JCMM
Available online at
www.jcmm.ro
www.jcmm.org

J. Cell. Mol. Med. Vol 10, No 3, 2006 pp. 716-726

A new approach to tissue engineering of vascularized skeletal muscle

A. D. Bach, A. Arkudas, J. Tjiawi, E. Polykandriotis, U. Kneser, R. E. Horch, J. P. Beier *

Department of Plastic and Hand Surgery, University of Erlangen, Erlangen, Germany

Received: May 9, 2006; Accepted: July 21, 2006

Abstract

Tissue Engineering of skeletal muscle tissue still remains a major challenge. Every neo-tissue construct of clinically relevant dimensions is highly dependent on an intrinsic vascularisation overcoming the limitations of diffusion conditioned survival. Approaches incorporating the arteriovenous-loop model might bring further advances to the generation of vascularised skeletal muscle tissue. In this study 12 syngeneic rats received transplantaion of carboxy-fluorescine diacetate-succinimidyl ester (CFDA)-labelled, expanded primary myoblasts into a previously vascularised fibrin matrix, containing a microsurgically created AV loop. As control cells were injected into fibrin-matrices without AV-loops. Intra-arterial ink injection followed by explantation was performed 2, 4 and 8 weeks after cell implantation. Specimens were evaluated for CFDA, MyoD and DAPI staining, as well as for mRNA expression of muscle specific genes. Results showed enhanced fibrin resorption in dependence of AV loop presence. Transplanted myoblasts could be detected in the AV loop group even after 8 weeks by CFDA-fluorescence, still showing positive MyoD staining. RT-PCR revealed gene expression of MEF-2 and desmin after 4 weeks on the AV loop side, whereas expression analysis of myogenin and MHC_{embryo} was negative. So far myoblast injection in the microsurgical rat AV loop model enhances survival of the cells, keeping their myogenic phenotype, within pre-vascularised fibrin matrices. Probably due to the lack of potent myogenic stimuli and additionally the rapid resorption of the fibrin matrix, no formation of skeletal muscle-like tissue could be observed. Thus further studies focussing on long term stability of the matrix and the incorporation of neural stimuli will be necessary for generation of vascularised skeletal muscle tissue.

Keywords: tissue engineering • myoblasts • skeletal muscle • vascularisation • arteriovenous loop

Introduction

Tissue engineering of skeletal muscle *in vitro* or *in vivo* holds promise for the treatment of skeletal muscle defects and might once provide an alternative to host muscle transfer. Skeletal muscle tissue engineering is based on the regenerative properties of the satellite cells or myoblasts and their potential for proliferation and differentiation, which can be harvested from adult and neonatal muscle of different species. Successful cultivation of these cells *in vitro* was established more than nearly two decades ago [1]. Cultures of primary satellite cells from myofibers grown *in vitro* are the preferred source of myoblasts used for Tissue Engineering purposes, since they recapitulate muscle development more precisely than commonly used immortal myogenic cell lines like C2C12 or L6 [2]. Studies aiming at the replacement of muscular tissues using tissue engineering methods have only recently started and many investigators have focussed on the creation of functional muscle tissues *in vitro* [3, 4]. However despite certain advantages of *in vitro* studies such as

* Correspondence to: J. P. BEIER (MD)
Department of Plastic and Hand Surgery, Head of Dpt. Prof. R. E. Horch, University of Erlangen Medical Center, Krankenhausstr. 12, 91054 Erlangen, Germany.
Tel.: ++49-9131-8533277
Fax: ++49-9131-8533297
E-mail: Justus.beier@chir.imed.uni-erlangen.de

Reprinted from:
Journal of Cellular and Molecular Medicine
doi:10.2755/jcmm010.003.14

controlled conditions and easier reproducibility certain limitations have to be considered: many results gained *in vitro* might not be transferred directly into the clinical setting, especially regarding the "matter of size". Every construct of neo-muscle, even if the demands for cell expansion and differentiation are met *in vitro,* has to be transplanted *in vivo* at one point. If the size of these constructs exceeds the limits of diffusion connection to a vascular axis becomes be necessary for sufficient oxygen supply. Hence the problem of pre-vascularisation, which arises with tissue engineering of every vascularised tissue, has been addressed by some investigators via the microsurgical arteriovenous-loop (AV loop) model [5]. So far these studies have shown the successful vascularisation of soft and hard matrices, but have not been combined with a clinically applicable approach using a biocompatible matrix together with primary expanded cells.

In the presented study we aimed at establishing a clinically feasible approach to the generation of vascularised skeletal muscle tissue: in syngeneic rats we microsurgically created AV loops which where embedded within a clinically approved fibrin matrix. After prevascularisation of the fibrin matrix expanded primary myoblasts, which were tested for expression of MyoD and labelled with carboxy-fluorescine diacetate-succinimidyl ester (CFDA) staining prior to transplantation, were injected into the axially vascularised matrix. Animals were sacrificed at three different time points and constructs were evaluated for presence and differentiation of transplanted myoblasts by fluorescence microscopy, immuncytochemistry and RT-PCR analysis.

Material and methods

Myoblast cell culture

Myoblasts were harvested as described previously for neonatal myoblasts [6]. Briefly primary rat myoblasts were obtained from hind limbs of adult syngeneic Lewis-rats. Satellite cells were dissociated from the minced muscles by digestion with 0.1% collagenase type III (Biochrom, Berlin, Germany) for 60 min and 0.25% trypsin (Viralex™ Trypsin / EDTA(1x), PAA Laboratories, Linz, Austria) for 45 min at 37°C. These cells were filtered through a sterile cell strainer (Beckton Dickinson, Franklin Lakes, NJ USA). Cells were grown in Dulbecco's modified Eagle's medium (D-MEM) containing 1% penicillin/streptomycin-solution (both from Life Technologies, Paisley, UK) and 10% foetal bovine serum (FBS). Medium was changed every three days. Cells were expanded through four passages by detachment using 0.25% trypsin, followed by resuspension, collection and seeding in new culture flasks at a ratio of 1:3.

Imunofluorescence staining for MyoD

Cultures were fixed in 100% methanol for 20 min at –20°C, washed with PBS and permeabilized with TRITON X-100™ (Sigma-Aldrich, Irvine, UK) 0.25% in PBS for 30 min. The cultures were then incubated with blocking solution (5% goat serum and 0.1% TRITON X-100™ in PBS) for 30 min. After being washed with PBS, the cultures were incubated with rabbit anti-MyoD polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:50 in blocking solution (2h at 20°C). The cultures were then washed with PBS and incubated with Cy™3-conjugated anti-rabbit IgG for 2h diluted 1:1500 in blocking solution. After being washed with PBS and mounted with Aquatex Mounting Media (EM Science, Gibbstown, NJ, USA), anti-MyoD-stained cultures were viewed and photographed by fluorescence microscopy (Leica Microsystems, Bensheim, Germany).

CFDA labelling

Myoblasts were detached as described above and incubated for 15 minutes with 10 mM freshly prepared CFDA (carboxy-fluorescine diacetate-succinimidyl ester, Vybrant CFDA SE Cell Tracer-Kit, Molecular Probes, Eugene, OR) reconstituted in DMSO and PBS according to the manufacturer's recommendations. After several washing and incubation steps cells were resuspended in fibrinogen solution and prepared for implantation into the vascularised fibrin matrix within the isolation chamber.

Animal model and surgical procedures

Syngenic male Lewis rats (Charles River Laboratories, Sulzfeld, Germany) served as donors and recipients. German regulations for the care and use of laboratory animals have been observed at all times. All experiments were approved by the animal care committee of the

University of Erlangen and the Government of Mittelfranken, Germany. The animals were housed in the veterinary care facility of the University of Erlangen Medical Center and submitted to a 12 h dark/light cycle with free access to standard chow (Altromin, Hamburg, Germany) and water. Operations were performed by the same micro surgeon under an operative microscope (Karl Zeiss, Jena, Germany). Prior to surgery, all rats received an injection of 0.2 ml of a broad spectrum depot antibiotic (Tardomycel Comp III, Bayer, Leverkusen, Germany). Operations were performed under Isoflurane (Baxter, Unterschleiβheim, Germany) inhalational anesthesia.

A total number of 12 rats were operated. 4 animals were sacrificed 2 weeks, 5 animals at 4 weeks and 3 animals were sacrificed 8 weeks after secondary (*i.e.* cell implantation) surgical procedure.

For AV loop construction, the surgical site was shaved, prepped and draped for sterility. Through a 3–4 cm long skin incision from the groin to the knee, the femoral vessels were fully exposed bilaterally. Dissection of the vessels extended from the pelvic artery in the groin to the bifurcation of the femoral artery into saphenous and popliteal arteries in the knee. A 20 mm vein graft was harvested from the right femoral vessels. Interposing the vein graft in original blood flow direction, an arteriovenous (AV) loop was created between the proximal end of the left femoral artery and vein using 11-0 non-resorbable microsutures (Fig. 2a). The AV loop was placed into the sterile cylindrical isolation chamber (inner diameter 10 mm, height 6 mm) made out of heat resistant medical grade Teflon, which was already half filled with a 1:1 mixture of fibrinogen (fibrinogen 20 mg/ml in normal saline solution) and thrombin 40 IU/ml (thrombin in 40 mM calcium-chloride solution; both from Baxter, Vienna, Austria). Subsequently the AV loop was covered with a second layer of fibrinogen/thrombin solution, thus forming a solid and opaque fibrin matrix embedding the AV loop (Fig. 2b). The lid was closed and the chamber was fixed in the left groin using Prolene 5-0 (Ethicon, Norderstedt, Germany) sutures. Hemostasis was assured. The wound was closed using Vicryl 5-0 (Ethicon, Norderstedt, Germany). In the right groin of each animal the same kind of isolation chamber containing the fibrin-matrix only and closed with a lid was fixed in the same way.

For secondary procedure involving cell transplantation, the wounds were re-exposed 14 days after primary surgery and the lids of the isolation chambers were lifted. Then $1x10^6$ CFDA labelled myoblasts suspended in fibrinogen solution were injected together with the thrombin (same concentrations as above) component using a two-way syringe with a blunt needle directly into the AV loop / fibrin-constructs. Subsequently lids were reattached to the isolation chambers in the groins and all wounds were closed as described above.

India ink injection and explantation of the matrices

After induction of anesthesia, the aorta was exposed through a longitudinal laparotomy. The aorta was then canulated using a 24-gauge catheter and flushed with 100 ml heparin solution in Ringer (100 IU/ml). The inferior vena cava was severed to allow blood and heparin solution to drain. Then 30 ml of India ink solution [50% v/v India ink, Rohrer, Germany in 5% gelatin (Roth, Karlsruhe, Germany) and 4% mannitol (Neolab, Heidelberg, Germany)] was injected into the abdominal aorta. After setting of the gelatin solution, specimen were macroscopically inspected, explanted and subjected to histological processing.

Histological analysis

Specimens were explanted in toto and embedded in Tissue Freezing Medium® (Leica Instruments, Nussloch, Germany). After being frozen on a liquid nitrogen cooled plate, they were sectioned in a Leica CM3050 cryostat at a thickness of 10 µm (Leica Microsystems, Bensheim, Germany). Sections were stained using hematoxylin eosin (HE) according to standard protocols. Every other section underwent immunofluorescence staining for MyoD as described above, followed by nuclear counterstaining with DAPI (Sigma Aldrich, Irvine, UK). For nuclear counterstaining sections were incubated with DAPI 10µg / 1 ml aqua bidest. for 30 min at room temperature followed by several washing steps. Microphotographs were taken using Leica fluorescence microscope and Leica digital camera (Leica Microsystems, Bensheim, Germany). Digital overview images were generated by using the "photomerge" - function of Adobe® Photoshop® CS2, Version 9.0.

RNA isolation, cDNA synthesis and PCR

After homogenisation of parts of the explants, total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was

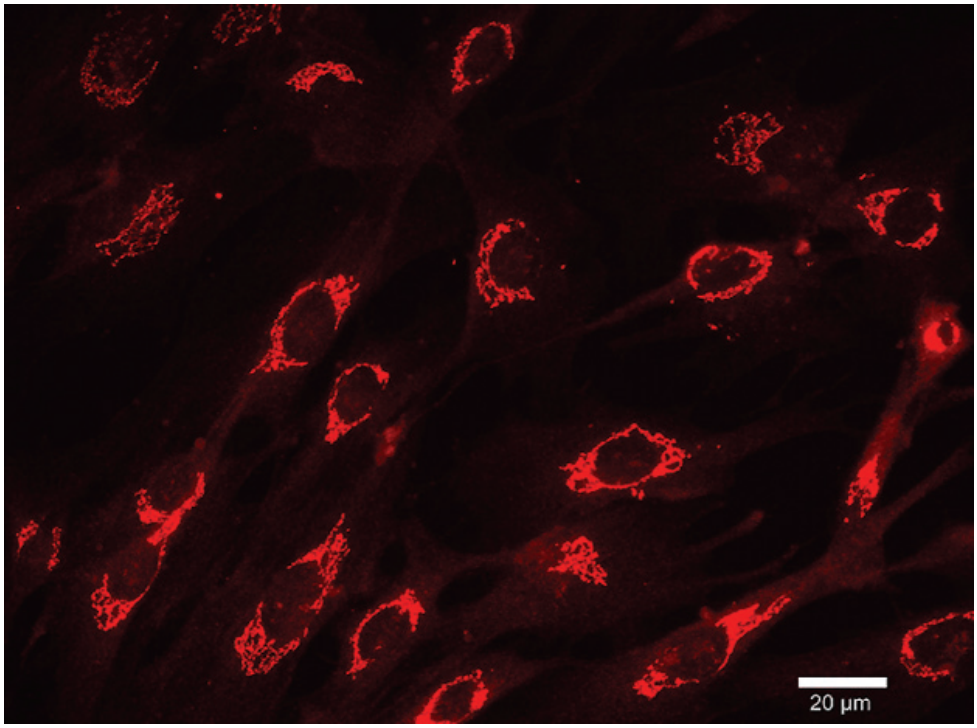


Fig. 1 Expanded primary rat myoblasts showing positive perinuclear staining for MyoD, indicating the preservation of myogenic phenotype.

treated with DNase I (Qiagen, Hilden, Germany), and both purity and concentration was determined by A_{260} and A_{280} (A_{260}/A_{280} = 1.7–2.0) measurements using a Ultraspec 1000 UV/Visible Spectrophotometer (Amersham Pharmacia Biotech, Buckinghamshire, UK). In order to detect RNA degradation, 1 µg of total RNA was fractionated by electrophoresis on 0.9% formaldehyde agarose gel and visualized under UV light. Aliquots of 5 µg total RNA per explant were reverse-transcribed by using oligo(dT)-primed first-strand cDNA synthesis with the ProSTAR First-Strand RT-PCR Kit (Stratagene, La Jolla, USA), according to the manufacturer´s recommendations. From all study groups, a no RT-control was preserved. All cDNA probes confirmed glyceraldehyde-3- phosphate dehydrogenase (GAPD) expression and were analysed for expression of the following muscle specific genes:

1. myogenin (primers out ACCAGGAGCCCCACTTCTAT and CAGGACAGCCCCACTTAAAA, in CGGGGAAAACTACCTTCCTG and TGGCAGACAATCTCAGTTGG)
2. desmin (primers out CGAGCTCTACGAGGAGGAGA and AAGGTCTGGATCGGAAGGTT, in TGGAGCGTGACAACCTGATA and ACATCCAAGGCCATCTTCAC)
3. MEF-2d (primers out CACTCCTTCCCTGGTGACAT and ACTTGGGGACACTGGTTCTG, in CAGCAGCCAGCACTACAGAG and GGGATGAGGTTGCTGAGAGA)
4. embryonic myosin heavy chain (primers out ACTCTGGAGCAGACGGAGAG and TGACGTTGGATTGTTCCTCA, in GGAAGATTGCAGAGCAGGAG and GGCCTCAGCATTACGTTTCT).

PCR was performed as a nested 25 cycle standard PCR, using Taq DNA polymerase with Q-Solution (Qiagen, Hilden, Germany) and 5 µl RT products as templates, in a PTC-200 DNA Engine Peltier Thermal Cycler (MJ Research, Incline Village, USA). Electrophoresis was carried out in 1.5% agarose gels containing ethidium bromide. Products were visualized and photographed under UV light.

Results

Expanded primary rat myoblasts keep their myogenic phenotype and can be CFDA-labelled

After establishing a primary rat myoblast culture, derived from the hindlimbs of adult syngeneic rats, an expansion through 4 passages was performed. High purity with myogenic specificity of these expanded cells was confirmed by positive anti-MyoD immunocytochemistry (Fig. 1). Prior to transplantation *in vitro* evaluation of CFDA labelling revealed best results in terms of signal strength *vs.* background ratio with a final CFDA concentration of

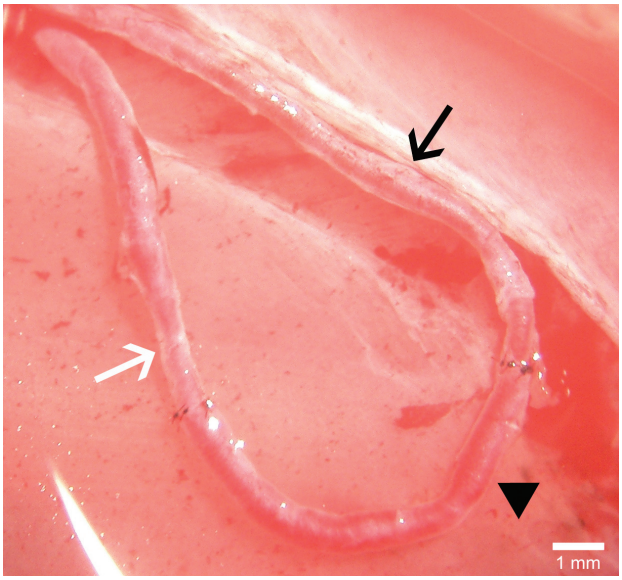


Fig. 2a Microsurgically created AV loop in the groin of the rat, consisting of femoral artery (black arrow), interpositional vein graft (arrow head) and femoral vein (white arrow).

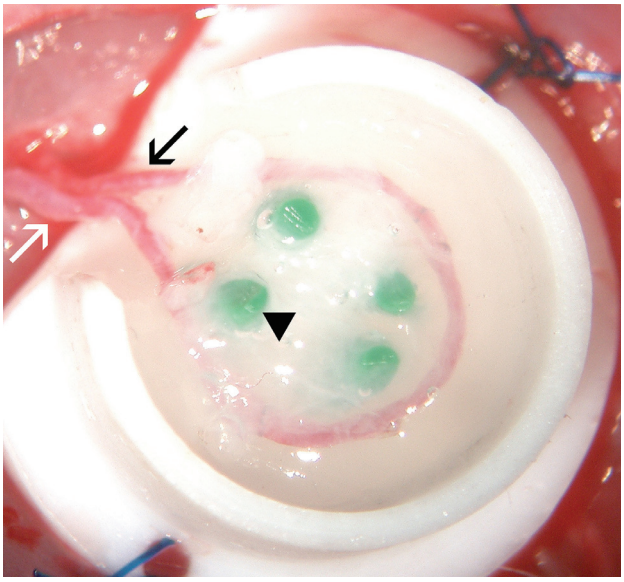


Fig. 2b Teflon chamber (inner diameter 10 mm, height 6 mm) filled with fibrin, containing the AV loop (femoral artery = black arrow, interpositional vein graft = arrow head and femoral vein = white arrow) wrapped around the fixing stacks (arrow head).

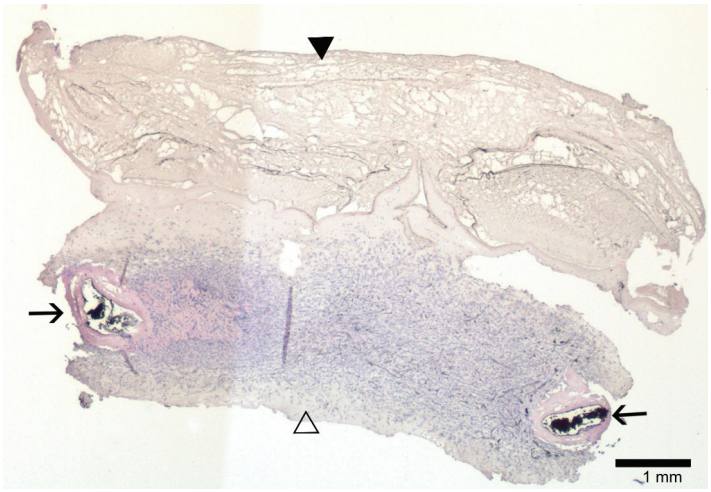


Fig. 2c H&E staining of cross section through AV loop fibrin construct following India Ink injection. Black arrows indicating patent (perfused) loop vessels. The bottom part of the matrix is already vascularised (white arrow head), the upper part not yet (white arrow head).

10 mM. CFDA fluorescence could be detected within *in vitro* cultures for several weeks.

AV-loops induce vascularisation of fibrin matrix

All animals survived the microsurgical procedure of generating the loop and recovered without any complications. Operation was only terminated when the AV loop was patent (Fig. 2a), which was evaluated intra-operatively by common microsurgical procedures (such as the ballooning-test or distal refill test). Positioning of the loop within the Teflon chamber was followed by direct injection of fibrinogen simultaneously with thrombin, resulting in an opaque fibrin gel embedding the AV loop, with the AV loop kept in place by four small stacks (Fig. 2b).

Two weeks after the first operation, animals were operated again, receiving an injection of $1x10^6$ previously CFDA-labelled myoblasts into the now vascularised fibrin-matrix. By using a two-way syringe connected to a 14" blunt needle, safe and sterile injection of the cell-suspension could be performed

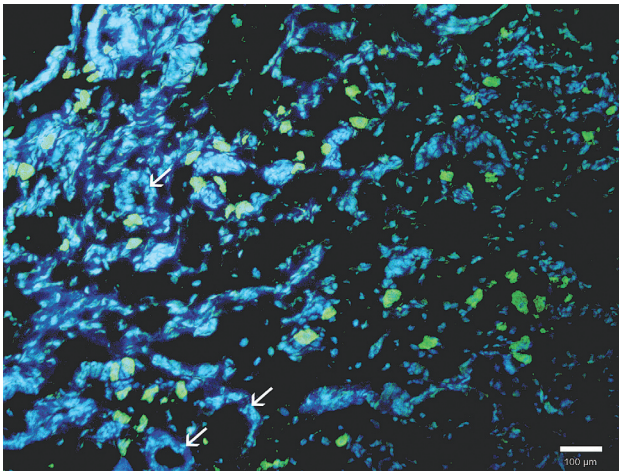


Fig. 3a 2 weeks after myoblast injection into AV loop constructs: donor myoblasts detected by CFDA labelling (green fluorescence), surrounded by host tissue (blue DAPI nuclear staining) within vascularised fibrin matrix (note cross section of vessels = white arrows).

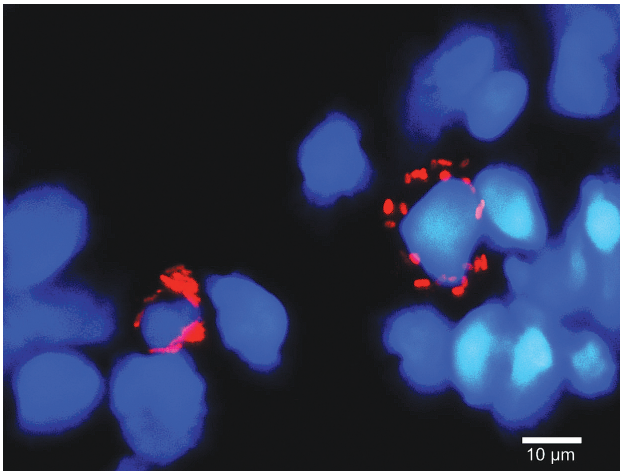


Fig. 3b 2 weeks after myoblast injection into AV loop constructs: myoblast showing perinucelar MyoD expression (red fluorescence, blue nuclear counterstaining with DAPI)

without the necessity of primarily implanting spacers, while avoiding puncture of the AV loop.

H&E staining of the chambers' contents, which were explanted after India Ink injection, revealed patency of most of the AV loops (10 out of 12) (Fig. 2c). Animals showing occluded loops were excluded from the study, resulting in group sizes of 4 (2 weeks), 4 (2 weeks) and 2 (8 weeks) animals per group. These stainings also revealed good vascularisation, but varying resorption rates of the fibrin matrix, thus resulting in different remaining matrix volumes.

Transplanted myoblasts survive in the AV loop constructs and display *MyoD* expression

Donor myoblasts were identified by positive CFDA staining even after 8 weeks and could be detected in all explants derived from the AV loop side at all explantation dates: after 2 weeks (Fig. 3a), 4 weeks (Fig. 4a + c) and after 8 weeks (Fig. 5 a + b). Myoblasts were not detectable in any of the control explants of the contralateral groin without the AV loop.

Positive anti-MyoD staining of these CFDA-positive myoblasts demonstrated that the cells kept their myogenic phenotype after transplantation 2 weeks (Fig. 3 b), 4 weeks (Fig. 4 b + d) and 8 weeks (Fig. 5 c). In the controls, where cells had been injected into fibrin matrix only, these observations were absent: no CFDA positive nuclei were detectable. Results of rats sacrificed at the same dates after transplantation did not show any significant difference.

MEF-2 and desmin is expressed in the AV loop constructs after 4 weeks

Analysis of mRNA expression of myogenic transcription factors myogenin and MEF-2 after 4 and 8 weeks revealed expression of early transcription factor MEF-2 after 4 weeks within the AV loop, whereas MEF-2 expression after 8 weeks and expression of late transcription factor myogenin after 4 and 8 weeks was negative in all other explants.

Expression of muscle intermediary filament desmin could be detected after 4 weeks in the AV loop, while it was negative after 8 weeks in the AV loop and all other groups. There was no expression of myosin heavy chain at any date in any group (Fig. 6).

Discussion

The purpose of this study was to evaluate a clinically feasible approach to tissue engineering of vas-

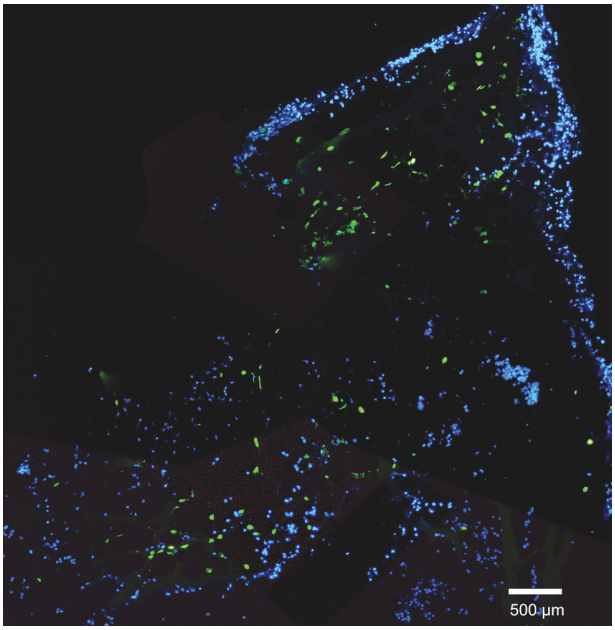


Fig. 4a 4 weeks after myoblast injection into AV loop constructs: overview of vascularised fibrin matrix containing donor myoblasts (green CFDA fluorescence) and host cells (blue DAPI fluorescence).

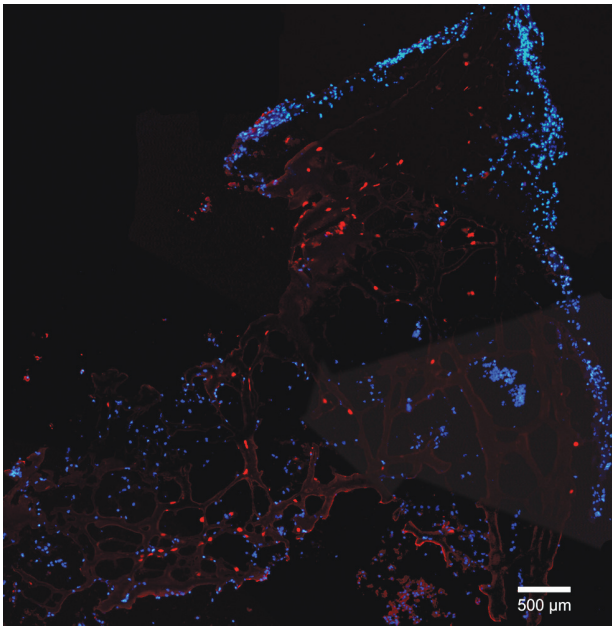
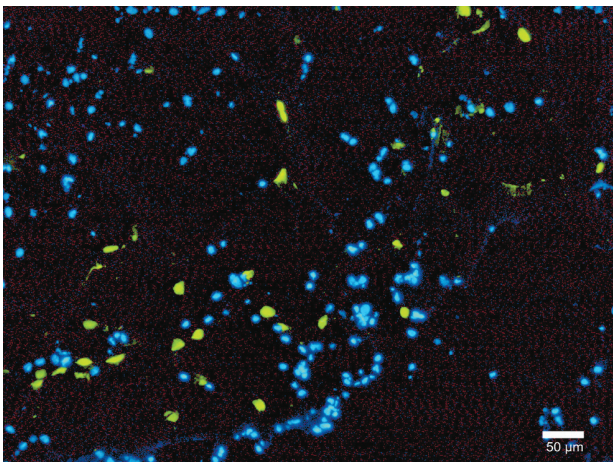


Fig. 4b 4 weeks after myoblast injection into AV loop constructs: same overview section as Fig 4a, showing MyoD positive donor myoblasts (red fluorescence) surrounded by host cells (blue DAPI stain) within vascularised fibrin matrix.



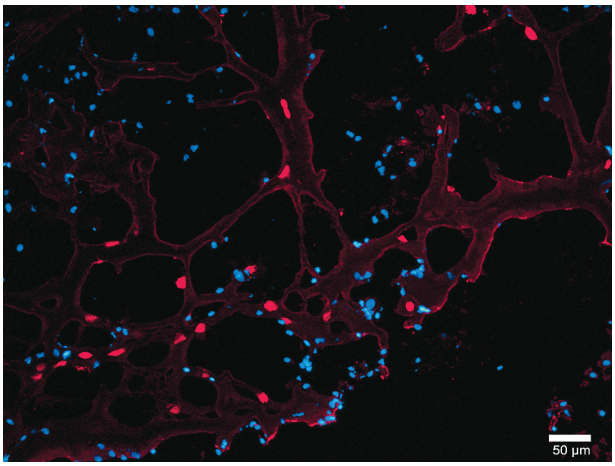


Fig. 4c + d 4 weeks after myoblast injection into AV loop constructs: higher magnification of 4 a + b, demonstrating the correlation between CFDA labelling (green fluorescence) and MyoD expression (red fluorescence) by the very same cells (*i.e.* donor myoblasts).

cularised skeletal muscle. Since sufficient vascularisation is an essential prerequisite for the creation of muscle neo-tissue, a rat model of vascularised tissue generation was used to investigate the pattern of pre-vascularisation within a clinically approved 3D fibrin matrix in conjunction with the differentiation process of expanded primary myoblasts.

The engineering of muscle tissue *in vitro* or *in vivo* holds promise especially for the treatment of skeletal muscle defects and might once provide an alternative to host muscle transfer. Skeletal muscle tissue engineering is based on the regenerative properties of the satellite cells or (in case of neonatal organisms) myoblasts and their potential for pro-

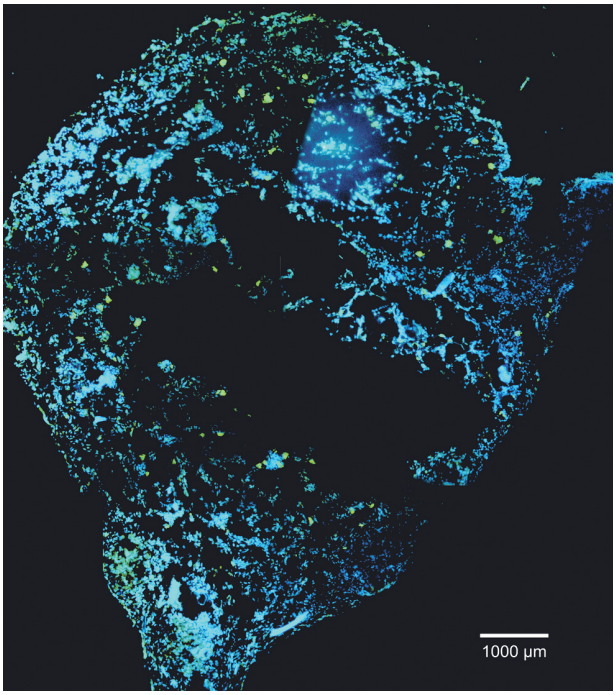


Fig. 5a 8 weeks after myoblast injection into AV loop constructs: overview demonstrating the survival of donor myoblasts (green CFDA fluorescence) within the fibrin matrix even after 8 weeks (blue DAPI stain = host cells).

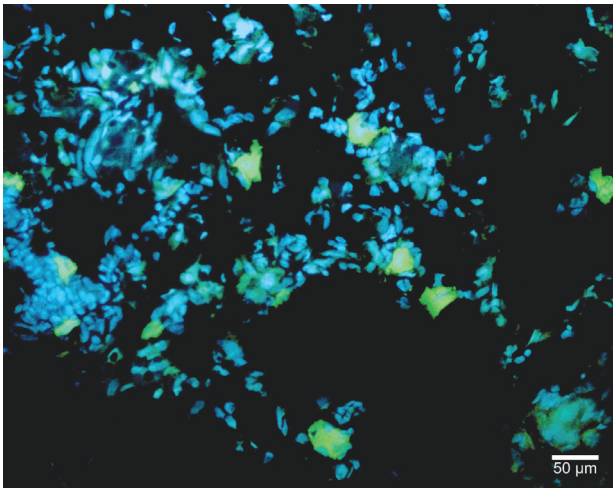
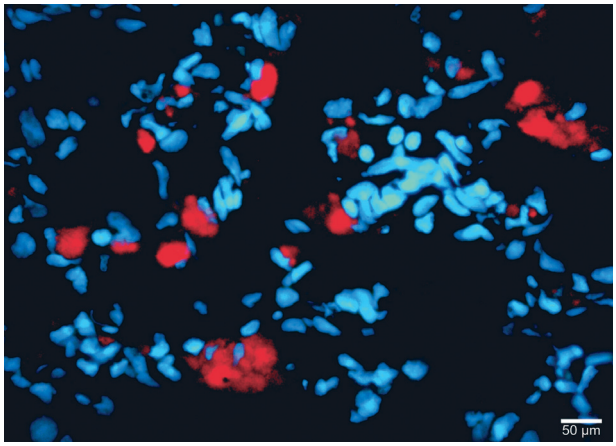


Fig. 5b + c 8 weeks after myoblast injection into AV loop constructs: section of Fig. 5 a at higher magnifcation, indicating that implanted CFDA-positive cells (green fluorescence, Fig. 5 b) are still expressing MyoD (red fluorescence, Fig. 5 c).



liferation and differentiation. These primary muscle precursor cells can be easily harvested from adult and neonatal muscle of different species. Successful cultivation of these cells was established more than nearly 25 years ago [1]. Among the desired features of engineered skeletal muscle tissue, like myoblast fusion and a parallel alignment of resulting myotubes, vascularisation is most crucial [7]. The engineered neo-tissue should also be biocompatible, which has to meet with the requirement of a three-dimensional culture matrix that should be bioresorbable as well [8].

The first step for obtaining sufficient volumes of engineered skeletal muscle is an efficient expansion of the source cells. Furthermore primary cultures derived from satellite cells from myofibers grown *in vitro* are the preferred source of myoblasts because they recapitulate muscle development more precisely than immortal myogenic cell lines [2]. Even after the necessary expansion we could confirm the myogenic phenotype of primary myoblasts after 4 passages by positive MyoD staining of nearly 100% of cultivated cells. In contrast to many other studies we exclusively used primary myoblasts derived from adult rats. For instance Levenberg *et al.* reported on the generation of vascularised skeletal muscle tissue by means of a three-dimensional multiculture system consisting of immortalized mouse myoblasts (cell line C2C12), mouse embryonic fibroblasts and human endothelial cells [9]. From the tissue engineering as well as from the clinical point of view the use of a xenogenic culture system (human and mouse) in combination with embryonic cells is more than questionable [7].

Coming to the desired features of the 3D culture matrix biocompatibility is an important prerequisite. Many other groups reporting on tissue engineering of skeletal muscle tissue still take the advantage of

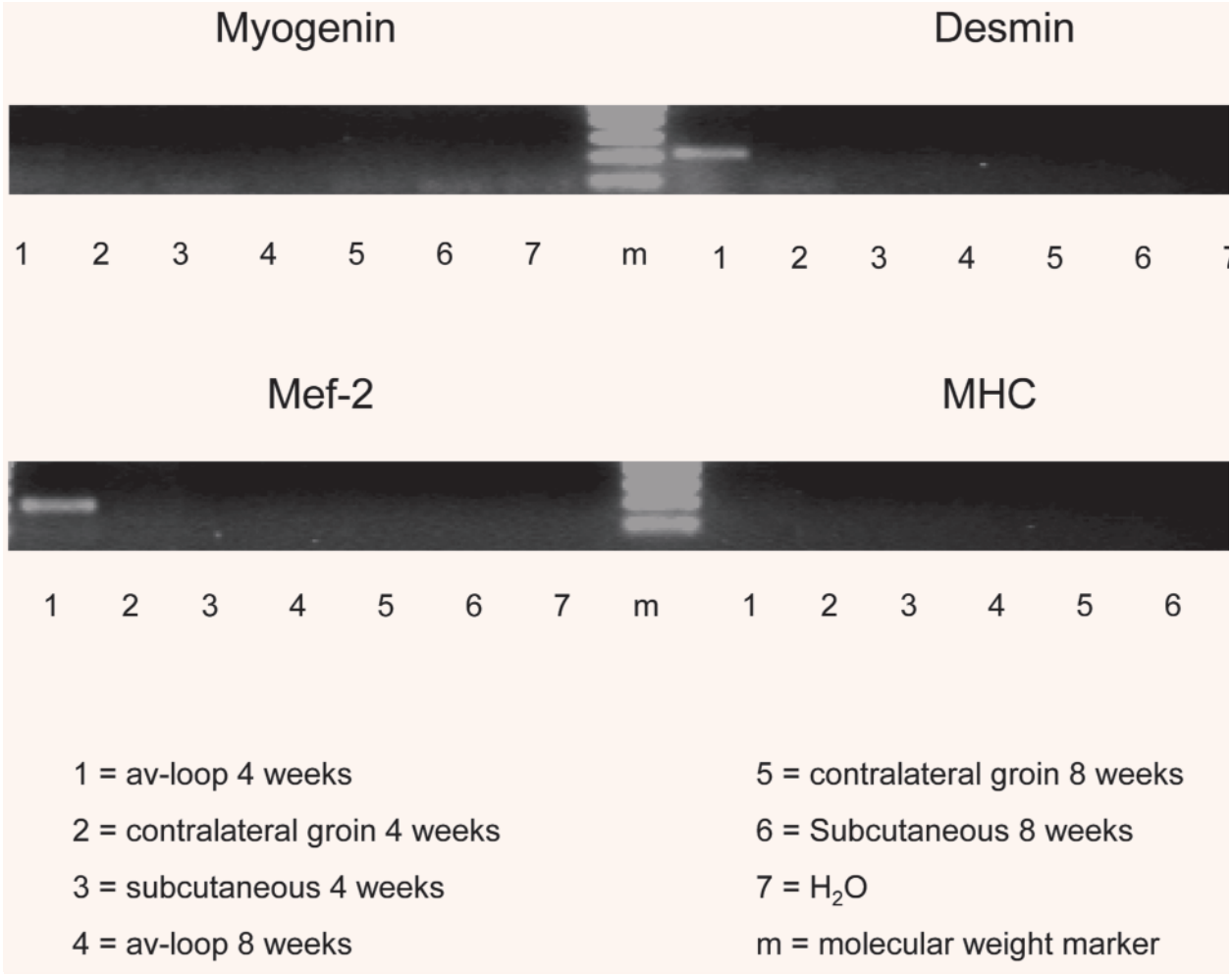


Fig. 6 Results of RT-PCR analysis, demonstrating mRNA expression of MEF-2 and desmin after 4 weeks in the AV loop constructs only.

using a potentially hazardous tumour-derived matrix like Matrigel™. In contrast to the authors approach of using a biocompatible matrix like fibrin for previously demonstrated successful *in vitro* engineering of 3D skeletal muscle tissue engineering [6], many other groups tend to use potentially hazardous matrices. Among these Messina *et al.* are the only group until today who have been reported on the use of the AV loop model in combination with skeletal muscle derived myoblasts [10]. They describe the implantation of these cells within a 3D matrix containing Matrigel™. Again from a clinical point of view this matrix is not acceptable due to its source (the Engelbreth-Holm-Swarm mouse sarcoma) and undefined composition. Another matrix propagated by the group of W. Morrison for the vascularisation in the AV loop model is PLGA [11]. However PLGA induces a strong foreign body reaction, thus not complying with the demand for biocompatibility [12]. In accordance with Birla *et al.*, who also used fibrin for their approach to vascularised cardiac tissue engineering [13], we prefer to use fibrin as a 3D matrix since it is biocompatible and bioresorbable and even clinically approved [14, 15].

The Teflon isolation chamber containing the AV loop which was used in this study has previously been described before by Kneser *et al.* for tissue engineering of bone [5]. It is based on the AV loop model first described more than two decades ago by Erol and Spira for the successful vascularization of a full thickness skin graft [16]. The model was enhanced by Morrison and coworkers who inserted the loop into isolation chambers resulting in a successful vascularisation of polymer- and gel matrices [11, 17]. This

group also demonstrated the superiority of the AV loop as vascular carrier in comparison to the vascular bundle in terms of vascular density and capacity for generation of new tissue [18]. Based on these findings we decided to generate AV loops for induction of vascularisation instead of the distally ligated femoral pedicle (as used by Birla and coworkers [13]), though microsurgical preparation of the loop is far more challenging and meticulous. Our observations concerning the resorption rates of the fibrin matrix raise the suspicion that more vascularisation results in accelerated resorption. To test this hypothesis further studies covering the vascularisation and resorption kinetics of fibrin matrices with larger numbers of AV loop constructs are necessary. However Cassell and coworkers also reported on certain problems coming along with fibrin as AV loop matrix [11].

The main obstacle arising with autologous or syngeneic cell transplantation studies is the identification of transplanted cells. Thus several different approaches have been described to overcome this problem. One possibility is the permanent modification of donor cells prior to transplantation by viral mediated gene transfer, but by using this established strategy one cannot exclude the likelihood of altered cell fate, phenotype and differentiation due to genetic modification. Therefore we do not regard this approach feasible in a clinically relevant context. A very elegant method is the detection of Y chromosome positive (*i.e.* male) donor cells in syngeneic female host animals by *in situ* hybridization (Y-ISH) [19]. However this method is very elaborate and more recommendable when it comes to longer periods between implantation and explantation. Since in this study the longest interval covers 8 weeks, the straightforward technique of CFDA labelling prior to implantation was applied for a reliable short-term tracing of labelled donor cells.

Looking at the survival rates of the myoblasts within AV loop constructs, pre-vascularisation apparently plays an important role. No vascularisation between first operation and cell injection occurred in the contralateral groin chambers, which did not including an AV loop but were also sealed with a lid. Neither did we detect surviving donor myoblasts after final explantation of these constructs. Instead of only remnants of the fibrin matrix were found. To our knowledge this is the first study showing the superiority of pre-vascularisation for the survival of transplanted primary cells. Due to the small number of AV loop constructs per time point, statistical conclusion in terms of time dependant cell survival or even proliferation can not be drawn. Further studies with larger numbers of animals would be necessary to figure out this kinetics, the best time point for cell injection or the optimal amount of donor cells. However positive MyoD immunostaining revealed the persistant myogenic phenotype of all transplanted and CFDA positive myoblasts in the AV loop constructs, again arguing for the superiority of pre-vascularised matrices for cell-based tissue engineering strategies. This is in accordance with our RT-PCR findings: the only detectable mRNA expression of myogenic genes, namely the early transcription factor MEF-2 [20] and the intermediary filament desmin was found in the AV loop constructs. Further differentiation phenomenon, such as myotube formation, the expression of the later transcription factor myogenin or of the contractile apparatus gene MHC_{embryo} were not noted, indicating that beyond axial pre-vascularisation potent myogenic stimuli (like neural activity *e.g.*) might be necessary.

Conclusion

One major obstacle for skeletal muscle tissue engineering still remains the vascularisation, as soon as it comes to clinically relevant dimensions. In this study we could show for the first time a successful combination of primary myoblast transplantation in the rat AV loop model within a clinically approved 3D fibrin matrix. Axial pre-vascularisation seems to promote myoblast survival and preservation of myogenic phenotype. In the future microsurgical vascularisation models might play the predominant role in the field of vascularised, cell based tissue engineering strategies in general. Beyond that skeletal muscle tissue engineering in particular will have to address the complex problem of differentiation induction by clinically feasible means.

Acknowledgments

This study was supported by a research grant from the University of Erlangen (ELAN Program).

References

1. **Blau HM, Webster C**. Isolation and characterization of human muscle cells. *Proc Natl Acad Sci USA*. 1981; 78: 5623–7.
2. **Blanco-Bose WE, Yao CC, Kramer RH, Blau HM**. Purification of mouse primary myoblasts based on alpha 7 integrin expression. *Exp Cell Res*. 2001; 265: 212–20.
3. **Kosnik PE, Faulkner JA, Dennis RG**. Functional development of engineered skeletal muscle from adult and neonatal rats. *Tissue Eng*. 2001; 7: 573–84.
4. **Neumann T, Hauschka SD, Sanders JE**. Tissue engineering of skeletal muscle using polymer fiber arrays. *Tissue Eng*. 2003; 9: 995–1003.
5. **Kneser U, Polykandriotis E, Ohnolz J, Heidner K, Grabinger L, Euler S, Amann KU, Hess A, Brune K, Greil P, Stürzl M, Horch RE**. Engineering of vascularized transplantable bone tissues: induction of axial vascularization in an osteoconductive matrix using an arteriovenous loop. *Tissue Eng*. 2006; in press.
6. **Bach AD, Beier JP, Stark GB.** Expression of Trisk 51, agrin and nicotinic-acetycholine receptor epsilon-subunit during muscle development in a novel three-dimensional muscle-neuronal co-culture system. *Cell Tissue Res*. 2003; 314: 263–74.
7. **Bach AD, Beier JP, Stern-Staeter J, Horch RE**. Skeletal muscle tissue engineering. *J Cell Mol Med*. 2004; 8: 413–22.
8. **Bach AD, Stem-Straeter J, Beier JP, Bannasch H, Stark GB**. Engineering of muscle tissue. *Clin Plast Surg*. 2003; 30: 589–99.
9. **Levenberg S, Rouwkema J, Macdonald M, Garfein ES, Kohane DS, Darland DC, Marini R, van Blitterswijk CA, Mulligan RC, D'Amore PA, Langer R.** Engineering vascularized skeletal muscle tissue. *Nat Biotechnol*. 2005: 23: 879–84.
10. **Messina A, Bortolotto SK, Cassell OC, Kelly J, Abberton KM, Morrison WA**. Generation of a vascularized organoid using skeletal muscle as the inductive source. *FASEB J*. 2005; 19: 1570–2.
11. **Cassell OC, Morrison WA, Messina A, Penington AJ, Thompson EW, Stevens GW, Perera JM, Kleinman HK, Hurley JV, Romeo R, Knight KR**. The influence of extracellular matrix on the generation of vascularized, engineered, transplantable tissue. *Ann N Y Acad Sci*. 2001; 944: 429–42.
12. **Cao Y, Mitchell G, Messina A, Price L, Thompson E, Penington A, Morrison W, O'Connor A, Stevens G, Cooper-White J**. The influence of architecture on degradation and tissue ingrowth into three-dimensional poly(lactic-co-glycolic acid) scaffolds *in vitro* and *in vivo*. *Biomaterials* 2006; 27: 2854–64.
13. **Birla RK, Borschel GH, Dennis RG, Brown DL**. Myocardial engineering *in vivo*: formation and characterization of contractile, vascularized three-dimensional cardiac tissue. *Tissue Eng*. 2005; 11: 803–13.
14. **Stern-Straeter J, Bach A, Stangenberg L, Foerster V, Horch RE, Stark GB, Beier JP**. Impact of electrical stimulation on three-dimensional myoblast cultures - a real-time RT-PCR study. *J Cell Mol Med*. 2005; 9: 883–92.
15. **Bach AD, Bannasch H, Galla TJ, Bittner KM, Stark GB**. Fibrin glue as matrix for cultured autologous urothelial cells in urethral reconstruction. *Tissue Eng*. 2001; 7: 45–53.
16. **Erol OO,Spira M**. New capillary bed formation with a surgically constructed arteriovenous fistula. *Plast Reconstr Surg*. 1980; 66: 109–15.
17. **Mian R, Morrison WA, Hurley JV, Penington AJ, Romeo R, Tanaka Y, Knight KR**. Formation of new tissue from an arteriovenous loop in the absence of added extracellular matrix. *Tissue Eng*. 2000; 6: 595–603.
18. **Tanaka Y, Sung KC, Tsutsumi A, Ohba S, Ueda K, Morrison WA**. Tissue engineering skin flaps: which vascular carrier, arteriovenous shunt loop or arteriovenous bundle, has more potential for angiogenesis and tissue generation? *Plast Reconstr Surg*. 2003; 112: 1636–44.
19. **Beier JP, Kneser U, Stern-Strater J, Stark GB, Bach AD**. Y chromosome detection of three-dimensional tissue-engineered skeletal muscle constructs in a syngeneic rat animal model. *Cell Transplant*. 2004; 13: 45–53.
20. **Riquelme C, Barthel KK, Liu X**. SUMO-1 modification of MEF2A regulates its transcriptional activity. *J Cell Mol Med*. 2006; 10: 132–44.