Christiane Fuchs / Babette Maleiner / Philipp Heher / Andreas Teuschl / Heinz Redl / Dominik Rünzler

Generation of highly aligned skeletal muscle-like tissue through application of strain to a 3D fibrin scaffold

107 - Translationale Gesundheitsforschung – Brücken bauen von Grundlagenwissenschaft zu angewandter Forschung

Abstract

The natural hydrogel fibrin is considered a very suitable scaffold material for skeletal muscle engineering since its mechanical properties can be adjusted to match those of native skeletal muscle tissue. In addition, fibrin responds to uniaxial mechanical stimulation with fibril alignment along the axis of strain, thus leading to parallel cellular patterning. To exploit this feature in a biomimetic skeletal muscle engineering approach, we have developed a novel bioreactor system ("MagneTissue") for the rapid generation of highly aligned skeletal muscle-like tissue constructs. With this system, murine myoblasts encapsulated in ring-shaped fibrin scaffolds were subjected to static mechanical strain via magnetic force transmission, leading to cellular alignment concomitant with the patterning of the scaffold material into highly organized fibrin fibrils. Within 9 days, a parallel array of myotubes with a mature phenotype in terms of sarcomeric patterning, width and length was obtained using a daily stimulation protocol of 10% static strain for 6 hours and 3% for the residual 18 hours. Moreover, static mechanical stimulation enhanced myogenic differentiation on the gene expression level, demonstrated by the upregulation of the myogenic determination factor *MyoD* as well as the contractile structural marker *Troponin T1*. The MagneTissue bioreactor system provides a versatile platform for tissue engineering of soft tissue whose functionality requires parallel cellular patterning, such as skeletal muscle - with the advantage that strain protocols can be individually adjusted using a custom-made software. For future work, this will allow implementing mechanical stimulation using different strain regimes in the maturation process of tissue engineered skeletal muscle constructs and elucidating the role of mechanotransduction in myogenesis. The ultimate goal is to generate functional muscle-like tissue that can serve as suitable transplants for damaged or missing tissues in patients suffering from trauma or genetic diseases such as dystrophies.

Keywords:

Fibrin, scaffold, bioreactor, mechanical stimulation, skeletal muscle engineering

1. Background

Due to the ongoing demographic trend towards a rising portion of the elderly and also a change of people's behavior towards taking higher risks in sports, the percentage of traumatic injuries resulting in volumetric muscle loss increases. Besides, there is still no cure or treatment option for patients with muscular dystrophies. These patients' quality of life could be significantly improved if they could be provided with functional transplants to restore their locomotive system and help them stay in touch with their environment. Therefore the necessity for artificial transplants is increasing and alternatives to current conservative treatment methods are needed in health care. In tissue engineering (TE) three approaches exist to regenerate or induce regeneration of tissue and are often combined to ameliorate the healing process (Nau and Teuschl, 2015).

- A so-called **scaffold** is used to close the defect and to temporarily adopt the function of the tissue.
- **Cells** are introduced to the site of defect to help build new tissue. This is preferably done using autologous (the body's own) cells such as adult stem cells.
- **Signals** that usually govern the regenerative process like growth factors or mechanical stimuli, are used.

Still there are many challenges to overcome and researchers found that there is more to be taken into account than "simply" combining a biomaterial with cells to create transplantable tissue. The ultimate goal in tissue engineering is to generate 3-dimensional tissue constructs that mimic the functional and structural properties of the native tissue. *In vitro* preconditioning with comparable mechanical forces or stimuli that these tissues naturally experience *in vivo* presents a feasible strategy (Rowe et al., 2007; Heher et al., 2015). With the system introduced here– the "MagneTissue" bioreactor - it is not only possible to generate 3D muscle-like tissue constructs which, in the future, could be used in animal models to test their functionality, but also to gain deeper insight into the role of mechanotransduction and the signaling pathways involved during myogenic differentiation. This will certainly improve our understanding of the impact of mechanical stimuli and will therefore pave the way for the generation of functional tissue constructs by using defined strain regimes. Due to the mechanical training the engineered muscle tissue should reach a maturity that allows for transplant contribution to the damaged/missing tissue in patients with need for muscle replacement.

2. Hypothesis

Our hypothesis was to create skeletal muscle-like tissue constructs that closely mimic the properties of native muscle. Bioreactors are devices that allow for tissue generation with tight control over parameters such as force, movement, pH, gas, nutrients etc and are thus considered feasible platforms in TE. We constructed a novel bioreactor system, called "MagneTissue", which uses magnetic force transmission to apply mechanical stretch to the scaffolds.

The biodegradable and -compatible biomaterial fibrin, a natural protein involved in blood clotting and also used as tissue sealant in clinics, was used to generate ring shaped scaffolds with murine myoblasts (C2C12 cells) encapsulated in them (Fig. 1).

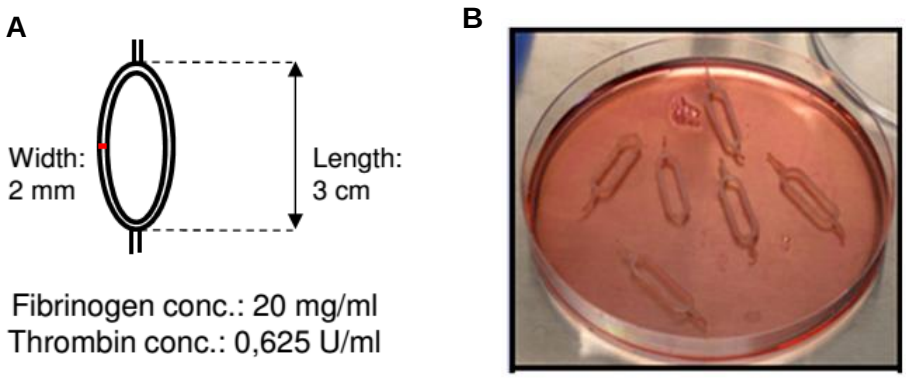

Figure 1: Fibrin scaffolds for "MagneTissue" bioreactor. A) Schematic representation of dimensions of fibrin constructs and composition. B) Ring-shaped fibrin scaffolds with encapsulated C2C12 cells after casting and polymerization.

The fibrin scaffolds are mounted onto a spool–hook system, attached to a tube inlet system that allows for gas exchange and media replacement. The hooks contain permanent magnets which are used to keep the constructs either at 0% strain in magnetic storage racks (representing the unstrained control group) or to apply mechanical strain (strain group). The bioreactor has magnetic combs that lock the constructs in a specific position for mechanical stimulation or resting (Fig. 2).

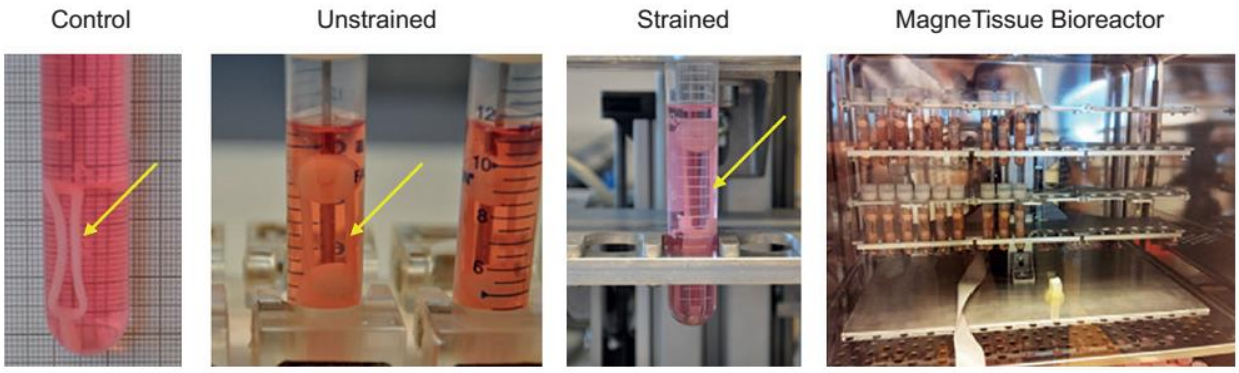

Figure 2: Different treatment groups of muscle tissue-like constructs. Left to right: floating control group; fibrin scaffold, mounted to spool-hook system receiving no strain (unstrained); scaffold in the custom made bioreactor system receiving strain (strain group); MagneTissue bioreactor used for mechanical stimulation of scaffolds (Figure adapted from Heher et al., 2015).

A custom-made software allows to control the movements of the bioreactor using a stepper motor. The user can define either a static or cyclic training program or a combination thereof. This allows the user to set-up unique training/mechanical stimulation patterns to mimic specific conditions ranging from developmental stimulation patterns to training patterns or even overuse to simulate disease-specific wear and tear. We chose a mechanical stimulation protocol that starts with a proliferation period for

the first two days, followed by a training pattern that resembles the natural growth (3% strain) of the musculoskeletal apparatus during development (Stewart, 1972; Vandenburgh and Karlisch, 1989) for 18 hours per day and a 10% strain period for 6 hours, which simulates isometric training. This protocol was performed for 7 consecutive days (Fig. 3). Then end point analysis via qPCR (myogenic markers: early – *Pax7, Myf5, MyoD*; mid – *Myogenin*; late – *Troponin T1*) and immunofluorescence microscopy (desmin and MHC fast) was performed to investigate the effect of mechanical stimulation on the cells within the scaffolds.

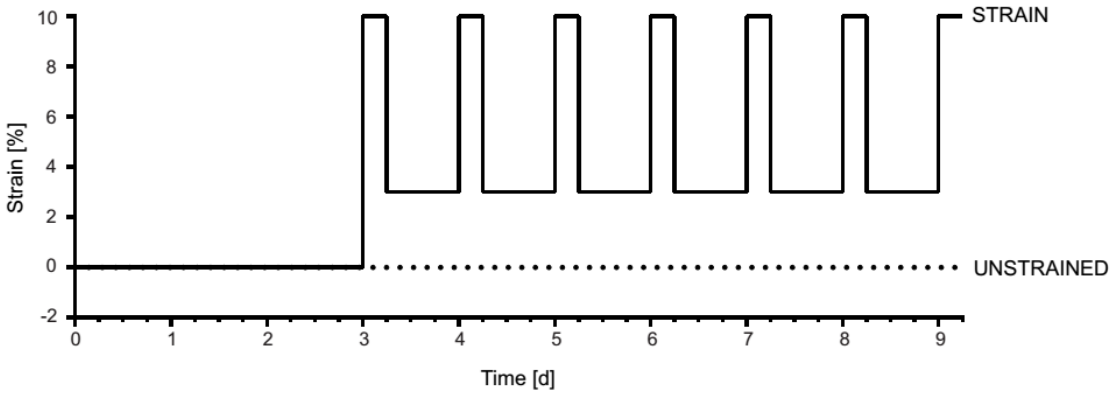

Figure 3: Scheme of the applied mechanical stimulation protocol with the MagneTissue bioreactor: constructs were cast and 3 days later 10% static strain for 6 hours (exercise phase) followed by 18 hours at 3% strain (rest phase) were applied daily until day 9 (Heher et al., 2015).

3. Results

The composition of a biomaterial strongly influences cell behavior in terms of proliferation and differentiation, thus different fibrinogen concentrations were tested. The fibrin composition of 20 mg/ml fibrin and 0.625 IU/ml thrombin turned out to be the best as the stiffness of the material was in the range of native muscle (Engler et al., 2006) and myoblasts were viable within the scaffold over the whole culture period of 9 days. Already after a single training period of 10% strain for 6 hours fibrin fibrils as well as cell morphology were altered compared to the unstrained and the control group, which was detected by scanning electron and immunofluorescence microscopy analysis. The fibrin fibrils were aligned along the axis of strain and cell morphology was also altered showing an elongated appearance of both cytoplasm and nucleus. Muscle differentiation is a highly orchestrated process where muscle-specific genes and transcription factors are expressed in a spatiotemporal manner. Over the differentiation period of six days gene expression followed a pattern similar to what is reported for native myogenesis (Bentzinger et al., 2012). However, the unstrained and strain group always displayed higher expression levels compared to the untreated controls. The static training significantly enhanced expression of the myogenic transcription factor *MyoD*, which initiates differentiation of myoblasts into myotubes (Yokoyama and Asahara, 2011). A late marker during myogenesis is the structural protein *Troponin T1 (TnnT1)* which can be used as a read out for assembly of the contractile apparatus and hints to the maturity of the developed muscle (Wei and Jin, 2011). Again the expression of *TnnT1* follows that of normal development in the control group and

increases significantly over time. More importantly, the unstrained group - where the fibrin rings are only mounted to the system but do not experience any further mechanical stimulation except for the cells own contractile forces generated due to anchoring - shows a higher degree of *TnnT1* expression from day 6 on compared to the controls. The highest expression, however, is observed in the strain group, which has at least a 2-fold higher induction compared to the control. Thus, we conclude that additional mechanical stimulation significantly increases myogenic marker expression and therefore differentiation of muscle tissue-like 3D constructs.

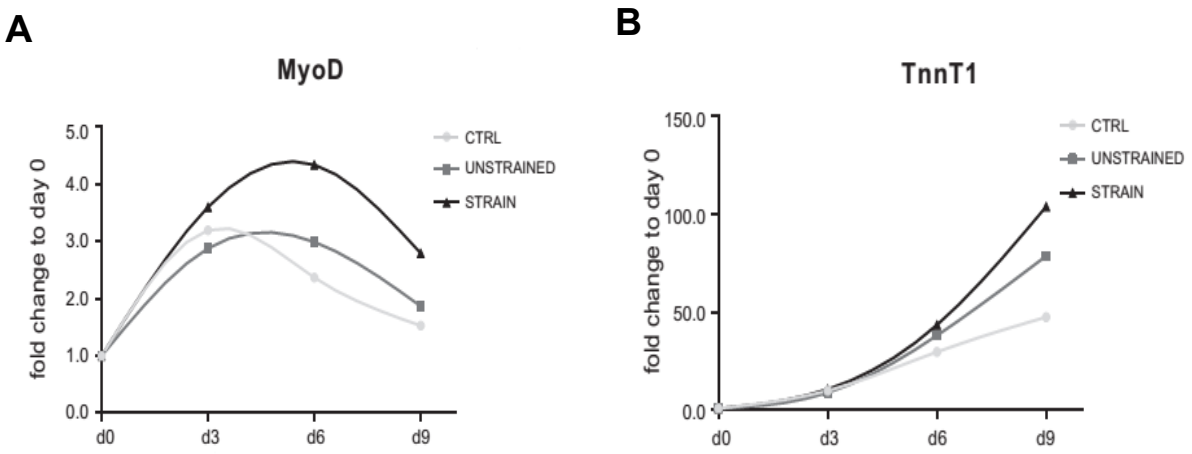

Figure 4: Mechanical strain positively influences myogenic marker expression during differentiation. A) Relative gene expression of *MyoD* over time. Fold induction was normalized to day 0 of each group. B) Relative gene expression of *Troponin T 1 (TnnT1)* over time. Fold induction was normalized to day 0 of each group (Figure adapted from Heher et al., 2015).

As we had already demonstrated that a single training session of 10% static strain for 6 hours had a massive impact on the alignment of the biomaterial and the encapsulated C2C12 myoblasts, we investigated the effect of repetitive daily training for 7 days at the end of the culture period (day 9). To assess the maturity of the constructs we analyzed the structural protein myosin heavy chain (MHC fast) which also plays an essential role in muscle contraction. The control group revealed a random orientation of myotubes within the scaffold (Fig. 5A, upper image). The unstrained group showed a higher degree of alignment compared to controls, which is mainly due to the contractile force the muscle cells themselves generate while differentiating (Fig. 5A, middle image). The strain group showed the highest extent of mytobues aligned along the axis of strain, with less than 15° deviation from the axis of strain (Fig. 5A, lower image). The myotubes in the strain group were also thicker and longer compared to their control counterparts. Besides these parameters of muscle maturity, strained constructs also showed a more pronounced sarcomeric patterning compared to control and unstrained samples (Fig. 5B). Obviously, myotubes do form in all experimental groups, indicated by the fusion index representing the percentage of nuclei within myotubes and thus the performance of myotube formation. We could demonstrate that mechanical stimulation of scaffolds with static strain results in

an almost 2-fold higher fusion index compared to floating controls, which again highlights the beneficial effect of mechanical stimulation in myogenesis.

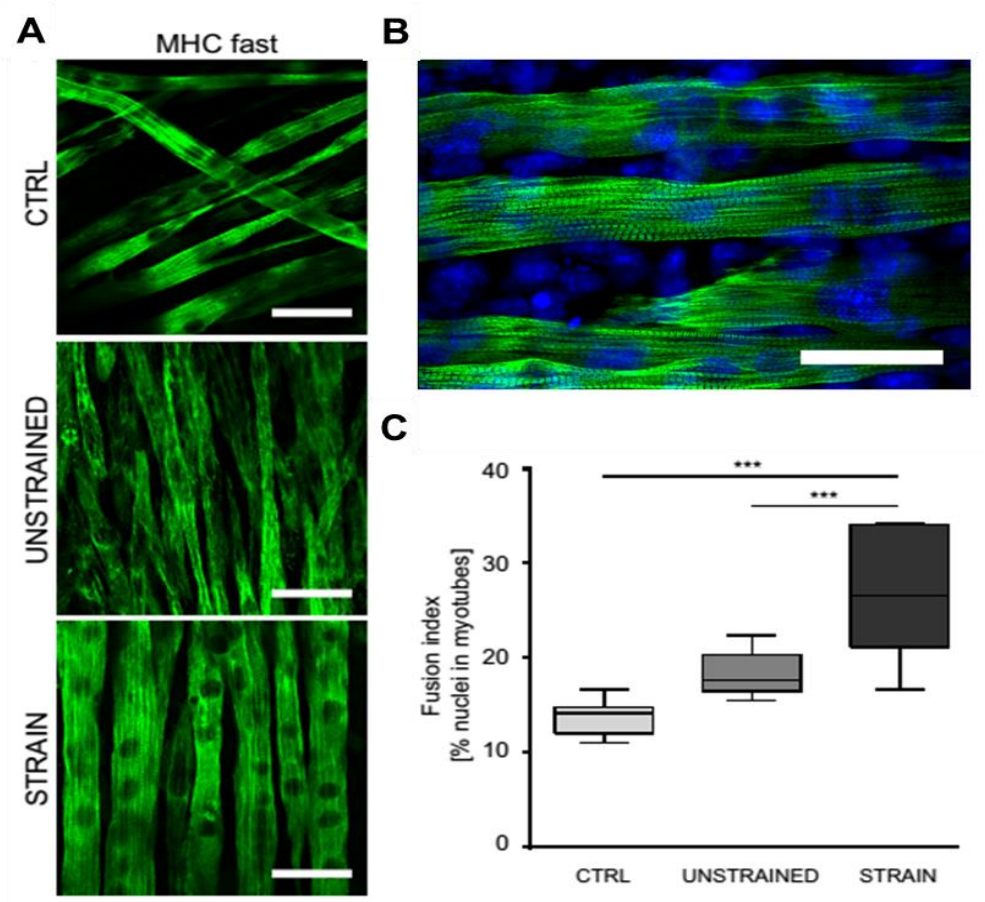

Figure 5: Mechanical stimulation leads to aligned formation of myotubes along the axis of strain and to a higher degree of differentiation. A) Scaffolds were strained for myosin heavy chain fast (MHC fast) protein for control (CTRL), unstrained and strain group on day 9. B) MHC fast (green) straining and nuclear counterstrain with DAPI (blue) of a representative strain group scaffold on day 9. MHC straining reveals typical sarcomeric patterning of muscle tissue, a read-out for muscle contractility. C) Fusion index, which gives the percentage of nuclei within a myotube, was calculated for control (CTRL), unstrained and strain groups (Figure adapted from Heher et al., 2015).

4. Conclusion

Tissue engineering of functional transplantable tissue has recently evolved as a promising strategy to reconstitute damaged or lost tissue in regenerative medicine. Despite tremendous advances in bone, cartilage or skin TE (Horch et al., 2000; Vangsness et al., 2004; Black et al., 2015) the *in vitro* generation of sufficiently mature, volumetric skeletal muscle tissue that can contribute to regeneration upon transplantation still remains a major challenge (Koning et al., 2009). Consequently, patients suffering from myopathies or muscle loss cannot be treated accordingly and the clinical outcomes with standard treatments are at best suboptimal (Qazi et al., 2015). It is the recent development of

sophisticated culture systems that now allow scientists to precisely control culture conditions and provide the skeletal muscle tissue with the right stimuli at the right time to increase the biomimicry compared to native tissue. In this respect, the “MagneTissue” bioreactor system provides a novel platform for the rapid engineering of skeletal muscle under close-to-physiological conditions. Since native muscle is constantly subjected to mechanical load, we designed our system to allow for application of individually adjustable strain regimes to generate muscle that resembles the native tissue. The strategy we used led to the formation of a parallel array of myotubes with improved structural and functional characteristics, which is a crucial factor as the constructs are expected to generate sufficiently large forces when transplanted *in vivo*. Moreover, the bioreactor system will allow us to gain deeper insight into the role of mechanotransduction in myogenesis and therefore will broaden our understanding of the effects of mechanical stimuli in organogenesis.

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References:

Bentzinger, C. F., Wang, Y. X. and Rudnicki, M. A. (2012) 'Building muscle: molecular regulation of myogenesis', *Cold Spring Harb Perspect Biol* 4(2).

Black, C. R., Goriainov, V., Gibbs, D., Kanczler, J., Tare, R. S. and Oreffo, R. O. (2015) 'Bone Tissue Engineering', *Curr Mol Biol Rep* 1(3): 132-140.

Engler, A. J., Sen, S., Sweeney, H. L. and Discher, D. E. (2006) 'Matrix elasticity directs stem cell lineage specification', *Cell* 126(4): 677-89.

Heher, P., Maleiner, B., Pruller, J., Teuschl, A. H., Kollmitzer, J., Monforte, X., Wolbank, S., Redl, H., Runzler, D. and Fuchs, C. (2015) 'A novel bioreactor for the generation of highly aligned 3D skeletal muscle-like constructs through orientation of fibrin via application of static strain', *Acta Biomater* 24: 251-65.

Horch, R. E., Debus, M., Wagner, G. and Stark, G. B. (2000) 'Cultured human keratinocytes on type I collagen membranes to reconstitute the epidermis', *Tissue Eng* 6(1): 53-67.

Koning, M., Harmsen, M. C., van Luyn, M. J. and Werker, P. M. (2009) 'Current opportunities and challenges in skeletal muscle tissue engineering', *J Tissue Eng Regen Med* 3(6): 407-15.

Nau, T. and Teuschl, A. (2015) 'Regeneration of the anterior cruciate ligament: Current strategies in tissue engineering', *World J Orthop* 6(1): 127-36.

Qazi, T. H., Mooney, D. J., Pumberger, M., Geissler, S. and Duda, G. N. (2015) 'Biomaterials based strategies for skeletal muscle tissue engineering: existing technologies and future trends', *Biomaterials* 53: 502-21.

Rowe, S. L., Lee, S. and Stegemann, J. P. (2007) 'Influence of thrombin concentration on the mechanical and morphological properties of cell-seeded fibrin hydrogels', *Acta Biomater* 3(1): 59-67.

Stewart, D. (1972) *The role of tension in muscle growth*, New York: Academic Press, INC.

Vandenburgh, H. H. and Karlisch, P. (1989) 'Longitudinal growth of skeletal myotubes in vitro in a new horizontal mechanical cell stimulator', *In Vitro Cell Dev Biol* 25(7): 607-16.

Vangsness, C. T., Jr., Kurzweil, P. R. and Lieberman, J. R. (2004) 'Restoring articular cartilage in the knee', *Am J Orthop (Belle Mead NJ)* 33(2 Suppl): 29-34.

Wei, B. and Jin, J. P. (2011) 'Troponin T isoforms and posttranscriptional modifications: Evolution, regulation and function', *Arch Biochem Biophys* 505(2): 144-54.

Yokoyama, S. and Asahara, H. (2011) 'The myogenic transcriptional network', *Cell Mol Life Sci* 68(11): 1843-9.