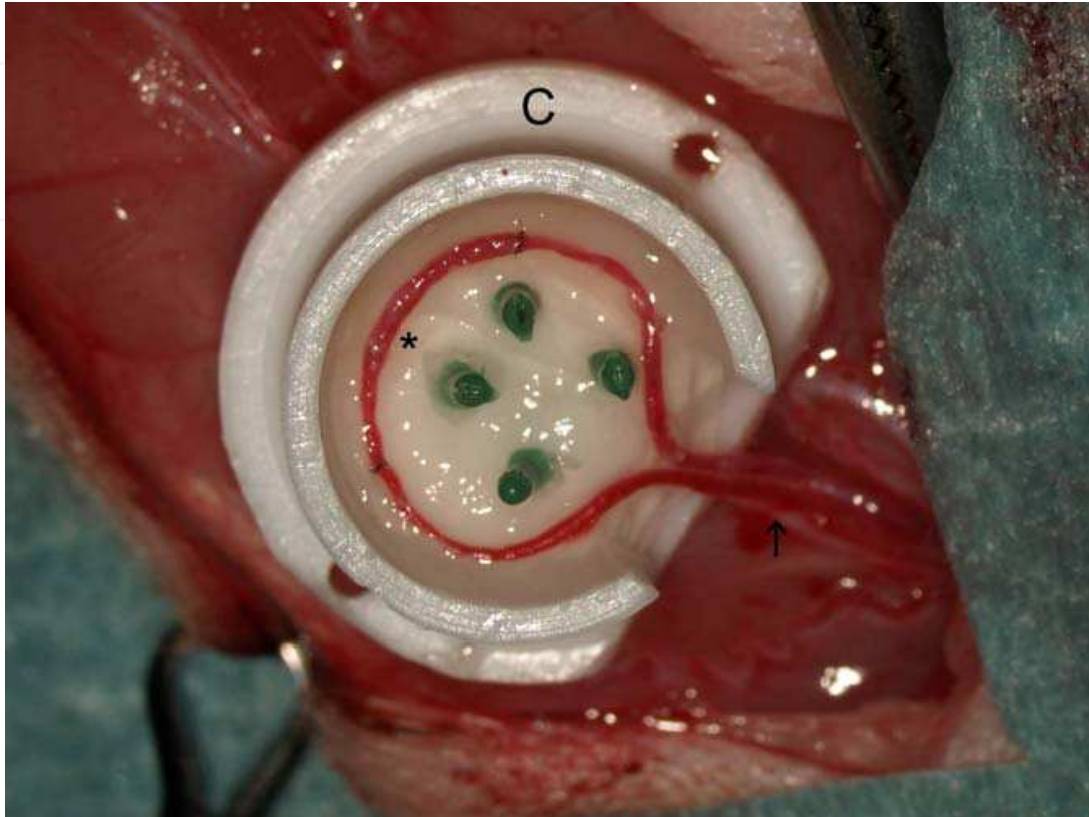

Fig. 5. Arterio-venous loop model in the rat as previously published by our group. The AV-loop (asterisk) is implanted into a Teflon chamber (C) filled with fibrin hydrogel. In this setting, the engineered tissue could be transplanted by anastomosing the pedicle (arrow) to the recipient site

Beside angiogenic growth factors, other factors such as insulin-like growth factor-1 (IGF-1) have been shown to increase survival rates of implanted cells *in vivo* (Wang, F. *et al.* 2009) and to improve myocardial regeneration (Davis *et al.* 2006, Padin-Iruegas *et al.* 2009). Thus, various methods exist to further ameliorate cell survival *in vivo* but still have to be analyzed in detail for their benefit for skeletal muscle TE.

5. Myogenic differentiation

5.1 Molecular factors for myogenic differentiation
Beside the improvement of cell survival rates, the myogenic differentiation of implanted muscle precursor cells into functional skeletal muscle tissue *in vivo* is another point that has to be addressed by future research. To date, many different molecular factors have been identified which support myogenic differentiation, e.g. akirin-1, muscle specific microRNAs and insulin-like growth factor (IGF-1).
The well-known factor IGF-1 has been demonstrated to increase the proliferation as well as myogenic differentiation of myoblasts *in vitro* by Allen and Boxhorn (Allen & Boxhorn

1989). Later on, this effect was affirmed in different *in vivo* experiments with overexpression of IGF-1 leading to muscle hypertrophy (Adams & McCue 1998) and improving muscle regeneration after trauma (Menetrey *et al.* 2000, Sato *et al.* 2003). Whereas many growth factors increase proliferation rates or differentiation of myoblasts only, IGF-1 enhances both, proliferation as well as myogenic differentiation of muscle precursor cells (Ten Broek *et al.*). Furthermore, IGF-1 overexpression improves survival rates of implanted cells *in vivo* (Wang, F. *et al.* 2009). These properties render IGF-1 as one of the most potential growth factors for myogenesis and skeletal muscle TE. Beside the mitogenic potential of IFG-1 and its positive influence on myogenesis, Haider *et al* have also shown that IGF-1 can mobilize stem cells and increase engraftment of implanted MSCs *in vivo* (Haider *et al.* 2008). The group explained this effect of IGF-1 through its activation of stromal cell derived factor (SDF)-1α and its receptor CXCR4. SDF-1α plays a crucial role in skeletal muscle regeneration and is therefore overexpressed after muscle injury as well as in dystrophic muscle to attract muscle precursor cells which express CXCR4 (Perez *et al.* 2009). Therefore, overexpression of SDF-1α through transfected MSCs (Haider *et al.* 2008) or via controlled drug release (Grefte *et al.*) improves regeneration of skeletal as well as cardiac muscle *in vivo*.

Another factor which influences early myogenic differentiation positively is akirin-1 (also known as Mighty) (Salerno *et al.* 2009). In skeletal muscle tissue, akirin-1 is known to activate quiescent satellite cells and thus promote proliferation of muscle precursor cells. Furthermore, akirin-1 consecutively induces the expression of IGF-2 and hence also increases myogenic differentiation indirectly (Marshall *et al.* 2008). Therefore, akirin-1 combines the activation of quiescent satellite cells with the promyogenic effect of downstream growth factors such as IGFs. Though promising for skeletal muscle TE, the mechanism and molecular pathways of akirin-1 still have to be analysed in detail in future. In addition, the administration of growth factors has to be critically analyzed concerning their risk of tumorigenicity *in vivo*.

Recently, a novel class of regulating factors of myogenesis has been analyzed for their promyogenic potential: Small non-coding RNAs, called microRNA (miRNA) which consist of approximately 20-22 nucleotides (Callis *et al.* 2008). Herein, certain microRNAs (miR-1, miR-133 and miR-206) have been demonstrated as muscle specific. Whereas miR-1 and miR-133 are also expressed in cardiac muscle, miR-206 is specifically expressed in skeletal muscle tissue and up-regulated in patients with muscular dystrophy (Eisenberg *et al.* 2009). Furthermore, the muscle-specific miRNAs differ in their effect on muscle precursor cells. MiR-133 increases proliferation of muscle precursor cells but also inhibits myogenic differentiation (Chen, J.F. *et al.* 2006). On the contrary, miR-1 and miR-206 have been shown to induce myogenic differentiation (Chen, J.F. *et al.* 2006, Kim, H.K. *et al.* 2006). In a recent study, Nakasa et al demonstrated that local injection of miR-1, miR-133 and miR-206 improves muscle regeneration and prevent fibrosis following muscle injury *in vivo* (Nakasa *et al.*). However, further studies are still necessary to analyze the promyogenic potential of muscle-specific microRNAs *in vitro* and *in vivo*.

5.2 Electrical stimulation and neurotization

Despite great efforts in the past and various molecular factors which regulate and enhance myogenesis, engineering of mature skeletal muscle tissue still remains a big challenge. Though contracting myotubes – which mark the differentiation and fusion of myoblasts in myogenesis - have been generated by various groups, the generation of adult muscle fibers depends on neural or electrical stimulation (Wilson & Harris 1993). The influence of electrical stimulation on further myogenic differentiation has been analyzed *in vitro*

(Donnelly *et al.*, Stern-Straeter *et al.* 2005) and *in vivo* (Fujita *et al.* 2007). For clinical applications, devices for electrical stimulation have to be implantable and suitable for long-term stimulation (Jarvis & Salmons 2001, Lanmuller *et al.* 2005). The *in vivo* experiments of Dennis and co-workers using implantable stimulation devices in the rat demonstrated that muscle mass as well as the maximum force of a denervated muscle can be maintained by electrical stimulation (Dennis *et al.* 2003). Thus, the physiologic stimulation via motoric innervation can be simulated to support further myogenic differentiation.

Liao *et al* showed that the combination of electrical stimulation with aligned micropatterned matrices even increases the positive effect on myogenic differentiation (Liao *et al.* 2008). Again, electrospun nanofibrous matrices offer the possibility to combine cell guidance through aligned matrix architecture with electrical stimulation via conductive nanofibers. Ghasemi-Mobarakeh and colleagues used electrospun PANi/PCL/gelatine-blend fibers as matrix for neural cell cultivation. Applying electrical stimulation to the matrices, they demonstrated enhanced cell proliferation and neurite outgrowth (Ghasemi-Mobarakeh *et al.* 2009). The use of PANi/gelatine-blend fibers offers an acceptable cell attachment *in vitro* and can be used for cultivation and electrical stimulation of muscle cells *in vitro* (Li *et al.* 2006). Finally, the influence of electrical stimulation on C2C12 murine myoblasts was analyzed by Jun and his group *in vitro*. In their experiments they found that electrical stimulation enhances myogenic differentiation via upregulation of myogenin which specifically marks early myogenesis (Jun *et al.* 2009).

However, engineering functional skeletal muscle tissue *in vivo* not only asks for highly differentiated and organised muscle fibers but also the formation of neuromuscular junctions and neurite ingrowth between the muscle fibers is necessary (Fig. 6).
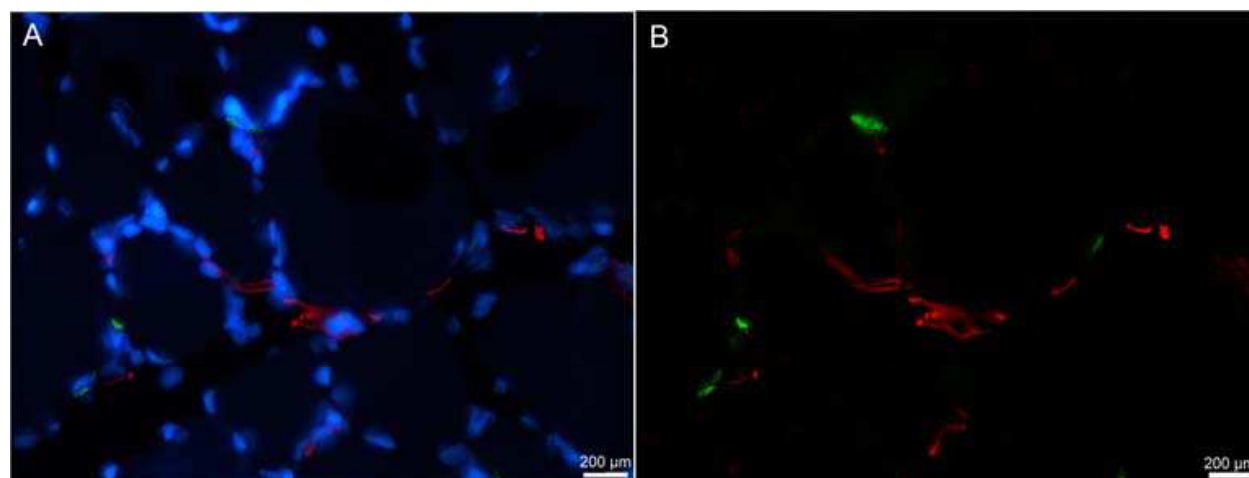

Fig. 6. Immunofluorescent staining of a cross-section of adult skeletal muscle. A: The nuclei are stained with DAPI (blue) and mark the outline of the muscle fibers. The acetylcholine receptors (specifically stained with α–bungarotoxin; green) are reached by nerve terminals (stained for specific neurofilament (NF-100), red) forming the neuromuscular junction (B: DAPI-filter excluded). Magnification 400x

The main component of the neuromuscular junction – the acetylcholine receptor (AChR) – is initially expressed in developing myofibers also in absence of neural cells, i.e. independent from motoric innervation (Witzemann 2006). The AChR clusters at this stage of myogenesis are located at the central regions of myofibers and this phenomenon known as "prepatterning", marks the development of mature myofibers in myogenesis. However, the

accumulation of AChR at synaptic sites and further development into functional neuromuscular junctions depends on the specific neuronal factor agrin. Thus, the molecular signalling between developing myofibers and motor neurons is necessary for the generation of functional neuromuscular junctions (Brockhausen *et al.* 2008). The motoric innervation even defines further maturation of the developing muscle tissue into slow- or fast-twitching muscle fibers (Nehrer-Tairych *et al.* 2000). Dhawan *et al* proved that motoric neurotization of implanted muscle precursor cells *in vivo* leads to the formation of neuromuscular junctions (Dhawan *et al.* 2007). In a comprehensive study, they showed nerve-induced contractions of the *in vivo* engineered skeletal muscle tissue after explantation and analysis *in vitro*. Therefore, a successful approach for skeletal muscle TE *in vivo* will necessarily include a motor nerve for neurotization of implanted muscle precursor cells. But to date, *in vivo* models combining motoric neurotization with a pre-vascularized matrix are still rare. Recently, a new AV-loop model in the rat including motoric neurotization has been developed by our group (unpublished data).

6. Conclusions

As a conclusion, the main challenges in skeletal muscle TE are therefore: (1) engineering a suitable matrix for muscle TE including a clinical application, (2) improving further myogenic differentiation *in vivo* and (3) enabling the transplantation of functional skeletal muscle tissue to the recipient site including microsurgical anastomosis of an adequate vasculature as well as motoric neurotization of the engineered muscle tissue. Despite these obstacles, the achievements of the recent years demonstrate an encouraging progress of skeletal muscle TE research. Therefore, Churchill's statement concerning skeletal muscle TE *in vitro* may still come true in the future.

7. References

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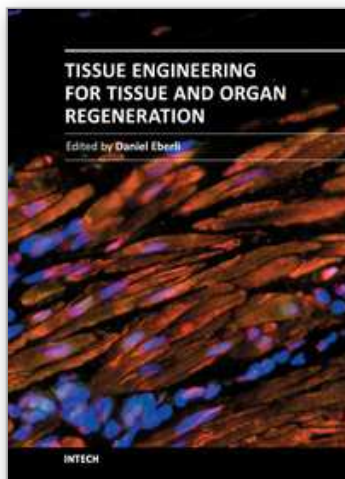
Tissue Engineering for Tissue and Organ Regeneration

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Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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