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Generation of 3D skeletal muscle-like fibrin constructs via the application of mechanical stimuli

104 – Biomedizin Innovativ – patientInnenfokussierte, anwendungs-orientierte sowie interdisziplinäre Forschung am Puls der Zeit

Abstract

Skeletal muscle tissue engineering demonstrates a promising tool for the creation of mature muscle tissue constructs in vitro and in vivo. The aim of engineering mature muscle-like constructs ranges from substituting lost tissue after traumata, cancer ablation to conduct research on in vitro muscle disease models. Therefore the interplay of three key factors is crucial, which constitute of the right biomaterial, myogenic cells moreover mechanical stimulation. In our 3D studies we were using fibrin based hydrogels with C2C12 mouse myoblasts incorporated, as fibrin constitutes a biomaterial suitable for skeletal muscle tissue engineering. The novel bioreactor system (MagneTissue) was used to apply mechanical stimuli to our ring shaped fibrin constructs. We were able to engineer skeletal muscle-like tissue constructs via parallel alignment of fibrin fibers and cells due to the applied strain causing them to differentate into myofibers. Static strain resulted in a positive effect on myogenic differentiation with an increase in the gene expression levels of myogenic markers such as MyoD, Myogenin and Troponin T. In more recent studies 10 % cyclic strain also seems to have a positive effect on gene expression levels of muscle markers for maturity like myosin heavy chain (MHC) and Troponin T. The MagneTissue bioreactor system provides a versatile tool for testing various training parameters, for engineering mature skeletal muscle-like tissue. A future goal is; to examine different mechanical stimulation protocols, also over a longer period of time, to achieve more mature skeletal muscle tissue constructs for putative transplantation.

Keywords:

Fibrin, skeletal muscle tissue engineering, myogenic cells, MagneTissue bioreactor system, mechanical stimulation

Introduction

Skeletal muscle tissue accounts for approximately 40 % of the whole body weight and is the largest organ in the human body (Juhas, Ye, & Bursac, 2015, Juhas et al 2015, Frontera W.R. & Ochala J. 2015). The functionality of the muscle contraction is inevitable for proper locomotion (Juhas et al 2015

& Lewis et al 2014). Therefore, if impaired, it clearly results in a pronounced reduction of quality of life. The hierarchical structure of skeletal muscle tissue is built of parallel aligned myofibers, which are made of previoulys fused myoblasts (Juhas et al 2015). Satellite cells are residing in the inner structure between myocytes and the connective tissue. They reside in a quiescent state only being activated to regenerate damaged muscle tissue in case of injury (Bursac, et al 2015, Bach, et al 2004, Frontera 2015, Vigodarzere 2014, Yang & Dong 2014). For force generation, small units, named sarcomeres, made of thin actin and thick myosin filaments, are responsible (Vigodarzere 2014, Cittadella V. & Mantero S). After an injury satellite cells get activated by re-entering the cell cycle. Furthermore they undergo asymmetric cell division to maintain the stem cell pool and also differentiate to form new 0myofibers (Bursac, et al 2015, Juhas et al 2015). When only a small percentage of skeletal muscle tissue is injured it normally regenerate itself. Whereas if the injury is too severe it might result in tissue loss accompanied by reduced functionality, fibrosis and the development of scar tissue (Choi et al 2015).

Therefore skeletal muscle tissue engineering would demonstrate a promising tool for the creation of mature muscle tissue constructs *in vitro* and *in vivo*. One application for engineered muscle tissue would be to substitute lost tissue for example after traumata or cancer ablation by the creation of autologous muscle constructs. Another putative scientific use would be investigate muscle disorders by using *in vitro* disease models (Juhas et al 2015).

State of the art is engineering of (free standing) 3D muscle tissue constructs for regenerative therapies and the attempt of creating models which mimic whole organ in physiological setting in such an environment (Bursac et al 2015). So far tissue engineering has only entered clinics in the sector of bone, cartilage or skin replacement and regeneration (Choi et al 2015). The creation of skeletal muscle-like tissue constructs relies on the interplay of three classical TE approaches: (1) favorable biomaterials or scaffolds, (2) cells with the potential of differentiating towards the myogenic lineage, which in addition ideally should be easy to expand, and (3), mechanical stimuli, which reportedly have a positive effect on the maturation of the tissue.

Biomaterials are one important component as they are supposedly having a positive effect on cell attachment and furthermore favor cell proliferation and differentiation. There is a broad spectrum of biomaterials, which ranges from synthetic materials like biodegradable polyesters of polyglycolic acid (PGA), polycaprolactone (PCL), poly(lactic-co-glycolic acid (PLGA) and poly-l-lactic acid (PLLA) (Lewis et al 2014, Quazi et al 2015, Grasman et al 2015) to natural materials such as alginate, collagen, matrigel and fibrin, or a combination of the mentioned - (Lewis et al 2014, Bursac et al 2015, Quazi et al 2015). Fibrin is biocompatible, biodegradable and non-toxic (Bursac et al 2015). Furthermore fibrin offers the attractive feature of tuneable mechanical characteristics, which means that it can be used to mimic the physiological stiffness of skeletal muscle tissue. Additionally, if mechanical stimulation is applied to fibrin, it´s fibrils align in the direction of the axis of the strain, which favors guided cellular structuring (Heher et al 2015).

Preferentially cells for muscle tissue engineering, can either be freshly isolated and expanded or immortalized muscle cells are used (Bursac et al 2015). The pool of cells scientists can choose from range from induced pluripotent stem cells (iPSCs) to myoblasts derived from (embryonic stem cells) (ESCs), human pluripotent stem cell-derived skeletal muscle cells (hPSCs), mesenchymal stem cells (MSCs) and immortalized muscle cell lines like C2C12 cells which are the most commonly used (Shadrin et al 2016).

Another crucial factor for muscle tissue engineering is the mechanical stimulation, as cells in general are affected by the forces they experience in their physiological environment. These mechanical stimuli

towards cells are known as mechanotransduction, which means that these physical stimuli are converted into biochemical signals ultimately leading to a biological response, influencing cellular behavior (Vigodazere & Mantero 2014). In myogenesis mechanical stimulation has been shown to have an impact on protein expression, gene expression, differentiation and maturation (Goldsplink G. 2003, Goldspink et al 1992, Powell et al 2002). To mimic the physiological conditions *in vitro*, exercise can be simulated by the application of certain mechanical stimulation protocols to prior engineered muscle-like constructs (Bach et al 2004) such as cyclic or static strain parameters by a bioreactor. Studies showed that exercise favors the fusion of myoblasts, furthermore it improves the alignment of those cells and leads to hypertrophy of skeletal muscle tissue (Bach et al 2004).

Material and Methods

In our studies we were using fibrin based hydrogels with C2C12 mouse myoblasts incorporated. Furthermore the novel bioreactor system (MagneTissue) was used to apply mechanical stimuli to our ring shaped fibrin constructs which were mounted on a spool-/hook system. Then a certain strain protocol was applied to the fibrin rings via magnetic force transmission. The custom made bioreactor software makes it possible to test different strain parameters, like cyclic, static strain or a combination of both.

Results

In our 3D *in vitro* studies we were able to engineer skeletal muscle-like tissue constructs by embedding C2C12 mouse myoblasts within fibrin hydrogels and cause them to differentiate towards myofibers and parallel alignment along the axis of strain, which is crucial for force generation in muscle tissue. Furthermore, we could demonstrate that myotubes were generated with a more mature phenotype concerning their sarcomeric patterning, width and length after mechanical stimulation of the scaffolds using 10 % static strain for 6 hours and 3 % strain for 18 hours within 6 consecutive days. Static strain had a positive effect on myogenic differentiation with an increase in the gene expression levels of myogenic markers MyoD, Myogenin and Troponin T. Myogenin as well as Troponin T have to increase over time of myoblast differentiation and eventually form myofibers. Myofiber formation was demonstrated by expression of myosin heavy chain (MHC) using immunofluorescence stainings on day 9 compared to non-strained and control fibrin rings (Heher et al 2015).

In recent stiffness studies, we could demonstrate that various fibrinogen (FBG) and thrombin (THR) concentrations (20 mg FBG/0,625 U THR, 15 mg FBG/ 3 U THR, 10 mg FBR/ 3 U THR) improve myofiber formation. This could be visualized by the expression of MHC via immunofluorescence stainings on day 9. Here static strain led to parallel aligned myofibers along the axis of strain in different material compositions (Fig.2). However unstrained floating control- as well as control fibrin rings indicated diffuse myofiber structure.

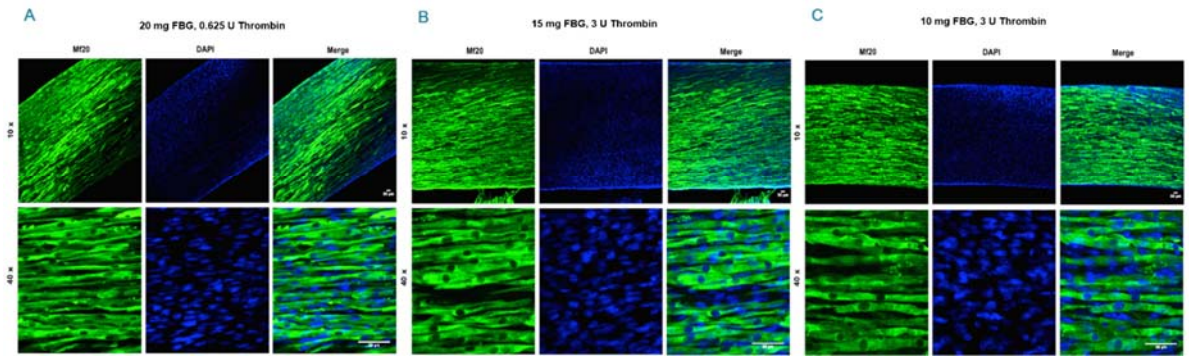

Fig.2: Myofiber formation in various material compositions: Immunofluorescence stainings of mouse myofibers for MHC fast (myosin heavy chain) in green, nuclei were counterstained with DAPI in blue on day 9. Fibrin scaffolds with varying material compositions 20 mg FBG/0,625 U Thrombin (A), 15 mg FBG/3 U Thrombin (B), and 10 mg FBG/3 U Thrombin (C) demonstrated parallel aligned myofibers along the axis of strain, FBG= Fibrinogen.

Furthermore, static strain led to increased gene expression levels of *TNT* as well as MHC within the various material compositions of FBG and THR compared to control groups where no strain was applied to (Fig.3)

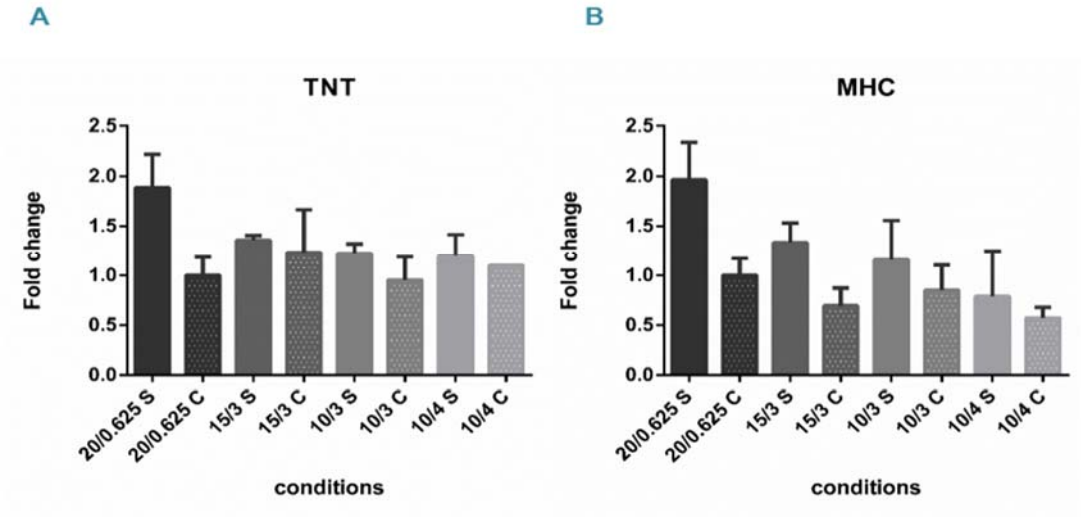

Fig.3: Increased levels of TNT and MHC due to application of strain: Gene expression levels of TNT (A) as well as MHC (B) were elevated when strain was applied, compared to non-strained fibrin rings (n=3, mean + SD, qRT-PCR was performed in triplicate, fold induction was calculated with the ΔΔCT method, S=Strain, C=Control).

Outlook

Latest experiments demonstrated that cyclic as well as static strain increased gene expression levels of mature muscle markers like MHC and Troponin T. Within a training phase of 9 days both training parameters resulted in aligned and more mature muscle-like constructs as compared to unstrained floating controls. Extensive training for up to 21 days seemed to have an enhanced effect on myogenic differentiation and maturation. Although further experiments will be needed.

The MagneTissue bioreactor system provides a versatile platform for testing various training parameters and regimes, which can be individually adjusted, using a custom made software, for the purpose of engineering mature skeletal muscle-like tissue. A future goal is, to examine different mechanical stimulation protocols to achieve more mature skeletal muscle tissue constructs for putative transplantation.

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