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Influence of Static Magnetic Fields Combined with Human Insulin-like Growth Factor 1 on Human Satellite Cell Cultures

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Abstract. *Tissue engineering represents a promising research field, targeting the creation of new functional muscle tissue in vitro. The aim of the present study was to show the influence of static magnetic fields (SMF) and insulin-like growth factor-1 (IGF1), as enhancing stimuli on human satellite cell cultures, which are preferred sources of stem cells in engineering skeletal muscle tissue. To detect effects on myogenic maturation and proliferation, AlamarBlue® proliferation, assay and semi-quantitative reverse transcription-polymerase chain reaction of following markers was performed: desmin (DES), myogenic factor-5 (MYF5), myogenic differentiation antigen-1 (MYOD1), myogenin (MYOG), myosin heavy chain (MYH) and α1 actin (ACTA1). As a distinct marker of differentiation, immuno-histochemical staining and fusion index determination was performed on satellite cell cultures stimulated with IGF1 and IGF1-plus-SMF with an intensity of 80 mT. Proliferation was increased by additional SMF application to IGF1-stimulated cell cultures on the first day of myogenesis. Relative gene expression of measured markers was increased by IGF1 application in the first days of myogenesis except for ACTA1. Additional SMF application enhanced this effect. Nevertheless we were unable to demonstrate the formation of contractile muscle tissue. Immunhistochemical staining verified muscle origin and all markers were displayed.*

Severe traumatic injury, excision of great tumor masses and congenital defects are still associated with suboptimal therapy. At present, the leading therapy concept for such defects is surgical treatment with muscle flaps. Unfortunately, this practice is accompanied with donor-site morbidity. Engineering of skeletal muscle tissue is a promising research field in regenerative medicine which attempts to deal with this problem by autologous grafting of new functional muscle tissue created *in vitro*. Satellite cells, also called myoblasts, are used as preferred stem cells. They are easily available by harvesting of muscle biopsies, can be very well classified and exhibit a consistent potential for differentiation into multi-nucleated myofibres (1). Due to these features, they are preferred for cell-based therapies to treat *e.g.* muscular dystrophies and other neuromuscular diseases (2).

Although these cells are well-defined, the creation of functional skeletal muscle *in vitro* is still a challenging process. It is difficult to obtain sufficient amounts of substitute tissue for functional restoration in clinical applications (3). Another important point is that *in vitro*-developed skeletal muscle myotubes must feature as many characteristics as *in vivo* skeletal muscle tissue. A permanent maturation progress is necessary for sufficient "neo-tissue" to maintain its potential following autologous transplantation. The optimal induction of this maturation is a methodologically challenging process and is still under investigation.

Our group investigated the influence of a static magnetic field (SMF) on the differentiation progress in human satellite cell culture. We found that the effect depends on the growth factor concentration in the medium used. Maturation of human satellite cells is enhanced by simultaneous treatment with SMF and growth medium (GM; containing high amounts of growth factors) but not with differentiation medium (DM; containing low amounts of growth factors) (4). Most recently, we showed that stimulation of human satellite cell cultures treated with hepatocyte growth factor (HGF) or HGF with SMF does not lead to the desired enhancement of myogenic differentiation in terms of increased myotube formation and generation of contractile muscle tissue. Analysis of marker gene expression revealed heterogeneous results for the different myogenic markers of differentiation (5). In addition, it has been shown that an SMF with an intensity of 80 mT increases the accumulation of actin and myosin, and the formation of large

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multi-nucleated myotubes. This correlates to a promotion of myogenic cell differentiation in the immortal rat cell line L6 (6).

The objective of the present study was to analyze the effect of an SMF in combination with insulin-like growth factor 1 (IGF1) on human satellite cell cultures. IGF1 is primarily expressed by the liver and skeletal muscle and is implicated in the myogenesis. Myogenesis can be divided into different steps. The first step is the activation of satellite cells; the second is the enhancement of proliferation and differentiation including the regulation of IGF1 receptor (IGF1R) (7-9). Three isoforms of IGF1 in human muscle are known: IGF1Ea, IGF1Eb and IGF1Ec [also known as mechano-growth factor (MGF)]. Mechano growth factor E peptide (MGF-E) activates human muscle progenitor cells and induces an increase in their fusion potential at different ages (10). The level of IGF1 is highly increased after muscle damage caused by extensive exercise. Concurrently the expression of myogenic differentiation antigen 1 (*MYOD1*), myogenic factor 5 (*MYF5*) and myogenin (*MYG*) is up-regulated (11). These findings were supported by the results of Liu *et al*. After six-week strength training IGF1Ec, IGF1 and myogenic factors increased (12). Pesall *et al*. revealed that the proliferation rate of turkey satellite cell cultures treated with IGF1 rises significantly (13).

In this study, we analyzed the maturation of human satellite cells treated with IGF1 with SMF as possible pro-myogenic stimuli. Custom-made magnets generated an SMF with an intensity of 80 mT (14). We studied the expression of desmin (*DES*), *MYF5, MYOD1, MYOG*, myosin heavy chain (*MYH*), α1 actin (*ACTA1*), which are well-known markers in the progress of maturation and the reference gene glyceralaldehyde-3-phosphate dehydrogenase (*GAPDH*); *MYF5, MYOD1* and MYOG, genes of the myogenic regulator family, controlling fusion of satellite cells to multi-nucleated myofibres, served as early markers of differentiation (15). *MYH* and *ACTA1*, which are both important proteins of the contractile apparatus in skeletal muscle tissue, also served as terminal markers of differentiation (4, 16). Furthermore we performed AlamarBlue® proliferation assay and immunohistochemical staining (IHC) of MYF5, MYOD1, MYOG, MYH and ACTA1. To complete the analysis, we calculated the fusion index (FI) as an independent marker of myogenic differentiation.

Materials and Methods

Cell culture. The study protocol was approved by the Ethics Committee of the Medical Faculty Mannheim of the University of Heidelberg, Germany (reference number: 2008-325NMA) and patients confirmed their written informed consent.

Satellite cell extraction was carried out by using chopped muscle tissue (collected from 15 patients during head and neck surgery) by digestion with collagenase B (Roche, Mannheim, Germany) for 60 minutes and 0.05% trypsin-0.02% EDTA (PromoCell, Heidelberg, Germany) for 45 minutes, filtration through a sterile 70-μm cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA), and finally purification with the pre-plating technique (17). Primary myoblasts were pooled from the 15 patients and expanded until passage three. We performed anti-desmin immunostaining to confirm culture purity (>80%). 0.2% Gelatine-coated culture flasks (Sigma, Deisenhofen, Germany) were used to grow cells in differentiation medium (DM) consisting of minimal essential medium (PromoCell) supplemented with 2% horse serum (PAA Laboratories, Cölbe, Germany) 2 mM L-glutamine, and antibiotic/antimycotic mixture Penicillin, Streptomycin, Fungizone (PSF) (PromoCell) after cells had reached approximately 60% confluence without or with 10 ng/ml recombinant human IGF1 (PeproTech, Rocky Hill, NJ, USA) changed every 72 h. Cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air for 1, 4, 8, 12, 16 or 21 days.

SMF exposure. SMF with a magnetic field of 80±5 mT was generated by 4 cm × 4 cm neodymium magnet. Magnetic power was measured with a gaussmeter and the magnetic field was axial to the magnetic North vector as Coletti *et al*. described (6). Experimental conditions were the same with and without SMF, and incubation of all cultures was performed simultaneously.

AlamarBlue® (Invitrogen, Darmstadt, Germany) proliferation assay. We used 0.2% gelatin-coated 96-well culture plates and plated each well with 5000 satellite cells. Cells with DM (negative control) without additives and DM plus IGF1 with or without SMF application were examined. We used measurements of existing fluorescence at a wavelength of 540 nm and absorbance was monitored at 590 nm. Proliferation was measured at days 1, 4, 8, 12, 16 and 21.

IHC. Immunocytochemical staining was performed on cells grown on chamber culture slides (BD Falcon, Franklin Lakes, NJ, USA). We used antibodies directed against: DES (Dako, Hamburg, Germany), MYF5, MYOD1 (both Santa Cruz, Biotechnology, Heidelberg, Germany), MYH (raised against full-length smooth muscle myosin heavy chain), MYHL (both Abcam plc, Cambridge, UK), and ACTA1 (Zymed Laboratories, Invitrogen, Karlsruhe, Germany). We used the following antibody dilutions: DES, 1:100; MYF5, MYOD1, MYH and ACTA1, 1:50. To this first specific antibody, a corresponding biotinylated secondary antibody was subsequently added. Control staining was carried out by using chamber culture slides without the first antibody. Aminoethylcarbazol (Dako) was used as a chromogen to complete the peroxidase reaction. Inhibition of endogenous peroxidase was performed for 30 minutes with 0.3% hydrogen peroxide. The sections were washed with phosphate buffered saline (PBS) and incubated with normal sheep serum in PBS for 30 minutes at room temperature with the objective of blocking non-specific antibody reactions. Nuclear staining was conducted with Harris' haematoxylin. We used a Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany) for light microscopic investigations. ACTA1 was examined on day 4, MYH on day 4 and 8; MYOD1, MYF5 and DES were exanimated on day 8.

RNA isolation. To isolate the RNA, we used RNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The total RNA concentration was determined by measurement of absorbance at 260 and 280 nm (A260/A280=1.7-2.0) using an Ultraspec 1000 UV/Visible Spectrophotometer (Amersham Pharmacia Biotech, Buckinghamshire, UK).

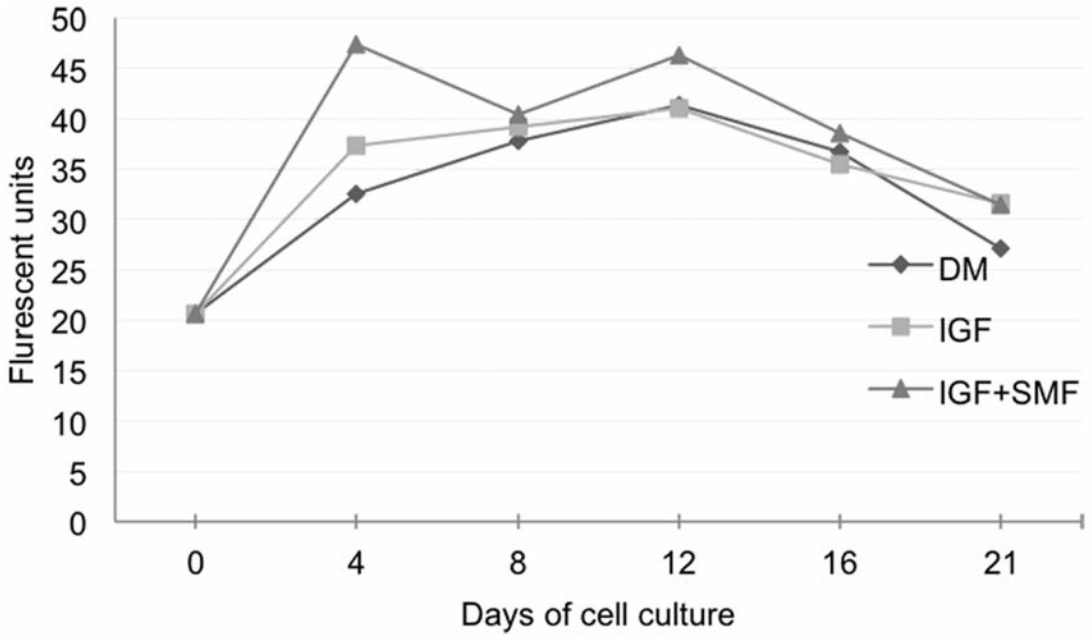

Figure 1. *AlamarBlue® proliferation assay of human satellite cell cultures stimulated with IGF1, IGF1+SMF and DM (control cell cultures). IGF1, Insulin-like growth factor 1; SMF, static magnetic fields; DM, differentiation medium.*

cDNA synthesis and polymerase chain reaction (PCR). We collected aliquots of 5 µg total RNA from cells after different treatments, then reverse transcription (RT) using an oligo(dT)-primed first-strand cDNA synthesis kit (Roche, Mannheim, Germany) was performed, according to the manufacturer's recommendations. All cDNA probes were analysed with a 30-cycle PCR procedure for *DES, MYF5, MYOD1, MYOG, ACTA1, MYH* and *GAPDH* by using *Taq* DNA polymerase (Amersham Pharmacia Biotech) and 2-5 µl RT products as templates in a Primus 96 Plus Thermal Cycler (MWG Biotech, Ebersberg, Germany). We used the same primer sequences as described elsewhere (18). The subsequent electrophoresis was run in 2% agarose gels containing ethidium bromide. Images of products were recorded under UV light. Relative gene expression was generated by using the densitometric scanning software ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Here *GAPDH* functioned as an internal standard.

FI determination. The number of nuclei in myotubes was counted and expressed as a percentage of the total number of nuclei analyzed. Counting was independently performed by two co-workers. FI determination was done on day 4 of culture in ACTA1-positive myotubes and on day 8 in DES-, MYOD- and MYH-positive myotubes by dividing the number of nuclei within the myotubes (with two or more nuclei) by the total number of nuclei ×100. Nuclear counterstaining was performed with haematoxylin.

Results

AlamarBlue® proliferation assay of human satellite cells treated with IGF1 and with/without SMF. IGF1 versus IGF1 plus SMF: Except for day 21, the fluorescence-units (FU) measured for cultures treated with IGF1 plus SMF were higher at all times than those for cultures treated with IGF1 only (Figure 1).

Control versus IGF1: The FU from cultures treated with IGF1 alone was higher at all times than those measured in the control culture (DM), except on day 12 and 16 (Figure 1).

RT-PCR analysis and gene expression. DES: We detected DES expression in all analyzed samples, which verified the myogenic phenotype of all cell cultures, and the expression remained constant in all samples. In cultures treated with IGF1, *DES* expression was higher in all samples at all time points compared to the control culture. DES expression was increased by SMF on day 4, 8 and 16 and decreased on day 12 and 21 when concurrently treated with SMF (Figures 2 and 3).

MYF5: Higher *MYF5* expression was detected in the control group except for day 4. *MYF5* expression was enhanced on day 4 and reduced on day 8 and day 21 by SMF application compared to the control (DM). No difference was detected on days 12 and 16 (Figure 3).

MYOD1: IGF1 did not lead to enhanced MYOD1 expression compared with the control group and the control group displayed higher expression on days 8-21 compared with the IGF1-treated group. Expression of *MYOD1* was enhanced by IGF1 plus SMF at day 4 and decreased on day 8 and 21; on day 12 and 16, no differences were found compared to sole addition of IGF1 (Figure 3).

MYOG: IGF1 treatment generally enhanced expression of *MYOG* days 8-21 compared to the control group. SMF treatment

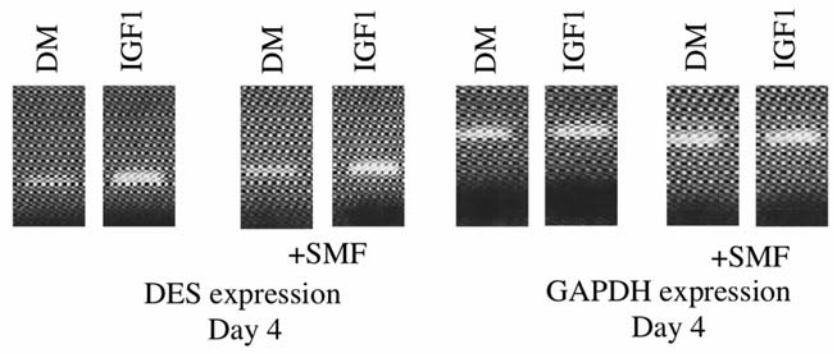

Figure 2. *Densitometric quantification of the DES and GAPDH gene expression. GAPDH was used as internal control. IGF1 and IGF1+SMF led to an increased DES expression in human satellite cell cultures on day 4. DES, Desmin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IGF1, insulin-like growth factor 1; SMF, static magnetic fields.*

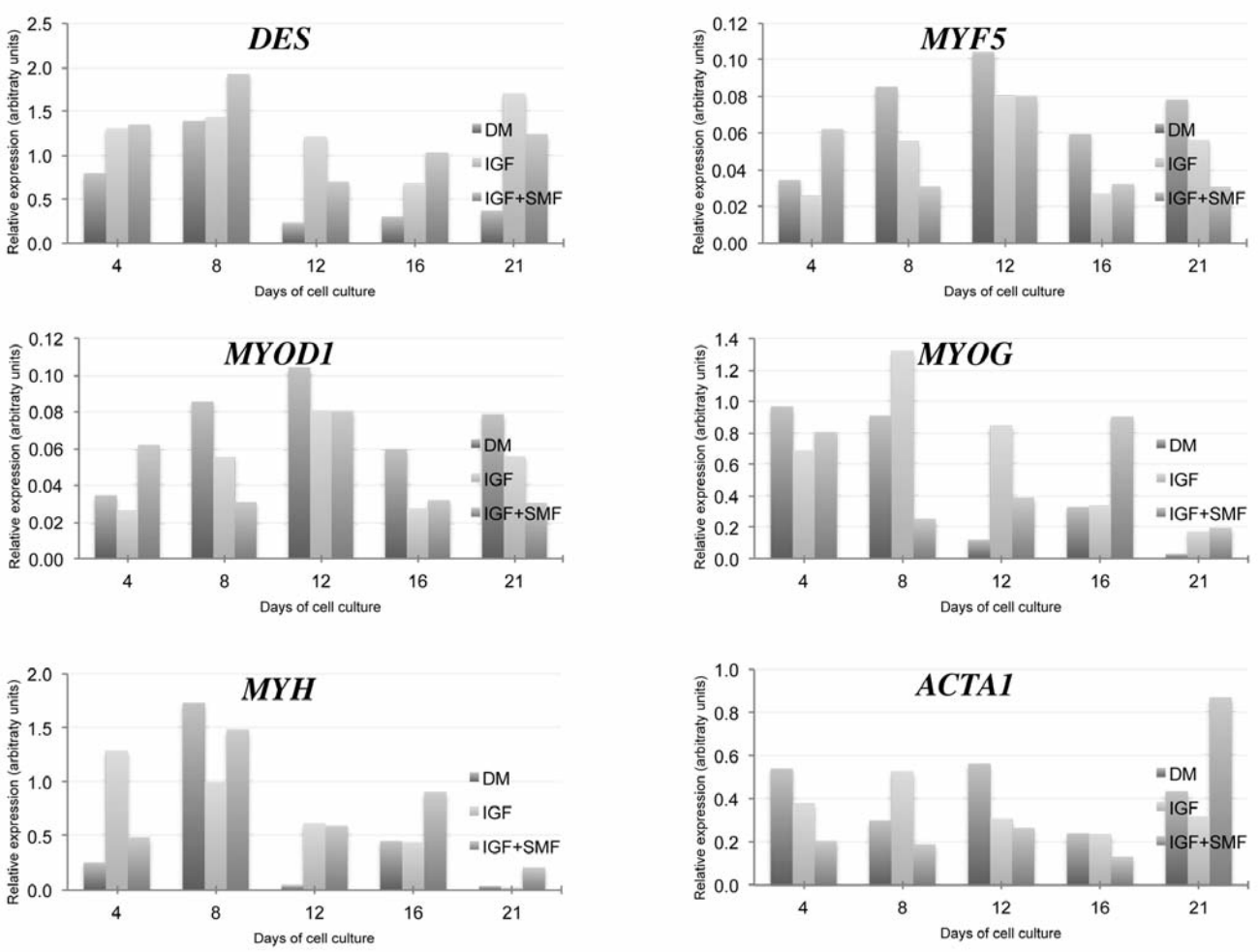

Figure 3. *Gene expression analyses of (A) DES, (B) MYF5, (C) MYOD1, (D) MYOG, (E) MYH and (F) ACTA1 in human satellite cell cultures stimulated with IGF1, IGF1+SMF and without additional stimulation (DM). GAPDH served as a reference gene. DES, Desmin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IGF1, insulin-like growth factor 1; SMF, static magnetic fields; MYF5, myogenic factor 5; MYOD1, myogenic differentiation antigen 1; MYOG, myogenin; MYH, myosin heavy chain; ACTA1, α1 actin; DM, differentiation medium.*

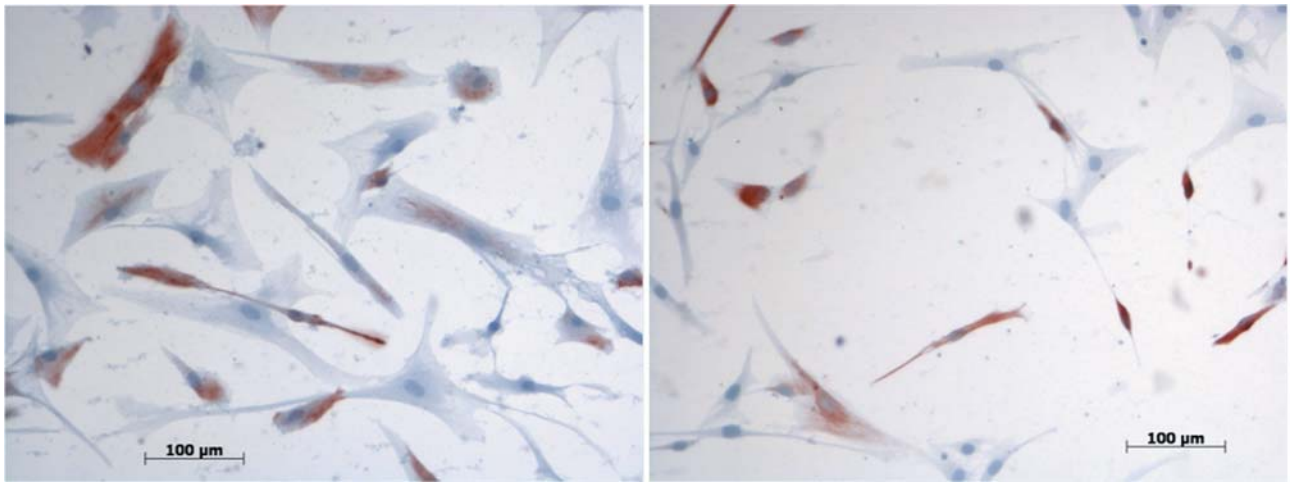

Figure 4. *Example of the immunohistochemical staining of DES in IGF1-stimulated cell cultures on day 8 (A) with and (B) without SMF treatment. DES, Desmin; IGF1, insulin-like growth factor 1; SMF, static magnetic fields.*

reduced *MYOG* expression in human satellite cell cultures on days 8 and 12 and increased MRNA levels on day 4, 16 and 21 in cultures simultaneously treated with IGF1 (Figure 3).

MYH: Compared to the control, IGF1-alone led to substantially increased *MYH* expression on days 4 and 12. SMF in combination with IGF1 led to increased MYH expression from day 16 (Figure 3).

ACTA1: Transcripts of α-actin were found in all analyzed cell cultures. Treatment with IGF1-alone did not lead to enhancement of *ACTA1* expression compared with the control group except on day 8. IGF1-plus-SMF treatment led to decreased relative expression in human satellite cells cultures from day 4-16 but to increased expression at day 21, compared to cultures threated only with IGF1 (Figure 3).

IHC and FI. To verify that the primary cells utilized were of muscle origin and did not change phenotype during cultivation, IHC was performed using specific monoclonal antibodies against myogenic markers DES and MYF5. By IHC, all investigated markers were detected. MYF5 was detected in cultures treated with IGF1-plus-SMF on day 8. Additionally, MYOD1 was expressed, verifying the myogenic phenotype on day 8. ACTA1-positive cells were found on day 4, with and without SMF stimulation. We could provide evidence of DES-positive cells at day 8 with and without SMF stimulation. FI of DES positive cells was higher in cells treated with IGF1 (but not with SMF) as in cells treated with both on day 8. ICC of ACTA1 showed more positively-stained cells in SMF-treated cell cultures on day 4, that is controversial to the relative gene expression results. On day 8 FI of ACTA1-positive cells treated with SMF showed a higher value than cells not treated with SMF. We could not detect contracting myofibrils at any time point (Figure 4).

Discussion

Engineering of skeletal muscle tissue aims to create new functional muscle tissue *in vitro* by utilising the myogenic differentiation potential of stem cells. In our case, satellite human myogenic stem cells were used, also called satellite cells. An important goal is to find preconditions which enhance cell maturation and proliferation. Two auspicious stimuli are SMF application and IGF1 addition to the satellite cell culture. With this study, the effect of IGF1 on human satellite cell cultures with and without combination of SMF was demonstrated.

The influence of SMF is still debated controversially, the mechanism is unclear and data involving human myogenic progenitor cell differentiation and proliferation are rare. However, it has been demonstrated that the impact of SMF is contingent on the magnetic field conditions. It was already shown by our group that an SMF is a strong stimulus which can enhance skeletal muscle tissue differentiation (4). Coletti *et al*. showed that application of SMF with an intensity of 80 mT promoted myogenic cell differentiation in the immortal rat cell line L6 In contrast, inhibition of proliferation and differentiation of skeletal muscle cells in newborn rat satellite cells was caused by *in vitro* reduction of earth magnetic field to 0.3 mT, while a low-intensity magnetic field (60-160 mT) had a stimulatory effect (19). Another investigation demonstrated that exposure to a strong SMF (10 T) leads to significantly orientated myotubes as the myogenic differentiation period increased in cultures from a mouse-derived myoblast cell line (C2C12) (20). SMF application at 2 T in C2C12 myoblast cells may delay cell growth and inhibit proliferation (21). Regarding the influence

of SMF (80 mT), the leading role of the growth factor concentration in the culture medium was demonstrated (4). It was also shown that stimulation of human satellite cell cultures with HGF or HGF with SMF did not lead to enhancement of myogenic differentiation, but led to a slight increase in proliferation in human satellite cell cultures from day 4-16 (5). Therefore, we assume that SMF application in addition to IGF1 stimulation may positively affect human satellite cell culture, for example, by enhancing expression of marker genes or cell proliferation.

The 70-amino-acid polypeptide IGF1 is synthesised under the control of growth hormone and, when it is found in the circulation, is primarily of hepatic origin, Other tissues, including skeletal muscle, also produce IGF1 (22, 23). IGF1 plays a critical role in skeletal muscle differentiation and growth. Absence of IGF1 and IGF2 receptor in mice led to muscle hypoplasia and death shortly after birth due to having insufficient muscle mass to inflate their lungs (24, 25). IGF1 overexpression leads to larger muscle fibres, enhanced muscle strength and prevents older mice from age-related muscle changes (26-29). IGF1 is also a crucial factor in adult muscle regeneration and hypertrophy, and both processes are related to muscle satellite cells. IGF-mediated action on skeletal muscle satellite cells has been followed with increasing interest (30). In transgenic mice with a local-acting isoform of IGF1 (expressed in skeletal muscle), no age-related muscle atrophy is known. Furthermore, the proliferative response to muscle injury is as characteristic as in younger mice and the repair of skeletal muscle damage was enhanced. This was up parents from the higher recruitment of proliferating bone marrow cells to the injured muscle and higher stem cell production (31, 32). Additionally, it has been shown that after acute muscle damage or chronic aerobic exercise, IGF1 production is increased locally while no change in circulating IGF1 is observed (33, 34).

Proliferation. Using the AlamarBlue® proliferation assay we showed that IGF1 addition at a concentration of 10 ng/ml to human satellite cell culture does lead to increased cell proliferation at day 4, 8 and 21, in contrast to the control culture. IGF1 with SMF leads to an increased proliferation in contrast to IGF1 at all time points except day 21. This culture also exhibits higher measurements in FU at all time points, in contrast to the control culture. This is in accordance with the results of Yang *et al*. who found enhanced proliferation for two days in C2C12 mouse myoblasts. They also showed higher proliferation with IGF1Ec addition than with native IGF1 (35). It was also shown that 2-week application of IGF1 onto atrophied gastrocnemicus muscle in rats dramatically increased proliferation and rescued lost muscle mass after immobilisation (36). In our case, SMF significantly enhanced the influence of IGF1 on satellite cell proliferation over 3 weeks in contrast to sole IGF1 application. This finding supports our hypothesis that SMF has a positive stimulatory influence on human satellite cell culture.

DES. DES-positive cells were found in all cultures by using IHC, verifying the myogenic phenotype. DES is an intermediate filament and part of the cytoskeleton. It is one of the earliest muscle-specific proteins expressed during myogenesis (37). Therefore, it can be used as a specific marker for early myogenic differentiation. While IGF1 treatment led to enhanced expression of *DES* mRNA, additional application of SMF did not lead to further enhanced expression, but rather to non-specific fluctuation without any clear tendency. In contrast, the FI of DES-positive cells decreased at day 8 in cultures treated with-IGF1-plus- SMF compared to cultures treated with IGF1 solely. This is in contrast to the findings of Czifra *et al*. who demonstrated that IGFI stimulation with 10 ng/ml or 100 ng/ml enhanced FI and the expression of DES on primary human skeletal muscle cells (38).

MYF5. Early satellite cell maturation can also be shown by proving the presence of myogenic regulator factor MYF5, which is up-regulated during the early course of differentiation (4, 39). IHC investigation showed MYF5 in all cultures. Transcripts of *MYF5* were greater on day 4, less on day 8 and 21 and about the same level at day 12 and 16 by IGF1 plus SMF treatment compared with sole IGF1 treatment. *MYF5* expression was not enhanced by IGF1 treatment compared to the control group, except for day 4. This could be explainded by its role as an early regulatory protein.

MYOD1. Another transcription factor, the myogenic determination factor MYOD1 induces differentiation by promoting expression of multiple muscle-specific genes. A prerequisite for myogenic differentiation is cross talking with cell cycle regulators. MYOD1 carries out this task, supports the withdrawal from the cell cycle, and its up-regulation represents the start of myogenic differentiation (40, 41). In our experiments, IGF1 had an inhibitory effect on *MYOD1* expression. Additionally SMF application did not lead to clear results. This is in contrast to the findings of Wu *et al*. who found that the IGF1-pathway proceeds as promyogenic cascade in myoblasts induced to differentiate (42).

MYOG. The relative expression of *MYOG* was enhanced from day 8-21 by IGF1 compared to the control group. MYOG is a transcription factor of the myogenic regulator family and operates during myotube development (4, 43). SMF application on IGF-stimulated cultures leads to varying results. Relative expression was enhanced on day 4, 16 and 21 and decreased on day 8 and 12. This is partly in accordance with our previous studies, where we showed that

SMF application led to lower relative MYOG expression (4) and that a stimulating effect of HGF on satellite cell cultures was supressed by SMF from day 4-21 (5).

MYH. mRNA levels of MYH, which is main part of the contractile apparatus and serves as a late marker of myogenic differentiation, were increased by IGF1 on day 4 and 12-21 (4, 44). It was shown that the loss of IGF1 isoforms (IGF1Ea or IGF1Eb) impairs myogenic differentiation and decreases MYH immunoreactivity by 70-80% (45). This indicates a higher grade of maturation. We found the same effect by HGF application to satellite cell culture (5). Additionally, SMF application on cultures stimulated with IGF1 led to enhanced MYH expression from day 16. We also detected MYH expression by Its, verifying cell differentiation.

ACTA1. ACTA1, another important structural protein of the contractile apparatus is ACTA1, which also acts as a marker of final stages of differentiation (46, 47). IGF1 did not lead to enhanced relative expression of *ACTA1* gene except on day 8, but SMF application caused a considerable increase on day 21, although it reduced the relative expression on day 4-16. This is in contrast to our findings that SMF application only reduces *ACTA1* gene expression but is in accordance with the results of Coletti *et al*., who also described an increase of ACTA1 in rat satellite cell cultures after SMF stimulation (4-6). We hypothesize that the discrepancy could be explained by the different origin of the utilized cells, but show that SMF application can also enhance the relative expression of *ACTA1* gene in human satellite cells. This is supported by the fact that we found higher FI for ACTA1-positive cells treated with SMF than in cells not treated with SMF.

In summary, SMF enhances IGF1-induced increase of satellite cell proliferation in the first days of myogenesis. IGF1 enhanced all measured markers except for *ACTA1*. Relative gene expression of *DES, MYF5, MYOD1, MYOG* and *MYH* was enhanced additionally by SMF. Relative gene expression of *MYH* and *ACTA1* was raised at late stages of myogenesis by IGF1 addition to the cell cultures. FI of ACTA1-positive cells was enhanced by IGF1-plus-SMF application in contrast to sole IGF1 application. Nevertheless we did not find contractile muscle tissue. Further investigation is needed and no categorical statement can be made regarding the influence of IGF1 and IGF1 with SMF.

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Conflict of Interest

There is no conflict of interest.

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