

Engineering skeletal muscle tissue – new perspectives *in vitro* and *in vivo*

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Abstract

Muscle tissue engineering (TE) has not yet been clinically applied because of several problems. However, the field of skeletal muscle TE has been developing tremendously and new approaches and techniques have emerged. This review will highlight recent developments in the field of nanotechnology, especially electrospun nanofibre matrices, as well as potential cell sources for muscle TE. Important developments in cardiac muscle TE and clinical studies on Duchenne muscular dystrophy (DMD) will be included to show their implications on skeletal muscle TE.

Keywords: skeletal muscle tissue engineering • satellite cells • electrospun nanofibre matrices • nanotechnology

Introduction

Tissue engineering (TE) represents a scientific approach focussing on the emulation of neo-organogenesis [1]. Research interest focuses on a variety of different tissues that might be recreated for replacement of lost tissues. After loss of skeletal muscle tissue new solutions might be brought about for the treatment of a variety of muscle diseases, including skeletal myopathies such as muscular dystrophy or spinal muscular atrophy [2, 3]. Furthermore localized loss of skeletal muscle tissue, as a result of traumatic injury, aggressive tumour ablation or prolonged denervation, *e.g.* are common clinical problems. In the last years, important developments have changed the field of skeletal muscle TE [4, 5].

Since Vandenberg *et al.* introduced the 3D cultivation of primary myoblasts in collagen gel and generated contracting muscle tissue *in vitro* for the first time in 1988 [6], there have been developments in the field of skeletal muscle TE and new approaches and techniques have emerged. However, collagen as well as fibrin gels have proven unsuitable for TE due to their fast degradation

in vitro [7]. With regard to material science, recent developments in the field of nanotechnology have brought up new possibilities which will be highlighted in this review. Two years after Vandenberg's 3D cultivation of myoblasts in collagen gels, Strohman and colleagues showed that monolayers of differentiating myoblasts detached from the membrane and formed 3D and contractible muscle tissues termed myoids [8]. Though the method of generating myoids was brought to perfection by Dennis and coworkers, the *in vitro* engineered muscle constructs did not exceed a diameter of 1 mm [9]. These *in vitro* studies led to a better understanding of myogenic differentiation and contractility. Yet, the nutrient supply for the cells in this setting depends solely on diffusion. Because diffusion capacity is limited to distances of less than 500 μm [10], nutrient supply in the central parts of engineered tissue or 3D constructs becomes deficient. Levenberg and coworkers showed that vascularized muscle tissue and recently also vascularized cardiac muscle tissue can be engineered *in vitro* by co-culturing myoblasts, embryonic fibroblasts

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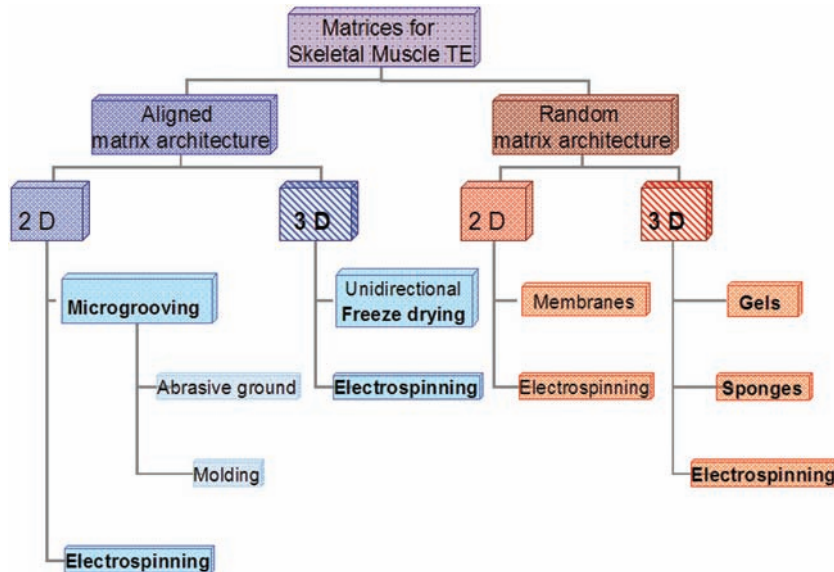


Fig. 1 Matrices for skeletal muscle TE can be divided into aligned and random matrices. Electrospinning enables the generation of aligned and random as well as 2D and 3D matrix architecture.

and endothelial cells in a 3D polymer scaffold [11–12]. The pre-vascularized constructs showed less apoptosis of myoblasts after transplantation *in vivo* [12]. However, in clinical application, the transfer of larger muscle constructs to the site of the defect requires an axial vascularization of the engineered muscle tissue. Furthermore, the use of embryonic cell sources is a critical issue in medical as well as in ethical terms. Therefore, several *in vivo* studies [13–15] have used the microsurgical AV-loop model in the rat first described by Erol and Spira in 1980 [16] to generate an axial vascularization. In combination with secondary cell transfer to axially pre-vascularized matrices this well-established model might be the most promising step towards a future application of large tissue-engineered constructs in a clinical scenario [17]. Recently, the rat AV-loop model was translated into the sheep AV-loop model for large constructs [18–19].

Despite these encouraging developments there are further obstacles for a bench-to-bedside approach of muscle TE which have to be addressed, a suitable cell source and myogenic differentiation being the most urgent. Recent developments in the field of myogenic differentiation *in vitro* and *in vivo* will be discussed in this review, including findings from clinical Duchenne muscular dystrophy research and TE of cardiac muscle.

Matrices for skeletal muscle tissue engineering – gaining orientation

Regarding the architecture, matrices can be divided into randomly orientated scaffolds and matrices with a certain alignment (Fig. 1). Commonly used matrices with random orientation, *e.g.* gels and sponges, can be used for a variety of tissues. However, in case of

skeletal muscle this tissue naturally consists of bundles of highly oriented muscle fibres in an extracellular 3D matrix to form an organized tissue with high cell density. The parallel orientation of muscle fibres guarantees the generation of longitudinal force after contraction that is induced by motoneuron activity [20] *in vivo*. Another key factor in TE is the composition of the extracellular matrix (ECM) which plays an important role in the alignment and differentiation of myoblasts [21–22]. Ideally the ECM constitutes the framework for cell adhesion and tissue growth, which includes cell proliferation and differentiation. The parallel alignment of the natural ECM in skeletal muscle tissue can be mimicked by matrix architecture. Different methods are in use to achieve parallel alignment depending on the question whether 2D or 3D matrices should be used. Regarding 2D scaffolds, alignment can be generated by electrospinning as well as microgrooving. The method of microgrooving uses either abrasives to directly grind microgrooves into the matrix as described by Shimizu *et al.* [23] or generating micropatterned moulds and casting the liquid matrix material onto the mould as described by Walboomers and others [24–25]. The fabrication of micropatterned matrices has been developed for *in vitro* analysis of cell behaviour and differentiation on aligned surfaces and showed orientated cell growth of, *e.g.* fibroblasts [26–27], myoblasts [28] and neural cells [29–30] along the microgrooves. This phenomenon commonly termed as ‘cell guidance theory’ [24, 31] encourages and facilitates myogenic differentiation *in vitro* [32]. Flaibani and coworkers analysed electrical stimulation in addition to microgrooved poly-(L-lactic acid) membranes on the differentiation of muscle precursor cells [33]. This setting increased the myogenic differentiation of myoblasts even more than cultivation on micropatterned membranes without electrical stimuli [33]. Though very valuable for *in vitro* studies analysing cell structure and differentiation, this 2D method is not suitable for engineering 3D muscle tissue. For

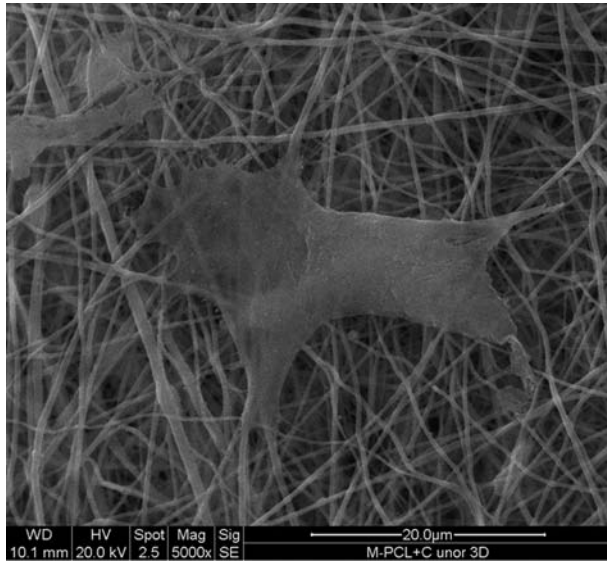


Fig. 2 Scanning electron microscopy: Myoblast growing on randomly electrospun 3D PCL-collagen matrix; 5000 \times magnification.

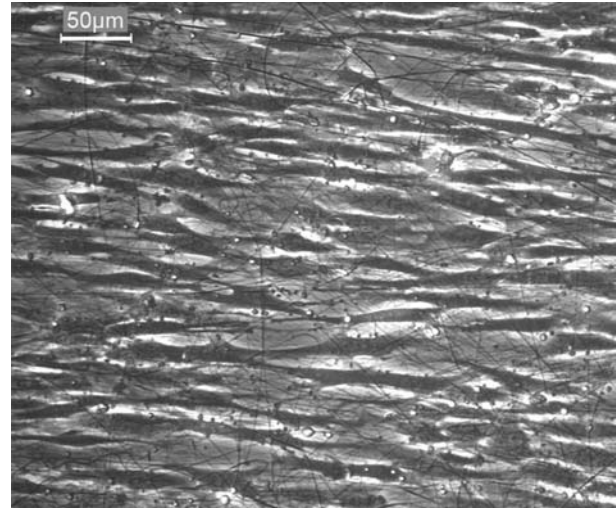


Fig. 3 Phase contrast microscopy: Myoblasts cultured on aligned PCL-collagen nanofibres; after 2 days in culture they align themselves along the nanofibres; 320 \times magnification.

engineering transplantable muscle tissue *in vitro* and for *in vivo* application of skeletal muscle TE a 3D approach is necessary.

Besides the electrospinning technique there are several alternative methods to generate spatially orientated structures in 3D matrices. Principally, the gradual formation of ice crystals induced by unidirectional freeze-drying of hydrogels results in highly porous scaffolds with aligned pores [34–35]. This method allows a fine adjustment of the average pore size by varying the freezing temperature [36]. A variety of different synthetic as well as biological materials can be processed as hydrogels by freeze-drying, *e.g.* collagen [34], fibroin [37] and composites [38]. Though these matrices have no limitations in their 3D size, the thickness and structure of matrix material between the pores is hardly controllable by the freeze-drying method. Therefore, further adjustments of the matrices including coating for controlled degradation or drug release as well as surface modification remain a challenge.

Electrospun nanofibre matrices

The electrospinning technique principally uses electrical voltage to form fibres in the range of micrometers down to a few nanometres. Though simple in its principle, electrospinning is a complex process that depends on a variety of parameters. This complexity elicits a tremendous spectrum of applications and variety of possibilities to adjust matrix properties as desired [39]. A great diversity of synthetic polymers as well as biopolymers can be used to generate electrospun nanofibres including proteins of the natural ECM such as collagen [40–41], elastin [40–41] and hyaluronic

acid [42]. Matrices composed of electrospun ECM proteins uniquely mimic the structure of the natural ECM which is of utmost importance for attachment, viability and differentiation of cells seeded into nanofibrous matrices [43–45]. The aforementioned advantages of biopolymers are often combined with the higher stability of synthetic polymers such as poly-(epsilon-caprolactone). Matrices solely composed of synthetic polymers, *e.g.* PCL [46] or poly-(ester-urethanex) [47] show high stability but also low elasticity and sparse cell attachment. Several methods exist to enhance cell attachment to synthetic polymer nanofibres. A commonly used method is to coat the nanofibre matrix with biopolymer solution after electrospinning. In the study of Riboldi *et al.*, microfibrillar randomly spun PU matrices were coated with Matrigel™, an extract from the Engelbreth–Holm–Swarm mouse sarcoma. Due to its origin, Matrigel™ is inappropriate for a clinical setting and its use is limited to experimental models only. Alternatively, synthetic polymers can be blended with biopolymers before electrospinning (Fig. 2). PCL-collagen blend nanofibres show a significantly increased cell attachment dependant on the percentage of collagen [44]. Furthermore, co-axial electrospinning of two different polymer solutions results in fibres surrounded by the second polymer. This core-shell technique enables the processing of synthetic polymers with a shell of biopolymers. Zhang and coworkers could show that cell attachment on electrospinning core-shell nanofibres of PCL (core) and collagen (shell) is even further increased as compared to the coating procedure [48].

As mentioned before, nanofibre matrices with parallel alignment (Figs. 3 and 4) prove as ideal scaffolds for cultivating myoblasts [49]. The generation of parallel fibre alignment can be easily achieved by using a rotating mandrel to collect the nanofibres [50]. However, the pore sizes of electrospun matrices with

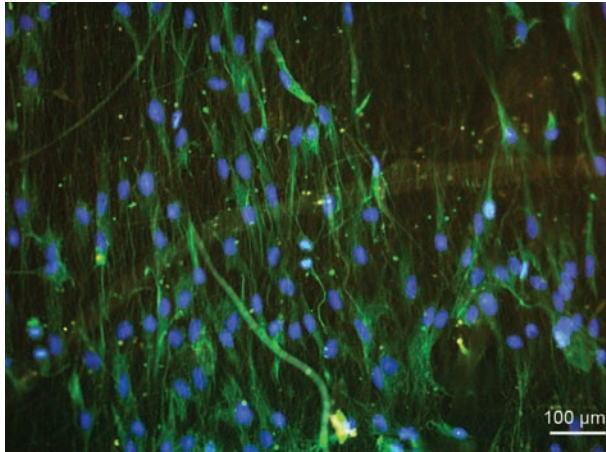


Fig. 4 Immunofluorescence staining: Myoblasts on aligned electrospun PCL-collagen nanofibres. Green: desmin; blue: DAPI counterstaining.

parallel fibre alignment are hardly controllable and have ever been a great challenge. Therefore, electrospinning of aligned nanofibres into 3D scaffolds usually results in densely packed nanofibres [51] leading to poor cell infiltration of slow degradable nanofibrous scaffolds *in vitro* and *in vivo* [52]. Baker and coworkers have introduced the method of co-spinning sacrificial fibres into 3D aligned nanofibre matrices [53]. The sacrificial fibres, *e.g.* water-soluble poly-(ethylene-oxide), are leached out after electrospinning and leave behind interspaces where cells can easily pass through [53]. Thus the interspaces between the aligned nanofibres can be augmented without increasing the fibre diameter.

Nanotechnology and smart matrices

The core-shell technique can also be used as drug delivery system (DDS), which has opened up a new opportunity to tailor 'smart' scaffolds with controllable drug release [54–55]. Nanoparticles and nanofibre matrices as drug delivery devices are an immensely emerging field in pharmacotherapy as well as for TE [56]. In case of Duchenne muscular dystrophy, the application of nanopolymer as DDS has already reached the stage of phase I clinical trials [57–58]. Nanofibre matrices as DDS also enable the administration of growth factors [59], angiogenic factors like VEGF [60–61], which can reduce the time of pre-vascularization of implanted matrices *in vivo* [62] as well as factors to enforce and promote cell differentiation. Regarding skeletal muscle TE, different factors are known to induce myoblast proliferation and myogenic differentiation, *e.g.* insulin-like growth factor-1 [63], nerve growth factor [64–65] or the synthetic pyrimidine MS-818 which is known as a neurotrophic factor but also accelerates muscle regeneration [66]. Smart nanofibrous matrices with controlled release of specific growth factors have already proven their potential in other fields

of TE [67–68], but for skeletal muscle TE this field still has to be investigated. However, due to the risk of tumorigenesis, the administration of growth factors in clinical applications is critical. Therefore, the definite risk has to be assessed in pre-clinical *in vivo* studies concerning local release of growth factors through DDS over distinct time periods.

Alternatively, electrical stimulation is a well-known factor for inducing differentiation in myoblasts [69] as well as cardiomyocytes *in vitro* [70]. Shafy *et al.* proved that simultaneous implantation of a pacemaker along with myoblasts into infarcted myocardium of sheep had a positive influence on differentiation of implanted myoblasts and led to better myocardial function [71]. As an alternative to the commonly used external electrical stimulation, Gaetani and coworkers analysed the influence of extremely low-frequency electromagnetic fields (ELF-EMF) on cardiac stem cells [72]. Their results show that exposure of cells to ELF-EMFs can induce differentiation of cardiac stem cells. Thus, the use of EMF could replace the implantation of pacemakers.

The electrospinning technique also provides the opportunity to generate electrically conductive matrices by spinning conductive polymers, such as poly(aniline) nanofibres (PANI). Ghasemi-Mobarakeh *et al.* showed that doped PANi blended with PCL and gelatine solution exhibit acceptable cell attachment [73]. Neural stem cells cultured on these PANi/PCL/gelatine-blend nanofibre scaffolds showed increased proliferation and neurite outgrowth when electrical stimulation was applied. A similar setting (PANI/gelatine blend nanofibres) was chosen by Li and coworkers to analyse the behaviour of rat cardiac myoblasts [74] on conductive nanofibrous scaffolds and by Jun and colleagues (poly(lactide-epsilon-caprolactone) [PLCL]/PANI blend nanofibres) using C2C12 murine myoblasts. Jun *et al.* showed that the expression of myogenin, a muscle specific protein that marks early myogenic differentiation, was significantly higher in myoblasts cultured on electrically conductive nanofibrous matrices than in myoblasts cultivated on PLCL scaffolds [75].

Cell sources for skeletal muscle TE

In opposite to the impressive developments in nanotechnology there are still great shortcomings on the cell side. Regarding the cell source, the satellite cell is the most prominent one (Fig. 5) [76–77]. The term 'satellite cell' is a histological description of undifferentiated cells residing between the sarcolemma and the basement membrane of muscle fibres but their exact biological characterization, their origin and differentiation potential and the question whether satellite cells are actually stem cells have been a point of discussion for a long time [78]. Today, it is common knowledge that satellite cells are characterized through the expression of the muscle-specific paired box (Pax) transcription factor Pax7 [79]. But it is also known that Pax7⁺ cells are a heterogeneous cell population consisting of a majority of Myf5⁺ cells and a minority (10%) of Myf5⁻ cells [80]. The expression of Myf5

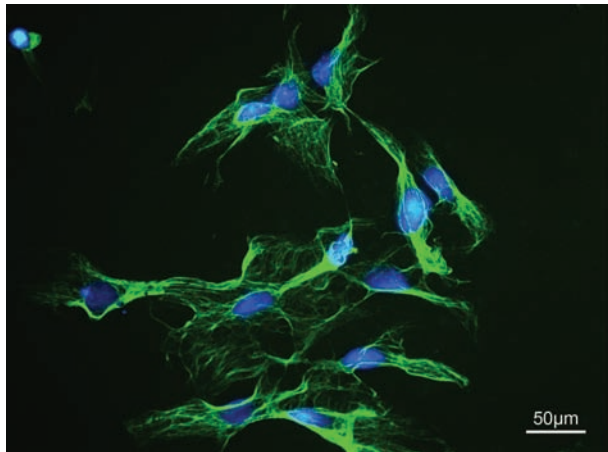


Fig. 5 Immunofluorescence staining of myoblasts isolated from skeletal muscle of the rat *in vitro*. Green: desmin (muscle specific cytoskeleton), blue: nuclei, DAPI counterstain, 400 \times magnification.

as an initiator of myogenic differentiation [81] marks the commitment of this cell population to the myogenic lineage [80]. Therefore, the majority of the satellite cell population actually consists of muscle precursor cells with a strong myogenic imprinting, whereas the Pax7⁺/Myf5⁻ subpopulation of the satellite cells (only 10%) show stem cell properties continuously renewing the Pax7⁺/Myf5⁺ cell population. High hopes have been built on this stem cell like subpopulation regarding their proliferative capacity *in vitro* because these cells are capable of allowing for regeneration of large parts of the musculature *in vivo* [82–83]. Yet, Yaffe and coworkers have demonstrated that isolated satellite cells undergo rapid dedifferentiation *in vitro* after few cell cycles [84]. Boonen *et al.* have explained this phenomenon by the loss of the highly specific stem cell niche which preserves normal function of satellite cells *in vivo* [85–86]. Therefore, *in vitro* expansion of satellite cells does not lead to an efficient amount of cells for muscle TE. Yet, the commitment to the myogenic line of the majority of satellite cells represents an unmatched myogenic potential and implies safety concerning clinical applications without the risk of tumour genesis.

As an alternative, mesenchymal stem cells (MSC) have been proposed for muscle TE [87]. Principally, MSCs can be derived from umbilical cord blood, bone marrow (BMSC) and adipose tissue. Though BMSCs have been commonly used, adipose-derived stem cells constitute an interesting alternative because they are easily accessible and show higher proliferation rates than BMSCs [88–89]. In addition to their high proliferation rates *in vitro*, MSCs have been shown to fuse with cardiomyocytes *in vitro* and *in vivo* [90]. Nevertheless, incorporation of MSC as well as transplanted satellite cells into skeletal myofibres *in vivo* remains a challenge. Brazelton *et al.* have reported an incorporation rate of 5% of transplanted MSCs into myofibres [91]. This low incorporation rate has contributed to the poor outcome of cytotherapy, *i.e.* the transplantation of satellite cells or MSCs with wild-type expression of dystrophin, as a treatment for DMD. Gussoni and coworkers

transplanted satellite cells into defect muscle tissue of Duchenne muscular dystrophy patients in a clinical trial. Their results revealed that only 10% of the transplanted myoblasts were left after 6 months [92]. However, MSCs impose an additional paracrine effect on differentiation and thus on tissue regeneration by different cytokines [93–94]. Thereby, transplantation of MSCs and satellite cells together could possibly augment the effectiveness of muscle TE *in vivo* by supporting satellite cell viability and myogenic differentiation. In the field of cardiac muscle TE, Gherghiceanu and Popescu have recently shown that a kind of interstitial Cajal-like cells, which are found in different tissues *in vivo* [95], function as ‘nursing’ cells for differentiating cardiomyocyte progenitors (CMP) [96]. These interstitial Cajal-like cells have been renamed by Popescu and coworkers as ‘telocytes’ due to their thin prolongations [95]. Telocytes in the cardiac muscle tissue seem to encourage and guide the fusion of differentiating CMPs to adult cardiomyocytes [96]. Further investigations may show, if similar mechanisms can be found in the skeletal muscle tissue.

In 2006, Takahashi and Yamanaka [97] have shown that adult fibroblasts can be transferred into pluripotent stem cells (PSCs) which by definition show differentiation into all three germ layers *in vitro* and form teratoma *in vivo*. Thus, these induced PSCs (iPSCs) opt as an alternative source to MSCs because cell proliferation and pluripotency of iPSCs are comparable to embryonic stem cells, without including the ethical difficulties that are implied with the use of embryonic stem cells. The role iPSCs could play in the future for regenerative medicine has been reviewed recently by Lee *et al.* [98] as well as Amabile and Meissner [99]. In case of cardiac regenerative medicine, iPSCs have been proposed by Yuasa and Fukuda [100] for the therapy of heart failure, though studies with clinical implications are still rare. Recently, Moretti and coworkers have isolated and induced adult dermal fibroblast from patients with long-QT syndrome, a hereditary heart disease which can cause sudden cardiac death [101]. The generated iPSCs were successfully differentiated into cardiomyocytes, but also showed prolonged action potential typical for the long-QT syndrome because the cells maintained their original genotype. Hanna and his group successfully combined iPSC cell therapy with gene therapy for the treatment of sickle cell anaemia in a humanized mouse model [102]. Before transplantation the mutation causing sickle cell anaemia was corrected and the generated iPSCs were differentiated into haematopoietic progenitor cells. Thus, iPSCs have already proven their potential for regenerative medicine. However, research on iPSC is still in its early stages and many shortcomings have to be addressed before clinical applications are conceivable [103]. One of these shortcomings is the use of lenti- or retroviral vectors for transfection of the four so called ‘Yamanaka factors’ – Oct4, Sox2, Klf4 and c-Myc – which are necessary for the reprogramming of adult dermal fibroblasts. To circumvent the risk of insertion mutation caused by lentiviral or retroviral transfection, Kaji *et al.* have used a virus-free vector for transfection [104]. Other methods for cell reprogramming like the use of microRNA are being discussed [105]. Another drawback is the risk of tumorigenicity that arises from the Yamanaka factors themselves because c-Myc and Klf4 are known oncogenes

[106–107]. Nagakawa and coworkers showed that c-Myc is not mandatory for the generation of iPSCs. Though fewer iPSCs are derived through this protocol, the group could also show that the tumorigenicity of c-Myc⁻ iPSCs is clearly lower. Whereas Okita *et al.* found tumours in approximately 20% of the F1 progeny mice derived from c-Myc⁺ iPSCs, the F1 mice derived from the Myc⁻ iPSCs in Nagakawa's study developed no tumours within 100 days after birth. Furthermore, Giorgetti *et al.* prove that iPSCs can even be generated by the use of only two factors, Oct4 and Sox2. Thus, iPSCs could play an important role in TE and regenerative medicine in the future. Apart from clinically orientated studies, further basic research is necessary to gain more security concerning tumour risk.

Conclusion

Essential problems of skeletal muscle TE such as cell expansion *in vitro* and myogenic differentiation *in vivo* are not solved to date, which hinders skeletal muscle TE from its clinical application. However, reviewing the recent developments in nanotechnology

and also taking into account other fields of research, *e.g.* cardiac muscle TE and clinical studies on Duchenne muscular dystrophy, huge leaps have obviously taken place. These results give rise to optimism regarding the clinical application of skeletal muscle TE in future.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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