Impact of electrical stimulation on three-dimensional myoblast cultures - a real-time RT-PCR study

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Abstract

Several focal skeletal muscle diseases, including tumours and trauma lead to a limited loss of functional muscle tissue. There is still no suitable clinical approach for treating such defects. A promising approach could be the tissue engineering of skeletal muscle. However, a clinically reliable differentiation stimulus for three-dimensional (3-D) cultures is necessary for this process, and this condition has not yet been established. In order to quantify and analyze the differentiation potential of electrical cell stimulation, primary myoblasts were stimulated within a 3-D fibrin-matrix. Gene expression of MyoD, myogenin and AChR- ϵ were measured by real-time RT-PCR over a time period of eight days, showing immediate down-regulation of all marker genes. For tissue engineering approaches, cell multiplication is crucial for acquisition of sufficient tissue volumes for reconstruction. Therefore, all experiments were performed with high and low passaged myoblasts, demonstrating higher transcript rates of marker genes in low-passage cells. Our findings strongly suggest a reconsideration of electrical stimulation in muscle tissue engineering.

Keywords: skeletal muscle tissue engineering • electrical stimulation • real-time RT-PCR • 3-D cell culture • fibrin • myoblast differentiation

Introduction

Since loss of skeletal muscle tissue, due to trauma or tumour ablation *e.g.*, often results in a scar forming healing process, the final aim of skeletal muscle tissue engineering would be the regeneration of skeletal muscle tissue. Different approaches to recreating skeletal muscle tissue *in vitro* and *in*

vivo have been established [1, 2], which stand besides the attempt to recreate tissues from embryonic stem cells [3]. The latter ones still faces the obstacles of legal, moral and biological issues coming along with the use of embryonic cells. Our approach of skeletal muscle tissue engineering focuses on the cultivation and replantation of autologous muscle precursor cells, namely myoblasts. One major difficulty is based on the need of expanding cells *in vitro* for subsequent autologous transplantation, in order to create sufficient volumes of neo-tissue [4]. After the expan-

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sion of myoblasts, one needs to establish techniques which induce the differentiation of these cells in the direction of functional myofibers. Ideally, these techniques should be feasible in a clinical scenario, which means the absence of mutagenic substances, genetic manipulation of cells, or methods that bear long-term risks (like retro-viral transfections). Therefore, our approach to skeletal muscle tissue engineering focuses on electrical stimulation of expanded myoblasts.

Differentiation of mononucleated myoblasts into functional skeletal muscle tissue is initiated and controlled by multiple regulatory factors and extracellular stimuli such as growth factors and mechanical or electrical stimulation. Some of these differentiation methods, such as the reduction of the serum content in culture media, have only been investigated in two-dimensional (2-D) cell cultures, which do not emulate the natural 3-D environment very precisely [5]. In previous studies, we showed that this stimulus neither induced differentiation in 2-D expanded myoblast cultures, nor in 3-D fibrin-myoblast-cultures [5]. Other often used differentiation stimuli are effective in non-clinical research studies but obsolete for later applications in clinical scenarios, *e.g.* the usage of Matrigel® [6, 7] or co-cultivation of myoblasts and neuronal tissue [5]. A clinically relevant differentiation stimulus, applied and evaluated in a defined 3-D environment, has not yet been investigated. Such an approach could provide further insight into the differentiation process of primary myoblasts and new perspectives in the field of skeletal muscle tissue engineering.

The use of chronic electrical stimulation to imitate neuronal activity during myogenesis has been shown to influence the expression of a myosin heavy chain (MHC) [8–12] and myogenic transcription factors [13–15], as well as promoting the maturation of 2-D primary muscle cultures, *in vitro* in terms of the expression of adult MHC isoforms. It has also been shown that cell proliferation increases in electrical stimulated cell cultures [16]. By evaluating different stimulation protocols, it has been demonstrated that gene expression of MHC isoforms can be modulated by the stimulation and burst frequency. As a result, stimulation protocols promoting differentiation of satellite cell cultures *in vitro* have been developed by Naumann and Pette [12]. The chronic electrical stimulation technique suits standards for later clinical applications and

can be applied in 3-D cell cultures. However, most of the previously conducted studies using electrical stimulation of myoblasts employed either 2-D cell culture methods [10, 12] or explant cultures of whole muscles, rather than expanded single myoblasts [13, 17]. In addition, no quantitative analysis of gene expression in three-dimensionally cultivated and electrically stimulated myoblasts has been performed yet.

Differentiation in myoblast cell cultures can be evaluated by investigating the expression of marker genes that play distinct roles during myogenesis. Among these genes, myogenic regulator factors (MRF) play a predominant role [18]. MyoD and Myogenin, both transcriptional factors of the MRF family, function as promoters of several muscle specific genes by heterodimerization with "E-proteins" and binding to the DNA-sequence CANNTG, termed E-box [19]. MyoD, the myogenic determination factor, appears in the early stages of myogenesis, induces fusion of myoblasts, and reduces proliferation by up-regulation of cell-cycle inhibitors (p21) and down-regulation of cell-cycle activators such as cyclins and cyclin-dependent kinases [20]. Myogenin appears later during differentiation, but acts more specifically in the creation of multinucleated muscle tissue. Myogenin deficient knock-out mice will fail to develop skeletal muscle due to insufficient myoblast fusion [21]. Both transcription factors are up-regulated during myogenesis [22] and, thus, can serve as marker molecules for the different stages of maturation.

Another important feature of differentiated myofibers is the expression of nicotinic acetylcholine receptors (nAChR) as part of the motor endplates. Expression of nAChR is also regulated by MRF [23]. The nAChR is a ligand-gated ion channel that exists in two forms: a fetal and an adult type. They contain either a γ -subunit or a ϵ -subunit, each of which results in different functional properties. The adult ϵ -subunit is only expressed in mature and differentiated skeletal muscle and can therefore function as a differentiation marker [5].

When aiming for the generation of neo-muscle tissue in order to reconstitute lost skeletal muscle by means of tissue engineering, large numbers of myoblasts must be obtained. This is only achievable through the expansion of myoblasts *in vitro*, resulting in a number of passages implying a low grade of myoblast differentiation [24]. There is not much

detailed and accurate data in the literature concerning the process of cell passaging and its influence on the ability of myoblast differentiation in 3-D cell culture systems.

The present study focuses on the influence of electrical stimulation on 3-D primary myoblast cultures and the potential of this method for clinical application in skeletal muscle engineering. Primary myoblasts were expanded up to two and four passages, cultivated in a 3-D, biocompatible and degradable fibrin matrix, and subsequently submitted to electrical stimulation, which was performed under constant current levels. Then, quantitative analysis of mRNA expression of MyoD, myogenin and AchR ϵ was conducted by using real-time RT-PCR (TaqMan[®]). Additionally, all samples were tested for MHC_{embryo} mRNA expression by conventional RT-PCR as a marker gene for later stages of myogenic differentiation.

Materials and methods

Cell culture

Primary rat myoblasts were obtained from hind limbs of three-day-old syngeneic Wistar-Kyoto-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 70 μ m 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), referred to as growth medium. Medium was changed every three days. Cells were expanded through two and four passages and purity of myoblast cultures (>95%) was evaluated by anti-desmin immunostaining as described in [5] including propidium iodide nuclei counterstaining.

Three-dimensional myoblast-fibrin culture

3-D myoblast-fibrin cultures were established by resuspending 500.000 trypsinized P2 or P4 myoblasts in

500 μ l growth medium, supplemented with 1000 KIU/ml aprotinin (Bayer, Leverkusen, Germany), and mixing with 500 μ l fibrinogen (20 mg/ml in isotonic sodium-chloride-solution (Sigma-Aldrich, Irvine, UK). The myoblast-medium-fibrinogen suspension was mixed with 80 μ l thrombin (60 IU/ml Thrombin S in 40mM calcium-chloride solution; Baxter, Vienna, Austria) and pipetted into a four-chamber culture slide (Beckton Dickinson, Franklin Lakes, USA). After one minute constructs hardened and were covered for five days with 1ml growth medium containing 1000 KIU/ml aprotinin.

Electrical stimulation of myoblast-fibrin cultures

Conductivity of fibrin matrix was confirmed by resistance measurements. Cell constructs (n=4) were continuously stimulated *via* platinum electrodes by a 8-channel stimulator (STG-1008[®], Multi-Channel-Systems, Reutlingen, Germany), which was programmed by computer-based control software (MS. Stimulus 1.8) and modified for higher current generation (up to 6.8mA). Electrodes were enclosed in a plexiglass cover and located in the outmost position of the culture-slides (Fig. 1a). Stimulation was performed with current at a constant 6.8mA (4ms duration), using biphasic stimuli in order to prevent electrolysis. As a result, changes in electric resistance due to media volumes, cell proliferation, etc. had no influence on the stimulation amplitude. Electric bursts lasted for 250ms and were delivered at intervals every 4s. Neither changes in the temperature of the cell culture medium nor cell death due to electrical stimulation were observed. Unstimulated control cultures were kept under identical conditions. Analysis and evaluation were performed on days 0, 2, 4 and 8 of the electrical stimulation.

At all observation times, the cell cultures were checked for morphologic changes. Yet there was no evidence of myoblast differentiation, such as fusion to myofibers (Fig. 1b).

RNA isolation and cDNA synthesis

After homogenisation, total RNA was isolated with TRIzol[®] (Life Technologies, Paisley, UK) according to the manufacturer's instructions. Total RNA was treated with DNase I (Qiagen, Hilden, Germany), and both purity and concentration was determined by A₂₆₀ and A₂₈₀ (A₂₆₀/A₂₈₀= 1.7-2.0) measurements using a

Fig. 1 a): Modified cell culture chamber slide for electrical stimulation of 3-D myoblast cultures with STG 1008 b): Phase photomicrograph of passage 4 mononucleated myoblasts in 3-D fibrin matrix after 8 days of electrical stimulation

Ultraspec 1000 UV/Visible Spectrophotometer (Amersham Pharmacia Biotech, Buckinghamshire, UK). In order to detect RNA degradation, 1 μ g of total RNA was fractionated by electrophoresis on 0.9% formaldehyde agarose gel and visualized under UV light. Degraded RNA was dismissed.

Aliquots of 5 μ g total RNA per experimental group (n=4) were pooled and 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 desmin and GAPDH expression and were analysed for MHC emb expression in a 30 cycle standard PCR, using Taq DNA polymerase with Q-Solution (Qiagen, Hilden, Germany) and 5 μ l 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.

Real-time RT-PCR (TaqMan)

TaqMan probes and primers were designed using the computer software Primer Express 1.0 (PE Biosystems, Forster City, USA), except for GAPDH. The specificity of each primer was confirmed by BlastSearch and PCR amplification, verified on a 4% agarose gel. Nucleotide sequences of primers and probes are shown in Table 1.

TaqMan PCR assays for the genes MyoD, myogenin, AChR- ϵ and GAPDH were performed in triplicate on cDNA templates in 96-well optical plates (Abgene, Surrey, UK) using an ABI Prism 7700 Sequence Detection System from Applied Biosystems (Foster City, USA). Standard amplification settings given by 7700 SDS were used, *i.e.* 10' at 90°C for activation of polymerase and then 15" at 90°C followed by 60" at 60°C for 40 cycles.

RNA quantification was performed by the standard curve method described in Applied Biosystems User Bulletin No. 2 (www.appliedbiosystems.com). Standard cDNA was obtained from RNA extracted from skeletal muscle biopsies of three-day-old rat pups. A dilution series from 40 ng/ μ l down to 0.05 ng/ μ l was prepared and used for calculations of RNA quantity. The real-time PCR reaction mixture contained 25 μ l of Absolute QPCR ROX Mix 2x (Abgene, Hamburg, Germany) mixed with 21 μ l DEPC-H₂O and 1 μ l forward primer (10 μ M), 1 μ l reverse primer (10 μ M) and 1 μ l probe (5 μ M). 1 μ l of a 20 ng/ μ l sample cDNA was added. The amplification of no-RT-PCR control templates was not detected at any time. The probe for GAPDH was fluorescence-labelled with VIC and TAMRA as quencher, where probes for the genes of interest were labelled with FAM and TAMRA. Results were expressed as ratios between the genes of interest and GAPDH fluorescence relative units. Primers and probe for GAPDH were commercially purchased (Applied Biosystem, sequences unknown), primers and probes for genes of interest from Qiagen (Hilden, Germany). Expression levels for each gene of interest

Table 1 Oligonucleotides and TaqMan fluorogenic probes.

Primer	Accession no	Corr. bp	Fragment size (bp)	Sequence	1) 5'-primer 1-3' 2) 5'-primer 2-3' 3) 5'-(FAM)-probe-(TAMRA)-3'
Desmin	452778	280-300 962-982	683	1)5' – GAC GCA GTG AAC CAG GAG TT – 3' 2)5' – ATC ATC TCC TGC TTG GCT TG – 3'	
GAPDH	X02231	224-244 821-841	598	1)5' – CGA TCC CGC TAA CAT CAA AT – 3' 2)5' – GTC CTC AGT GTA GCC CAG GA – 3'	
MHC_{emb}	NM_012604	2865-2885 3361-3381	497	1)5' – TGA CCT TGA GCT GAC ACT GG – 3' 2)5' – CTT CCT CCA GCT CCT CAA TG – 3'	
MyoD	M84176	568-584 635-653 593-618	86	1)5' – CGC CGC CT GAG CAA AG – 3' 2)5' – TGG TTC GGG TTG CTG GAC – 3' 3)5' – CCT TCG AGA CCC TTA AGC GCT GCA – 3'	
Myogenin	AF054894	311-332 389-411 334-357	101	1)5' – TGA AGA GAA GCA CCC TGC TCA – 3' 2)5' – TCA ATG TAC TGG ATG GCA CTG C – 3' 3)5' – TCA ATG ATG GTG GTG GCA CAG – 3'	
AChR-ε	X13252	308-328 374-396 330-352	89	1)5' – GGA CGA TTT TGC AGG CGTAG – 3' 2)5' – AAC AAT CTC TGG CAG CCA TAC A – 3' 3)5' – ATC CTG CGG GTC CCT TCC GAA C – 3'	
GAPDH				Sequence only known to providing company	

were calculated by normalising the quantified mRNA amount to the quantified mRNA amount of GAPDH.

Statistical analysis

The significance of the experimental differences reported here was determined using Student's t-test for non-paired variants. Significance was defined as $p < 0.05$.

Results

In this study, a reproducible method for the electrical stimulation of 3-D cell cultures was established by using an 8-channel electrical stimulator (STG-1008®) with custom made modifications to facilitate the generation of a homogeneous, longitudinal electric field within sterile cell culture chamber

slides. This device allowed the electrical stimulation of 3-D fibrin myoblast-cultures with currents up to 6.8mA under resistant-dependent voltage conditions. Prior to the electrical stimulation of 3-D myoblast fibrin-cultures, the current generation and conductivity of the cell-free fibrin matrix covered with growth medium was confirmed by resistance and current measurements.

To verify that the primary cells utilized were of skeletal muscle origin and did not change phenotype during cultivation procedures, myoblasts were stained for desmin (including propidium iodide counterstaining) revealing a >95% purity of myogenic cells and all prepared cDNA samples were confirmed for desmin expression as a specific marker of skeletal muscle tissue (data not shown). In all samples, degradation of isolated total RNA was excluded by formaldehyde-agarose-gel electrophoresis prior to reverse transcription. Sufficiently reverse-transcribed mRNA levels were

Fig. 2 Effect of electrical stimulation on primary myoblast gene expression of MyoD, myogenin and AChR ϵ -subunit measured by quantitative real-time RT-PCR with GAPDH as internal standard. EST = electrical stimulated myoblast fibrin-constructs. P2 = passage 2, P4 = passage 4

verified by reverse transcriptase (RT) PCR of the internal standard gene GAPDH before analysis by real-time RT-PCR (TaqMan[®]), thus confirming the quality of the prepared cDNA. Specificity of designed primers and probes was proven in real-time RT-PCR (TaqMan[®]) assays by using non-reversed transcribed RNA as control templates, which failed amplification.

Effects of electrical stimulation

MHC_{emb} expression in RT-PCR assays, in passage 2 (P2) as well as in passage 4 (P4) myoblasts within 3-D fibrin-cultures could only be detected on day 0, but not on the subsequent investigation dates, in both electrically stimulated and non-stimulated control cultures (data not shown).

The mRNA levels of the myogenic transcription factor MyoD could be quantified on all examination dates by TaqMan[®] real-time RT-PCR. Expression of MyoD in P2 myoblasts was significantly lower in the electrically stimulated cultures on days 2, 4 and 8, as compared to the

non-stimulated control cultures. In P4 myoblasts, expression of MyoD was lower on day 2 and 8 in electrically stimulated cultures; only on day 4 MyoD expression was higher in stimulated cultures (Fig. 2a).

Transcripts of the specific myogenic transcription factor myogenin could be quantified in all analysed samples, in electrically stimulated as well as non-stimulated myoblast-fibrin cultures. In all electrically stimulated cultures, expression of myogenin in P2 myoblasts was lower compared to non-stimulated constructs. On day 2 and day 4, differences in mRNA levels were significantly lower in stimulated cultures. In all P4 myoblast 3-D fibrin-cultures, expression of myogenin was lower in electrically stimulated cultures than in non-stimulated cultures (Fig. 2b).

P2 fibrin-myoblast constructs showed no significant difference in AChR- ϵ expression on day 2. On day 4 and 8, quantified gene expression was significantly lower in electrically stimulated cultures. P4 cultures showed almost equal expression on day 2, and lower mRNA levels on day 4 and 8, in electrically stimulated cultures (Fig. 2c).

Fig. 3 Influence of number of passages on primary myoblast gene expression of MyoD, myogenin and AChR ϵ -subunit in non-stimulated myoblasts cultivated within a fibrin-matrix. P2 = passage 2, P4 = passage 4

Influence of number of passages on primary myoblast gene expression

In order to investigate the influence of the number of passages on myoblast differentiation in a 3-D environment, myogenin, MyoD and AChR- ϵ transcript levels in P2 and P4 myoblast cultures were compared after TaqMan[®] real-time RT-PCR followed by Student's t-test. Though no significant differences were noted in electrically stimulated 3-D myoblast cultures (myogenin $p=52\%$, MyoD $p=60\%$, AChR- ϵ $p=6\%$), overall findings in non-stimulated myoblasts suggested a higher level of differentiation in P2 cells compared to cells of higher passages (P4). The transcript levels of MyoD in non-stimulated myoblasts were significantly higher in P2 cells, compared to P4 cells, on day 2 and day 4 and slightly lower on day 0 and day 8. Comparison of myogenin transcript levels in non-stimulated P2 and P4 cells showed significantly higher expression on day 0 and 4, equal expression on day 2, and slightly lower expression on day 8, in myoblasts of lower passage (P2) (Fig. 3a and 3b). Transcript levels of AChR- ϵ in P2 myoblasts, compared to P4 myoblasts, were also significantly high-

er on day 0 and day 4 and slightly higher on day 8, but slightly lower on day 2 (Fig. 3c).

Discussion

The emerging field of Tissue Engineering focuses on ways to isolate, cultivate and expand autologous cells *in vitro* and attempts to generate three-dimensional tissues of these cells, which subsequently could once be transferred to the patient. Novel sources for pluri- or even omnipotent cells could be the bone marrow [25], peripheral blood [26] or even tissues like the skin [27]. In case of skeletal muscle tissue engineering the commonly used cell source is skeletal muscle itself, since this tissue comprises its own regenerative cells, *i.e.* satellite cells, in the early stages termed myoblasts. Understanding and thus inducing the differentiation process of myoblasts to myofibers bears the potential for "re-generating" skeletal muscle tissue *in vivo*. Various stimuli have been investigated in terms of their differentiation potential. As neuronal co-cultivation [5], gene transfer methods [28], and other tech-

niques are not very feasible for a later clinical application, the electrical stimulation of myoblasts, myofibers or whole muscles have been subjects of investigation for several years.

Other electrical stimulation studies focussed on the effect of electrical stimulation on previously differentiated myofibers [10]. However, in a successful tissue engineering approach, expandable cells, harvested *via* patient biopsies (*i.e.* myoblasts), still have to be differentiated to myofibers *in vitro*. Furthermore, extensively expanded myoblasts tend to desist from differentiating to myofibers without very potent stimuli, as for example co-cultures with neuronal cells [5]. Therefore, our study focussed on the electrical stimulation of expanded primary myoblasts from rats, in order to gain results with high transferability to tissue engineering applications.

In most of the previous electrical stimulation studies, only 2-D cell culture systems were used [9, 10, 12]. As 3-D cell growth has been shown to differ significantly from monolayer cultivation [29, 30], a closer insight into myoblast differentiation might be gained by conducting these experiments in 3-D cell culture systems [16]. This also fulfills the requirements for a successful tissue engineering approach. Thus, we used a well defined and previously established 3-D myoblast-fibrin culture system [1, 31] for the electrical stimulation experiments. In our study, we evaluated the effects of electrical stimulation using a stimulation protocol which has been beneficial for the maturation of 2-D primary rat satellite cell cultures [12]. In particular, we established a stimulation protocol with bursts of constant current, independent of cell density culture media composition, filling, heights and 3-D matrix conductivity, thus ensuring that, in every culture, applied stimuli were identical. This was rendered possible by the modification of a commercial stimulus generator and ensured by the evaluation of applied current under every condition used in the different stimulation experiments.

Our results of myogenic gene expression in electrically stimulated 3-D myoblast-fibrin cultures, as obtained by real-time RT-PCR (TaqMan®), indicate a negative impact on the myogenic differentiation process. The early myogenic transcription factor MyoD appears to be down-regulated under electrical stimulation. The same observation can be made concerning the expression of myogenin, another myogenic transcription factor of the MRF-family

known to be expressed in the later stages of myogenesis. The third gene we analysed is the acetylcholine receptor ϵ -subunit (AChR- ϵ), which has been found to be part of the mature receptor isoform, as it is found in adult, terminally differentiated motor endplates [32]. In previous studies, we could show the induction of AChR- ϵ mRNA and protein under the influence of neuronal tissue within a fibrin-matrix [5]. Under electrical stimulation, by which we intended to mimic at least part of the neuronal activity, the contrary could be observed: with ongoing electrical stimulation the AChR- ϵ expression (mRNA) of myoblasts decreased. In summary, these results suggest a suppressing influence of electrical stimulation on three-dimensionally cultivated myoblasts *in vitro*. This stands in contrast to several earlier works [9, 10, 12], which proclaim a maturation-inducing influence of electrical stimulation. This may be due, in part to the differentiation markers chosen in those studies, such as myosin heavy chain (MHC) isoforms. In our study, no expression of the embryonic isoform of MHC could be observed by RT-PCR analysis (data not shown), possibly indicating a different initial situation. In some studies, already differentiated myotubes were stimulated, thus resulting in increased contractile activity, but not mimicking the situation of expanded mononuclear myoblasts. Another reason for the results obtained by Pette *et al.* might be the utilization of non-expanded (P0) myoblasts, which display a significantly different expression pattern, as confirmed in our study (see below). However, our findings are in accordance with the results of Neville *et al.*, which also stated a down-regulation of MyoD, myogenin and acetylcholine receptor subunits in chick neonatal muscle tissue under electrical stimulation [13]. In our study, for the first time, primary mammalian cells have been investigated for the expression of the MRFs MyoD and myogenin under electrical stimulation, showing a time-dependent decrease in expression patterns.

Very little is known about the process of cell passaging and its influence on the ability of myoblast differentiation in 3-D cell culture systems. In order to cultivate and regenerate tissues, it is necessary to expand cells in order to obtain large enough numbers of cells or tissue volume. Although the induction of differentiation appears to be more difficult with higher numbers of passages

[24], the affects of cell passaging on the differentiation process, in terms of myogenic gene expression, to our knowledge have never been accurately measured in a 3-D culture system. To explore this relationship, we evaluated the effects of electrical stimulation and fibrin matrix on myoblasts with cells of low passages (P2) and compared these results to those of high passaged (P4) cells. Our results indicate a strong influence of the number of passages on MyoD, myogenin and AchR- ϵ expression levels: In non-stimulated myoblast-cultures, the expression levels of MyoD and myogenin (and for the most part AchR- ϵ) are higher in low passage myoblasts (P2) compared to high passage cells (P4). This reflects the particular hazards of the differentiation process when it comes to tissue engineering, in which cell expansion is inevitable. For the first time, a quantified analysis of the dependence of myoblast gene expression on of the number of passages in a 3-D environment is presented.

The real-time RT-PCR technique (TaqMan[®]) we used in this study has been shown to be a highly sensitive method, enabling the processing of multiple samples simultaneously and the generation of results more rapidly than Northern Blotting [19, 33]. For electrical stimulation experiments, only Northern Blotting has been used as a quantitative method of analysis [9, 13]. This method is limited to situations in which very low amounts of mRNA are to be detected (as in 3-D cell cultures, compared to whole muscle experiments). Expression levels of MRFs (like MyoD and myogenin) under electrical stimulation have never been quantified before. Therefore, studies using real-time RT-PCR analysis methods might provide a more accurate insight into gene expression levels in stimulated cells.

In conclusion, the electrical stimulation of primary myoblasts downregulates the expression of the myogenic marker genes MyoD, myogenin and AChR- ϵ , as quantified by real-time RT-PCR. Cultivation of myoblasts in a 3-D fibrin-matrix appears to have a differentiation inducing potential, at least during the initial phase of cultivation. We have shown that the number of cell passages has a significant influence on myoblast gene expression. Quantitative analysis of gene expression patterns, as made possible by using real-time RT-PCR, should be applied in cell differentiation research. Future experiments should utilize primary cells, and the particular problem of extended cell expansion for tissue engineering applications should be addressed.

As electrical stimulation of expanded primary myoblasts leads to down-regulation of myogenic marker genes, further investigation might focus on alternative strategies for inducing myoblast to myofiber differentiation.

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