The myokine interleukin-7 may influence differentiation and migration of cultured satellite cells

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SUMMARY

Regular physical activity has beneficial effects on health. The relations between exercise and health are not fully understood. Myokines are secretory peptides of skeletal muscle origin with auto-, para- or endocrine functions. So far interleukin (IL)-6 is the best explored myokine. The identification of this myokine has made the basis for many new hypotheses. For example, can IL-6 or other muscle-derived factors explain some of the beneficial health effects of physical activity?

Recently, our group demonstrated production of IL-7 by cultivated skeletal muscle cells of human origin. The aim of this Master thesis was to investigate if IL-7 can influence skeletal muscle cell development in a paracrine manner.

To do this, we incubated skeletal muscle progenitor cells (satellite cells) with recombinant human (rh) IL-7. Gene expression analyses by real time polymerase chain reaction (RT-PCR), showed that rhIL-7 incubation during myogenesis significantly reduced the expression of the late differentiation marker gene myosin heavy chain 2 (MYH2) by 37 ± 4 % (mean ± SEM) at mRNA level after 7 days. Shorter incubation time (6 hours) with rhIL-7 significantly down-regulated the expression of early myogenic differentiating marker gene myogenic differentiation 1 (MYOD1) and the late myogenic differentiating marker gene myosin heavy chain 2 (MYH2). These findings indicate that IL-7 may influence differentiation of satellite cells into fully developed skeletal muscle cells. Radioactively labelled thymidine, glucose and oleic acid were used to monitor the influence of rhIL-7 on satellite cell proliferation, and on glucose and fatty acid metabolism. Recombinant hIL-7 incubation did not influence these parameters in satellite cells. Migration of satellite cells was measured using BD Falcon Insert Systems together with green fluorescence dye staining, picture analytical software and manual counting. Relative to control the migration was increased 40 ± 13 % (mean ± SEM) after 48 hours incubation with rhIL-7.

In conclusion: IL-7 may influence differentiation and migration of cultured satellite cells.

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Abbreviations

BSA, bovine serum albumin; cDNA, complementary DNA; CH, constant region of heavy chain; CL, constant region of light chain; DMEM, dulbecco's modified eagle's medium with low glucose; DMSO, dimethyl sulfoxide; ECM, extracellular matrix gel; ER, endoplasmatic reticulum;; EtOH, ethanol; FCS, foetal calf serum; GM-CSF, granulocyte macrophage colonystimulating; GSN, gelsolin; HAS, human serum albumin; HCl, hydrocloric acid; IL-7R, interleukin-7 receptor α chain; L-glut, L-glutamine; LD, living donor; M1, medium 1; M2, medium 2; M3, medium 3; MRF, myogenic regulatory factor; MYH2, myosin, heavy chain 2; MYOD1, myogenic differentiation 1; P, passage or p-value; PAX7, paired box 7; PBS, phosphate buffered saline; P/S, Penicillin/Streptomycin; rh, recombinant human; rhHGF**,** recombinant human hepatocyte growth factor; rhIL-7, recombinant human interleukin-7; rhIL-7R, recombinant human interleukin-7 receptor α chain; RNA, ribonucleic acid; RPLP0, targeting human large ribosomal protein P0; SE, standard error; SEM, standard error of the mean; SPARC, secreted protein, acidic, cysteine-rich (osteonectin); spm, scintillations per minute; SkGM, skeletal growth muscle medium.

1. INTRODUCTION

Skeletal muscle constitutes approximately 40 percent of the human body. Its main function is to posture the body, promote physical movement and breathing. Regular physical activity can enhance life-expectancy and reduce coronary heart disease, hypertension, colon cancer and diabetes mellitus (1). The same report also concludes that it is important for health of muscles, bones and joints, and it relieves symptoms of depression and anxiety and improves mood.

Although the health benefits are quite striking the mechanisms behind these positive effects are mostly unknown. In the later years it has been postulated that skeletal muscle can release myokines, "exercise factors." So, does skeletal muscle constitute endocrine properties, and if so, can this explain some of the positive health benefits? Some recent findings support the hypothesis that skeletal muscle releases exercise factors. During physical activity interleukine-6 (IL-6) is released from skeletal muscle and is shown to increase systemically (2). Due to its muscular origin Pedersen et al. refers to IL-6 as a "myokine" (3). IL-15 levels have also been shown to rise locally in response to physical activity (2;4). Recent findings in our laboratory have shown that human myotubes *in vitro* synthesize interleukine-7 (IL-7), and a 5-fold increase in total mRNA was found in skeletal muscle biopsies from 10 male individuals after an 11 week strength training programme (Haugen et al, 2009, submitted for publication). Our findings also indicated that expression of IL-7 receptor (R) mRNA was enhanced in undifferentiated myoblasts as compared to fully differentiated satellite cells. Based on these findings the present study was designed to investigate potential autocrine and paracrine actions of IL-7 functioning locally in skeletal muscle. To do so we have tested recombinant IL-7 incubation in an *in vitro* model of cultured human satellite cells/myotubes. We have monitored oxidation of fatty acids and glucose, proliferation and migration of satellite cell and changes in mRNA expression of differentiation markers in myoblasts during differentiation in the presence of recombinant human IL-7.

Interleukin-7 and the IL-7 receptor

Interleukin-7 is a glycoprotein and is a member of the type I cytokine family. It is known as a lymphopoetic cytokine and is vital for the development and survival of T-cells (5). Production of IL-7 has been shown in bone marrow stroma, thymic stroma, keratinocytes, neurons, lymph node follicular dendritic cells, and endothelial cells (6). The spleen may also be a significant production site in humans (7), and hepatic IL-7 expression regulates T cell responses in mice (8).

The IL-7 receptor α (IL-7R) is a heterodimer and belong to the γ-chain family of cytokine receptors, including IL-2, -4, -9, -15 and -21 receptors (9). The IL-7R in lymphocytes is a part of the Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) receptor niche and binding of IL-7 activates multiple pathways that regulate lymphocyte survival, glucose uptake, and cell proliferation and differentiation (10). IL-7R is expressed in resting T cells but is down-regulated in response to IL-7 (11). The majority of publications about IL-7 are related to its functions on the immune system. Its function in the skeletal muscle is, to our knowledge, unexplored.

Interleukin-7 and health

Mutations in the IL-7R ectodomain cause an under-stimulation of the IL-7 pathway and inhibits T and B cell development, as exemplified in patients with a form of severe combined immunodeficiency (SCID) (12). In murine models, IL-7 has demonstrated an ability to enhance antitumor immune response both *in vitro* and in mice previously injected with tumour cells. IL-7 may potentially have clinical use due to immunoenhancing properties (6). Increased levels of circulating IL-7 levels are observed in patients with HIV-infections and in T-cell lymphopenia (5). And in CD4(+) T cells from HIV-infected persons the IL-7R signalling is deficient (13).

It seems like a tight regulation of IL-7 expression is essential to avoid over-stimulation of immune response. Sawa et al. have demonstrated that excessive IL-7 production in non-hematopoietic cells, contributing to the development of arthritis (14). Elevated levels of IL-7 have been detected in synovial fluid from patients with rheumatoid arthritis compared with the levels in synovial fluid from patients with osteoarthritis (15), and IL-7 transgenic mice overexpressing IL-7 develop autoimmune diseases, such as colitis (16) and dermatitis (17). This may suggest that dysregulation of IL-7 is part of autoimmune pathogenesis. In a recent publication, Sawa et al. proposed that targeting the toll like receptor-type I interferon-IL-7 (TLR-IFN-I-IL-7) signalling axis may provide an effective therapeutic approach for autoimmune diseases, including multiple sclerosis (8).

The satellite cell

Skeletal muscle develops by the progressive specification, proliferation, migration, and fusion of myoblasts to form terminally differentiated, contractile, highly patterned myofibers. Skeletal muscle is repaired or replaced postnatally by a similar process, involving a resident myogenic stem cell population referred to as satellite cells (18). In human skeletal muscle, satellite cell content varies between muscles with different functional properties and between individuals with different physical activity level and age (19). In the human body the satellite cell is located between the sarcolemma (cell membrane of a muscle cell) and the endomysium of separate adjacent muscle fibres. Here it is found in a quiescent state and can be identified by its abundance of heterochromatin (20). In response to non- damaging muscle exercise or muscle damaging exercise, or skeletal muscle trauma, satellite cells are activated and recruited to the injured area. Release of inflammatory substances, cytokines, and growth factors from active skeletal muscle, and also from the surrounding connective tissue, probably represent important triggers of satellite activation. The healing process has been divided into four interrelated and time-dependent phases: 1) necrosis/degeneration; 2) inflammation; 3) repair; and 4) scar tissue formation (19). During this course the

satellite cells are activated, they migrate, proliferate, differentiate and finally fuse and mature into myofibers. *In vitro*, the satellite cell differentiation process can to some extent be monitored through changes in myogentic regulatory factors (MRFs), see attachment 1 (21), myocyte enhacer-binding factor 2 (MEF2s) and skeletal muscle specific proteins.

MRFs and skeletal muscle specific proteins

MRFs are a family of skeletal muscle-specific transcription factors which bind to DNA in control regions and thus regulate myogenesis. All members of this family contain a conserved helix-loop-helix motif. Members include the myoD protein, myogenin, myf-5, and myf-6 (also called MRF4 or herculin) (22). Expression of the paired box 7 (PAX7) gene, is one of the most common molecular markers for identifying quiescent satellite cells. Myogenic differentiation 1 (MYOD1) expression is initiated when the cells are activated, whereas myogenin (MYOG) is a late acting marker (21). Myosin heavy chain 2 (MYH2) motor protein is also expressed late in the differentiation process and is responsible for actin-based motility. Most of our current knowledge of MRFs is based on *in vitro* models of satellite cell development. It is important to keep in mind satellite cells do not differentiate into mature myofibers *in vitro*, but are halted as myotubes, see attachment 1.

Potential myokines

Pedersen et al have reviewed the published data relevant to the notion that skeletal muscle is an endocrine organ (23). Skeletal muscle has been proposed as an immunogenic organ by different scientists. In 1995 Legoedec et al demonstrated expression, by human myoblasts in vitro, of complement (C) alternative pathway components C3, factor B, factor H and factor I (24), and in 1997 they reported that human myoblasts in vitro express immunological properties by producing complement

components (CC). They reported biosynthesis of Clq, Clr, Cls, C2 and C4 constitutively. Based on these findings they postulated that human myoblasts may constitute a local source of CC and therefore the CC could be implicated in inflammatory or physiopathological processes developed in skeletal muscle (25). In his master thesis: "Myokines – peptide hormones from skeletal muscle," Frode Norheim reported that in vitro cultered myotubes have the potential to secrete albumin, haptoglobin, immunoglobulin (Ig) alpha-1 chain C, Ig gamma-1 chain C, Ig kappa chain C, Ig kappa chain V-III, retinol-binding protein, transferrin, IL-6, IL-7, IL-8, granulocyte macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor alpha (TNFα) (26). Bortoluzzi et al have also identified 319 putatively secreted proteins from skeletal muscle (27). Among these potential myokines several are complement components. However, IL-6 and IL-7 were not among their findings.

1.1 AIMS

The aim in this Master thesis was to investigate if IL-7 can influence skeletal muscle cell development in a paracrine manner. In all assays we used *in vitro* cultured satellite cells and recombinant human (rh) IL-7 incubation. Myogenic differentiation was analysed by total mRNA isolation and RT-PCR. To estimate satellite cells uptake and oxidation of glucose or oleic acid, radioactively labelled glucose and oleic acid were used and analysed by CO_2-trapping (28) and detection of radioactivity by scintillation analysis. Total protein quantification was used to assess protein content. Cell proliferation was monitored by incubation of radioactively labelled thymidine and detection of radioactivity by scintillation analysis. Satellite cell migration was investigated by the use of BD Falcon™ FluoroBlok™ Insert Systems together with green fluorescence dye staining, fluorescence microscopy photography, Kodak D1 3.6 picture analytical software and manual counting.

2. MATERIALS AND METHODS

2.1 Culture conditions

1) Extracellular Matrix Gel (ECM-gel)

5 mL Extracellular Matrix Gel (ECM) (Sigma, #E1270) was diluted to a working stock in 70 mL Dulbecco's Modified Eagle's Medium with low glucose (DMEM) (Sigma, #D6046). The dilution was transferred to 1.5 mL Eppendorf tubes, 1 mL per tube and put in a freezer (-20° C) for later use.

2) Medium 1 (M1) for seeding

500 mL Dulbecco's Modified Eagle's Medium with low glucose (DMEM) (Sigma, #D6046), 50 mL Foetal Calf Serum (FCS) (Sigma, #F7524), 5 mL L-glutamine (L-glut) (Sigma, #G7513) and 5 mL Penicillin/Streptomycin (P/S) (Gibco, #15140-144).

3) Skeletal muscle growth medium (SkGM)

500 mL SkBM (Lonza, #CC-3161), 5 mL L-glut and 5 mL Penicillin/Streptomycin. SkGM BulletKit (Lonza, #CC-3160): 10 ng/mL Epidermal Growth Factor (#CC-4017N), 0.5 mg/mL Fetuin Bovine (#CC-4140N), 0.39 µg/mL Dexamethasone (#CC-4150N), 0.5 mg/mL Bovine Serum Albumin (#CC-44160N), 50 µg/mL Gentamicin Sulphate (#CC-4018N), insulin not added.

4) Medium 2 (M2) for proliferation in wells

500 mL DMEM with low glucose, 10 mL FCS, 10 mL Ultroser G (BioSepra, #P/N 15950-017), 5 mL L-glutamin and 5 mL Penicillin/Streptomycin

5) Medium 3 (M3) for differentiation

500 mL DMEM with low glucose, 10 mL FCS, 5 mL L-glutamine, 5 mL Penicillin/Streptomycin and 25 pmol Insulin (⇒21 µL of 1000 x diluted Insulin Actrapid®, Novo Nordisk, 0.6 mM).

6) Freezing medium

1 mL DMEM, 1 mL FCS and 2 mL 20 % Dimethyl Sulfoxide (0.4 mL DMSO + 1.6 ml DMEM) (Sigma, #D5879).

2.2 Human Satellite cells

All *in vitro* experiments were performed using satellite cells isolated from muscle biopsies taken from musculus obliqus internis abdominis of healthy volunteering kidney donors aged 28-53 years, exhibiting body mass index (BMI), fasting glucose and insulin within normal range. The biopsies were obtained with informed content and approval by the National Committee for Research Ethics, Oslo, Norway. With minor modifications the freeze-stocks of fibroblast-free satellite cell cultures were established by the method of Henry et al, (29). Approximately 400 mg of muscle tissue were washed three times in Hams F-10 media (Gibco, #31550) and placed in a Petri dish with 20 mL Hams F-10 and cut into as small pieces as possible with a scalpel. 20 mL Trypsin-EDTA (Gibco, #25300-062) were added and the tissue solution was transferred to a 50 mL spin tube that was placed in a shaker for 20-30 minutes in rum temperature. The supernatant was removed and put on ice, and the

washing, adding of 20 mL Hams F-10, trypsination, shaking and removal of supernatants were repeated 2 more times. The supernatants were gathered, 10 % FBS (vol/vol) (Gibco, #10108-165) was added and centrifuged for 7 min at 1800 rpm (550g). The supernatant was removed and the cell pellet was re-suspended in 3 mL SkGM (SkBM + bullet kit with no insulin, 2.5 mL Fungibact (Gibco, #15290-026), 5 mL Glutamine (Gibco, 25030-024) and 2 % FBS. Cell suspension was then spread in a pre-coatet 25 cm^2 or 75 cm^2 flasks and placed in an incubator 37°C, 5 % CO_2. SkGM medium was first changed after 24 hours and there after twice a week. The cell cultures were then grown to until they reach 70 – 80 % confluence. Then the culture was split and reseeded or made into a freeze-stock.

2.2.1 Coating of flasks

1 mL of ECM-gel working stock was diluted in 4 mL DMEM to a solution containing 8-12 mg/mL basement membrane proteins; laminin, collagen type 4, heparan sulphate, proteoglycan and entactin. A thin layer of ECM was spread over the surface area where the cells should be cultured, excess ECM was removed and the coating was left for air drying for approximately 45 minutes.

2.2.2 Storing myoblasts in nitrogen at - 196º C

Making a freeze stock: A cell pellet was re-suspended in Freezing medium; 1 mL DMEM, 1 mL FCS and 2 mL newly made 20 % Dimethyl Sulfoxide (#D5879, Sigma) (0.4 mL DMSO + 1.6 mL DMEM). Cell number was counted and dilutions containing approximately 5 x 10^5 celles/mL were made, 1 mL was then distributed per 1.5 mL cryogen freezing tubes placed in ice and without delay put into a -70° C freezer. The next day the tubes were placed in liquid nitrogen (÷196° C) for later use.

2.2.3 Seeding and culturing myoblasts

Thawing of cells: The cryogen freezing tubes where placed in water bath 37°C for quick thawing. The cell suspension was then gently transferred to a prepared 75 cm²

ECM coated flask containing 10 mL M1, and put into an incubator 37°C, 5 % CO_2 over night. The next day the M1 and the possible harmful Freezing medium were substituted with 10 mL SkGM. The myoblas was then left for proliferation until 70 - 80 percent confluence was reached while the SkGM was changed every 3 - 4. day and proliferation was monitored by microscopy every 1- 2. day. Then the cell culture was split as described lower in the text and seeded in new flasks with greater growth surface to let the proliferation continue, or the cells was used in different test assays.

2.2.4 Splitting of cell cultures

Detaching of satellite cell/myoblast cultures grown in 75 or 150 cm² flasks; the medium was removed and the cells were washed with pre-heated (37°C) 10 mL DMEM or PBS. 5 – 10 mL trypsine-EDTA (trypsin) with 0.5 g percine trypsin and 0.2 g EDTA 4 Na/l HBSS (Sigma, #T3924) was added and the flask 75 cm^2 or 150 cm^2 was put into an incubator (37°C, 5 % CO_2) for about 5 minutes. After detaching, when all the satelitecells/myoblasts were detached from the growth surface, the trypsin was inactivated with an equal volume of 2 % FCS (vol/vol) in DMEM. The cell suspension was transferred to a 50 mL spin tube and centrifuged 3 minutes at 1500 rpm. The supernatant was removed and the cell pellet was solved in 2 mL SkGM. Cells were then counted, reseeded or put into freezing stock.

2.2.5 Counting cells

10 µL cell suspension (cells solved in 2 mL SkGM) was mixed with 10 µL Trypan blue. The external supports of the Bürker counting chamber was moistened with distilled water and the cover glass was gently pushed onto the counting chamber from the front. A pipette was used to dispose a few drops of the sample between the cover glass and the counting chamber. As a result of the capillary effect the gap between the cover glass and the chamber base filled up. The Bürker counting chamber was placed

under a microscope, and the myoblasts were counted in (12 x 3) x 2 squares (E-squares; 0.004 mm^3).

$$\frac{\text{Number of living cells (n) x 2 x 1000}}{0.3} = \text{Number of cells/mL}$$

2.2.6 Differentiating myoblasts into myotubes

When 70 – 80 percent confluence was reached the cells were washed once with an appropriate amount of PBS. Differentiation was initiated by substitution of growth medium SkGM or M2 with M3 (differentiation medium). The satellite cells were placed in an incubator 37°C, 5 % CO_2 for 7 - 9 days to differentiate into multinucleated myotubes and the M3 was changed on day 3 and 5. The differentiation process was monitored trough microscopy and documented trough photography and by day to day harvesting of cells to analyse mRNA expression of specific myoblasts differentiating genes. An example of satellite cell myogenesis in culture is pictured in figure 1.

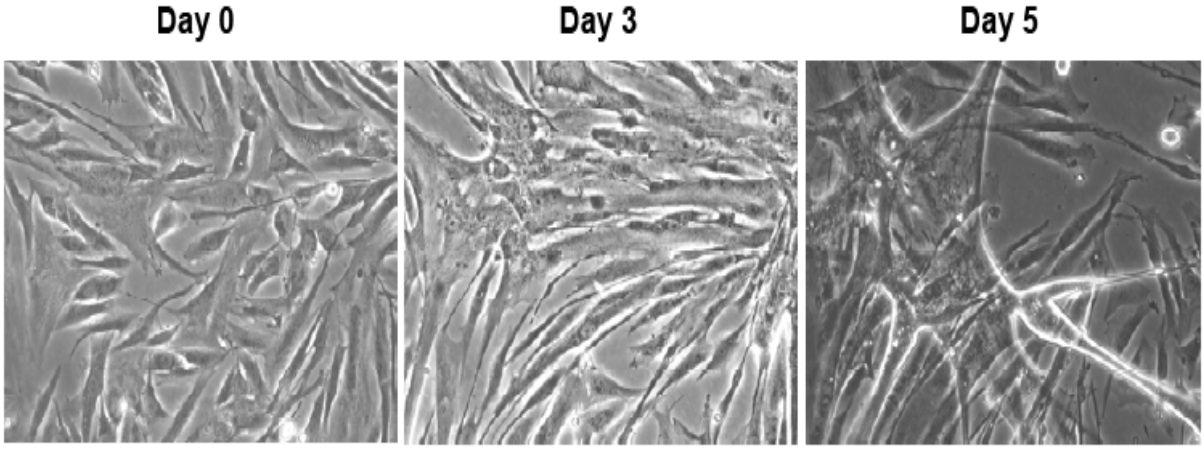

Figure1: Satellite cell differentiation. Light microscopy picture of undifferentiated myoblasts (left), after 3 days differentiation (middle), and fused multinucleated myotubes after 5 days differentiation (right) (40x magnification), Frode Norheim and Fred Haugen.

2.2.7 Relative quantification of mRNA by real-time RT-PCR

Total RNA from satellite cells and myotubes cultured *in vitro* was isolated using RNeasy™ Mini Kit (Qiagen, #74106) and with some modifications the manufacturer's protocol was conducted. In short; the cell culture plate was put on ice to inactivate the cells. The total medium was collected in 2 mL Eppendorf tubes and centrifuged 5000 rpm, 5 minutes 4ºC (Biofuge fresco, Heraeus Instruments). 1.7 mL of the medium was transferred to new 2 mL Eppendorf tubes and put in a -20ºC freezer for later analyses. The cells was lysated with 350 µL/well RLT lysis buffer (RNeasy Mini Kit) and the plate, still on ice, was placed on a Orbital shaker (KS 125 Basic, IKA Labortechnik) at speed 300 for 5 - 10 minutes. Then the lysate was collected in 1.5 mL Eppendorf tubes in ice and homogenized for 30 s using a RNase cleaned (RNase AWAY, Molecular BioProducts, #7005) rotor-stator homogenizer (Ultra-Turax T8, Labortechnik) and put for storage in a -70° C freezer for later total-RNA isolation. Later, frozen lyseates was incubated at 37° C in a water bath until the ice was completely thawed and salts dissolved. Prolonged incubation was avoided to minimise compromising RNA integrity. One volume of 70 % ethanol (Arcus, Norway) was mix into the lysate and the mixture was loaded in an RNeasy spin column placed in a 2 mL collection tube and centrifuged for 30 sec at 13000 rpm with in the temperature range 20 - 25°C. The flow-through was discarded at this step and in the following steps. 700 µl buffer RW1 was added to the RNeasy spin column which was centrifuged again. Then 500µl Buffer RPE was added to the RNeasy spin column and the column was spun again. This step was repeated once more but now spun in 2 min at 13000 rpm. The collection tube was changed and the spin column was spun for 1 min. To collect the total-RNA a new 1.5 mL Eppendorf tube was placed under the spin column and 30 µl RNase-free H_2O was pipetted directly to the membrane of the column which was spun a last time for 1 min. The total-RNA was then stored at -70°C before use. Quantification of total mRNA was preformed on a NanoDrop Spectrophotometer (ND-1000) with software. Real-time RT-PCR was performed using reagents and instruments from Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Samples from *in vitro* experiments were analyzed in a 96-well format. Up to 1 µg total RNA was reversely

transcribed into cDNA in 20 µL reactions on a GeneAmp™ PCR 9700 thermal cycler using High Capacity cDNA Reverse Transcription Kit. The thermal cycling program was sett to 10 min at 25°C initially; followed by 120 min at 37°C, then 5 sec at 85°C to inactivate the reverse transcriptase and finally rapid cooling down to 4°C holding temperature. The cDNA reactions were diluted with water before analyses and equivalent of 15 ng RNA was analysed in each well using the 96-well format. Duplicate or triplet PCR reactions were set up containing diluted cDNA template (10 µL), TaqMan™ Gene Expression Master Mix (12.5 µL) and different pre-developed TaqMan™ Gene Expression Assays (1.25 µL), and adjusted to a final volume of 25 µL with water. The pre-developed assays all included a primer pair targeting separate exons and an intron-spanning TaqMan™ probe, which was 6-carboxy-fluorescein phosphoramidite (FAM™) labelled in the 5' end and contained a minor groove binder and non-fluorescent quencher at the 3' end. A pre-developed endogenous control assay targeting human large ribosomal protein P0 (RPLP0) (Applied Biosystems, # 4333761) was used as control for RNA loading and reverse transcription efficiency. Real-time PCR was performed in a 7900HT Fast instrument using the SDS 2.3 software and the default thermal cycling program: 10 min at 95°C initially; followed by 15 sec at 95°C and 1 min at 60°C for 40 or 45 cycles. Fluorescence acquisitions were made real-time at the end of each cycle and were analyzed with the RQ manager software. Briefly, data were curve-fitted, and the theoretical cycle number values (Ct) associated with fluorescence signals exceeding software determined threshold levels were calculated on this basis. When non-parametric tests were used, Ct-values were set to 40 or 45 for mRNA levels below the detection limit. Relative target mRNA expression levels were calculated as $2^{-[Ct(target)-Ct(RPLP0)]}$, thereby normalizing the data to endogenous control RPLP0 (Fred Haugen).

2.3 Assays

2.3.1 Differentiating myoblasts into myotubes

Satellite cells from three different Living Donors (LD); LD13 at passage (P) 6; LD16, P3; LD30, P5 were obtained as in previous mentioned and seeded out in pre-coated 6 well plates with Corning CellBIND Surface (Corning Inc, Costar 3335), at 10 000 cells/cm² in 2 mL SkGM. Nine wells were seeded per donor, one for each day of harvesting. The cells was grown in SkGM for to days before it was substituted with 2 mL differentiation medium M3. This event was named Day 0. M3 was changed on day 3 and 5. Cell cultures and medium was harvested form Day -1 until Day 7. During the assay differentiation was monitored by microscopy. Increased number of cells with 2 nuclei or more was interpreted as differentiation had initiated. Total mRNA isolation from the myoblasts/myotubes was done as previously described with RNeasy™ Mini Kit. cDNA was made and real-time PCR (RT-PCR) was also done according to previously described procedures. The following target genes (the official gene symbol in parenthesis) were monitored: paired box 7 (PAX7), Hs00242962_m1; myogenic differentiation 1 (MYOD1), Hs00159528_m1; myogenin (MYOG), Hs00231167_m1; desmin (DES), 01090875_m1; myosin, heavy chain 2 (MYH2), Hs00430042_m1; interleukin-7 (IL-7), Hs00174202_m1; interleukin-7 receptor α chain (IL-7R), Hs00904814_ml, (Applied Biosystems, TaqMan™ Gene Expression Assays). RPLP0 was used as endogenous control.

2.3.2 Effect of recombinant human IL-7 incubation on satellite cell differentiation

Satellite cells from 6 different Living Donors; LD11, P6; LD13, P7; LD16, P2; LD30, P7; LD29, P2; LD37, P6, were seeded in duplicates, in 6 well plates with Corning CellBIND Surface, at 10 000 cells/cm² in 2 mL SkGM. Tree of the donors was used in pilot study. The tree next was used to repeat the study. The myoblasts were incubated for 24 hours to fix to the growth surface. Then the SkGM was removed, the cells were washed 4 times with 1 mL pre-warmed (37º C) low glucose DMEM. In the control

wells 2 mL differentiation medium M3 was provided. In the experiment wells, 100 ng/mL recombinant human Interleukin-7 (rhIL-7) (R&D systems, #207-IL/CF) solved in 0.1 % Low endotoxic bovine serum albumin (BSA) (Sigma, #A-2058) in PBS was added to the M3. In the negative control wells, 20 ng/mL rhMyostatin (ProSpec, #CYT418A) solved in 0.1 % BSA/PBS was added to the M3. To minimise possible BSA induced biases all wells received the same amount of BSA. At day 3 and 5 the M3 was replaced and collected. At day 7 the medium was collected, cells were inactivated on ice, lysated and total-RNA was isolated. cDNA was made and RT-PCR was done according to mentioned procedures. The following target genes (the official gene symbol in parenthesis) were monitored: interleukin-7 (IL7), Hs00174202_m1; interleukin-7 receptor α chain (IL-7R), Hs00159528_m1; paired box 7 (PAX7), Hs00242962_m1; myosin, heavy chain 2 (MYH2), Hs00430042_ml; myogenin (MYOG), Hs00231167_m1. RPLP0 was used as endogenous control. As the only mRNA, MYOG dCt-values was in this assay calculated with Ribosomal protein L27 **(**hRPL27), Hs01652274_gH, as endogenous control instead of hRPLP0.

2.3.3 Effect of short-term incubation with recombinant human IL-7 on satellite cell differentiation

Satellite cells from 4 different Living Donors; LD11, P7; LD16, P4; LD30, P8; LD37, P7, were seeded out in 6 well plates with Corning CellBIND Surface, at 26 000 cells/cm² in 2 mL M2. Four wells were seeded per donor (2 for total-RNA isolation and 2 for protein quantification). The myoblasts were put in an incubator for 24 hours to fix to the growth surface. Then the M2 was discarded and 1 mL M2 ± 50 ng/mL rhIL-7 was added for 6 hour incubation. The parallels for total-RNA was, as previously described, harvested with RNeasy Mini Kit. Eventually cDNA was made and expression of the following genes was analysed with RT-PCR (the official gene symbol in parenthesis): myosin, heavy chain 2 (MYH2), Hs00430042_m1; paired box 7 (PAX7), Hs00242962_m1; myogenic differentiation 1 (MYOD1), Hs00159528_m1; myogenin (MYOG), Hs00231167_m1; interleukin-7 (IL7), Hs00174202_m1; interleukin-7 receptor α chain (IL-7R). As previously described RPLP0 was used to

control for RNA loading and reverse transcription efficiency. Cells for protein analysing assay were wash 3 times with 1 mL PBS on ice, lysated with 200 uL lysisbuffer/well (PBS + 1 % Triton X-100 (Sigma, #T8787) + Complete protease inhibitor cocktail (Coplete Mini, #11 836 153 001). To ensure that all cells for protein collection were completely lysated and as much protein as possible were collected, scrapes were used. Collected lysate was put to storage at -70°C for later use.

2.3.4 Effect of recombinant human IL-7 incubation on cell proliferation rate and survival

Satellite cells from 3 different Living Donors; LD11, P7; LD30, P8; LD37, P7 were used in the cell division rate assay. Cells was treated as previously mentioned and seeded out in 12 well plates with Corning CellBIND Surface (Corning Inc, Costar 3336), at 5 000 cells/cm² (~ 20 000 per well) in 1 mL SkGM and put in incubator to fix to growth surface over night. Four duplicates x 5 plates were seeded per donor. The next day the myoblasts were washed 4x with 0.5 mL PBS and incubated ± 50 ng/mL and 18.8 µL radioactive labelled thymidine [methyl-^{14}C] (Amersham Pharmacia Biotech, Thymidine, 1.85 MBq, 50 µCi, #CFA532-50UCI) in 0.5 mL M2 growth medium. Time of incubation was set to 0 hours. Every 24 hour the +IL-7 wells was boosted with 50 ng/mL IL-7 (25 ng per well). Cells and media were harvested at 0, 24, 48, 72 and 96 hours. When harvested the cells were chilled on ice, media was discarded, cells were washed 2 times with 1 mL PBS per well and 2 times with 1 mL trichloroacetic acid (TCA) 5 %. To confirm that the myoblasts were not washed away microscopy was used. To lysate the cells 500 µL NaOH 0.2M was added per well. The lysate was homogenized by a ultrasonic processor (Labsonic, B. Braun Biotech) at 0.6 cycle and 60 % amplitude, 3 cycles per well. 400 µL of the lysate was transferred to a 6 mL Pony Vial counting tube (Perkin Elmer, #6000292) and mixed with 3 mL scintillation fluid, ULTIMA GOLD TX (Perkin Elmer, #6013119). Scintillations per minute (spm) were measured in a WinSpectral, 1414 Liquid Scintillation Counter (Wallac). Protein content was analysed accordingly: 50 µL of the lysate samples were transferred to a 96 well plate. 1 mL Bio-Rad Protein Assay (Bio-Rad Laboratories,

#500-0006) was mixed in 4 mL MQ water, and 200 µL was added to the lysate and the incubation went for 5 min in rum temperature before the absorbance was measured at 590 nm in a photo spectrometer (EFLAB, Titertek Multiscan PLUS, MK II). The Bio-Rad Protein Assay dilution was used as control.

2.3.5 Effect of recombinant IL-7 on satellite cell migration

Living donor LD11, P7; LD13, P7; LD29, P3; LD30, P8 was used in this assay, two donors in a pilot study and two in a repeat of the study. BD Falcon™ FluoroBlok™ Insert Systems (BD Biosciences, #351152) was used and the insert membranes were coated on the underside with 50 µl ECM and airdryed for the minimum of one hour before use. The satellite cells were stained with 10 µM green fluorescence dye (CellTracker™ Green CMFDA, Invitrogen, #C7025). With some modifications this was done according to manufacturers instructions. Seeding density was 13 000 per insert (~ 41 000 per cm²). Recombinant human hepatocyte growth factor (rhHGF) (R&D systems, #294-HG-005/CF) was used as positive control. Two hours before incubation triplets of each donor were seeded out in 300 µl low glucose DMEM with 1 % FCS (vol/vol) in the top compartment. In the bottom compartment 700 µl low glucose DMEM with 1 % FCS (no cells) was added (attachment 2). At 0 hours the 1 % FCS in the bottom compartment was replaced with low glucose DMEM containing either: 10 % FCS (control), 10 % FCS + 100 ng/mL rhIL-7 or 10 % FCS + 10 ng/mL rhHGF (positive control). For one donor different rhIL-7 incubation concentrations (1, 10 and 100 ng/mL) were tested out to asses the optimal IL-7 amount. The cells were investigated with a fluorescence microscope (Nikon Elippse TS 100 with C-SGH Mercury Illuminator and Epi-Fluorescence attachments) and pictures were taken with a Nikon digital camera DXM 1200 with ACT-1 software at 0-, 24- and 48 hours. Kodak D1 3.6 picture analyse software and manual counting of cells on one picture per insert (4x magnification which equals 2.66 mm² (~ 7.4 %) of the total 36 mm² membrane area) taken of the centre of the insert membrane, was used to document migration.

2.4 Presentation of data and statistical analysis

Data that were normalized to control are presented as means ± standard error of the mean (SEM). Data that were not normalized are presented as mean ± standard deviation (SD). Data were compared using Paired-Samples or Non-paired Student's t-test. Statistical significant difference was set at $p < 0.05$.

3. RESULTS

3.1 Differentiating myoblasts into myotubes

Total-RNA was isolated daily during 8 days of differentiation of primary human myocytes from tree donors. Expression of IL-7 and IL-7R and the known myogenic differentiation markers PAX7, MYOD1, MYOG, DES and MYH2 (21), were analyzed by RT-PCR. This experiment was conducted to validate the differentiation protocol and to monitor the IL-7 and IL-7R mRNA expression during myogenesis. Mean IL-7 mRNA increase was not significant for the three donors (Fig. 2A). Living donor 13- and 30´s mRNA expression increased 2-3 folds during the differentiation assay. Results from LD 16 were less clear, fluctuating between 0.5-1 folds. IL-7R mRNA was significantly decreased by approximately 54-81 % (mean) from day 5 ($P < 0,005$), day 6 ($P = 0.002$,) to day 7 ($P = 0.006$) (Fig. 2B). The primary human myocytes differentiated into myotubes according to the time-dependent manner (Fig. 2C and 2D) described by Zammit et al. (21), see attachment 1. The data also suggest that expression of IL-7 tends to increase and IL-7R decrease during myogenesis, in a time-dependent manner.

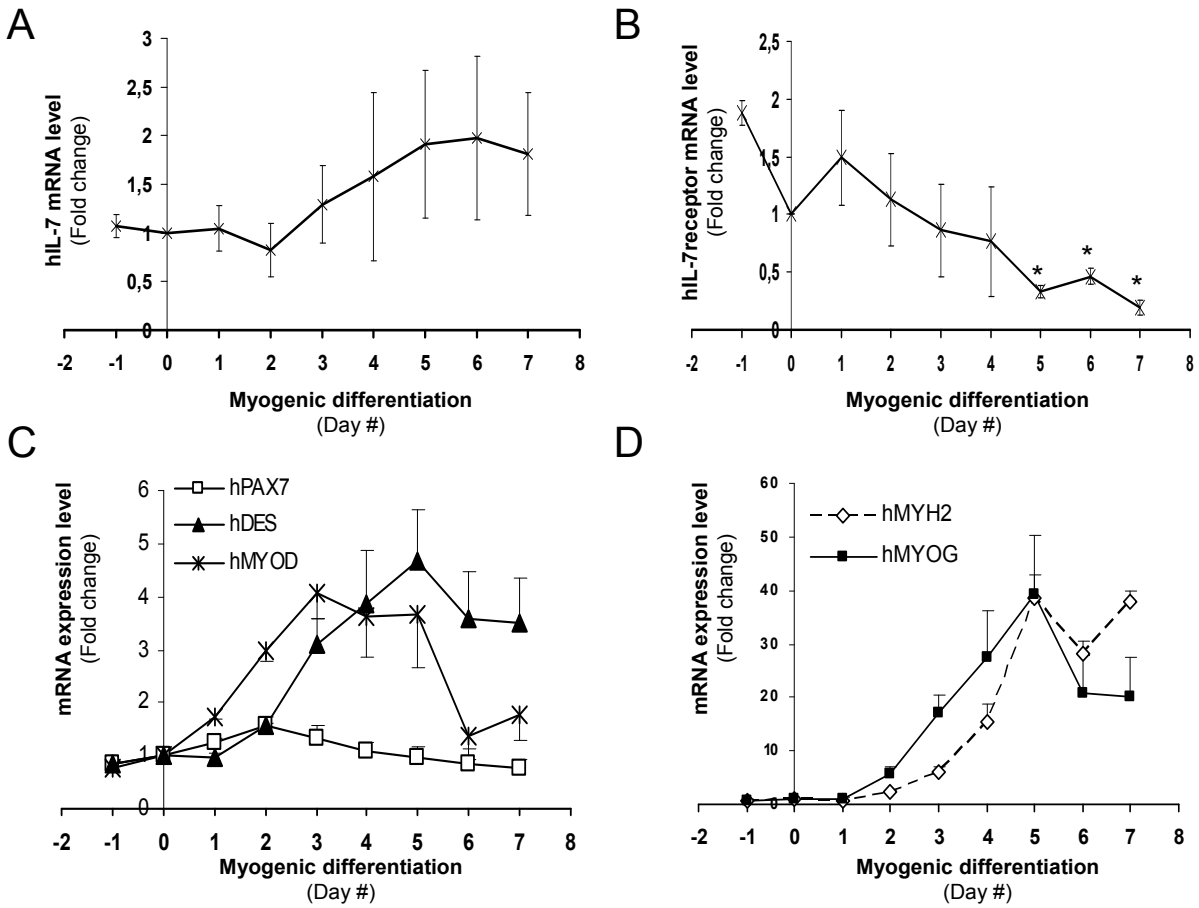

Figure 2. Expression of interleukin-7 (IL-7) and IL7 receptor (R) and myogenic marker genes, during myogenesis. Myocytes were harvested at: one day prior to (day -1); and different number of days after (day 1-7) the initiation (day 0) of differentiation. Relative mRNA levels were determined by RT-PCR of genes encoding; (A) interleukin-7 (IL-7); (B) interleukin-7 receptor (IL-7R); (C) paired box 7 (PAX7), myogenic differentiation 1 (MYOD1), desmin (DES); (D) myogenin (MYOG), and myosin heavy chain 2 (MYH2). Data are relative to RPLP0 and were normalized to day 0. Each point represents mean ± SEM from 3 separate donors grown in differentiation medium M3. *$P < 0.05$ using paired Student´s t-tests.

3.2 Recombinant human IL-7 incubation down regulates expression of myosin heavy chain 2 and IL-7 at mRNA level during myogenesis

The observations that mRNA expression of IL-7 tended to increase and IL-7R decreased during myogenesis led us to hypothesise that IL-7 took part in satellite cell differentiation. Thus, we tested if recombinant IL-7 incubation could influence expression of known myogenic differentiation markers at the mRNA level during myogenesis. Relative to control, incubation with IL-7 showed no significant change in the satellite cell marker gene PAX7 (Fig. 3A) or MYOG after 7 days of differentiation (Fig. 3B). Significant decrease in the transcription of the differentiation gene MYH2 was found ($P = 0.045$) (Fig. 3C). The reduction was 37 ± 4 % (mean ± SEM). The down regulation of MYH2 mRNA expression indicates that IL-7 influence satellite cell differentiation. The positive control, myostatin incubation, significantly increased PAX7 and decreased MYOG and MYH2 mRNA expression (Fig. 3 A-C). During this assay we also examined if IL-7 regulates its own expression. Relative to control IL-7 incubation significantly decreased the expression of IL-7 mRNA ($P = 0.020$) by 42 ± 4 % (Fig. 4A). IL-7R mRNA expression was unchanged (Fig. 4B). These findings indicate that IL-7 down regulate its own expression at mRNA level and that IL-7 may influence satellite cell differentiation.

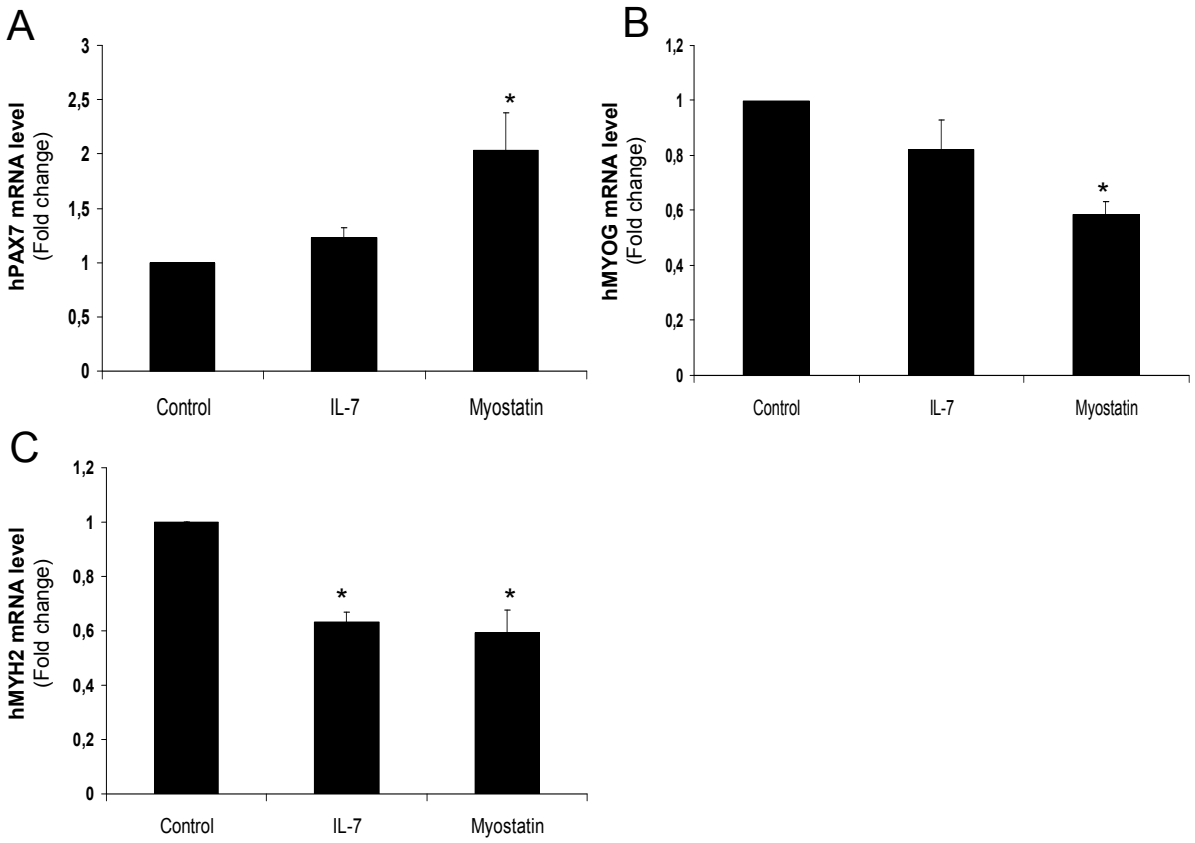

Figure 3. Recombinant human IL-7 (rhIL-7) influence mRNA expression of differentiation markers during myogenesis. Satellite cells were harvested after 7 days of rhIL-7 incubation during cell differentiation. Relative mRNA levels were determined by RT-PCR of the genes encoding; (A) paired box 7 (PAX7); (B) myogenin (MYOG); (C) myosin heavy chain 2 (MYH2); Data are relative to RPLP0 and were normalized to control. Each point represents mean ± SEM from duplicates of 6 separate donors. *P < 0.05 using paired Student's t-test.

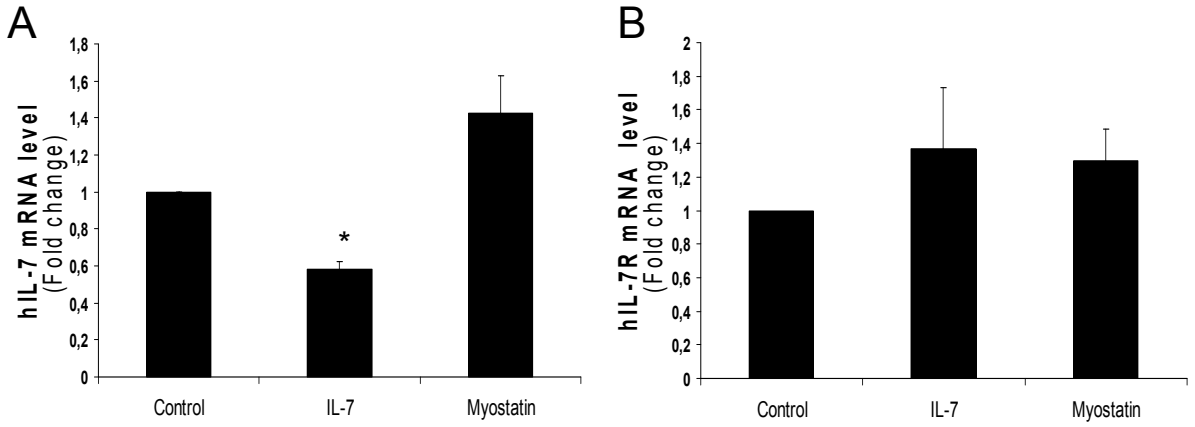

Figure 4. Recombinant human IL-7 (rhIL-7) reduces its own expression. Satellite cells were harvested after 7 days of rhIL-7 incubation during cell differentiation. Relative mRNA levels were determined by RT-PCR of the genes encoding; (A) interleukin-7 (IL-7) and (B) interleukin-7 receptor (IL-7R). Data are relative to RPLP0 and were normalized to control. Each point represents mean ± SEM from duplicates of 6 separate donors. *P < 0.05 using paired Student's t-test.

3.3 Short-term incubation with recombinant human IL-7 influences mRNA expression of satellite cell differentiation genes and reduces IL-7

Based on the findings that IL-7 may influence satellite cell differentiation, we examined if short term incubation with recombinant IL-7 would influence differentiation markers (PAX7, MYOD1, MYOG and MYH2) in satellite cells in proliferation medium M2. We also tested mRNA expression of IL-7 and IL-7R. Results are relative to RPLP0 and normalized to control. Relative to control all four differentiating markers were decreased, although the changes were small (10-14 % compared to control) and for PAX7 and MYOG the change was not significant (Fig. 5A). The other two markers were significantly changed: MYOD1 ($P = 0.025$) and MYH2 ($P = 0.046$). IL-7 mRNA was significantly reduced ($P = 0.030$), whereas IL-7R mRNA was not significantly changed (Fig. 5B). These results support the previous findings that IL-7 may influence satellite cell differentiation and that IL-7 may reduce its own expression.

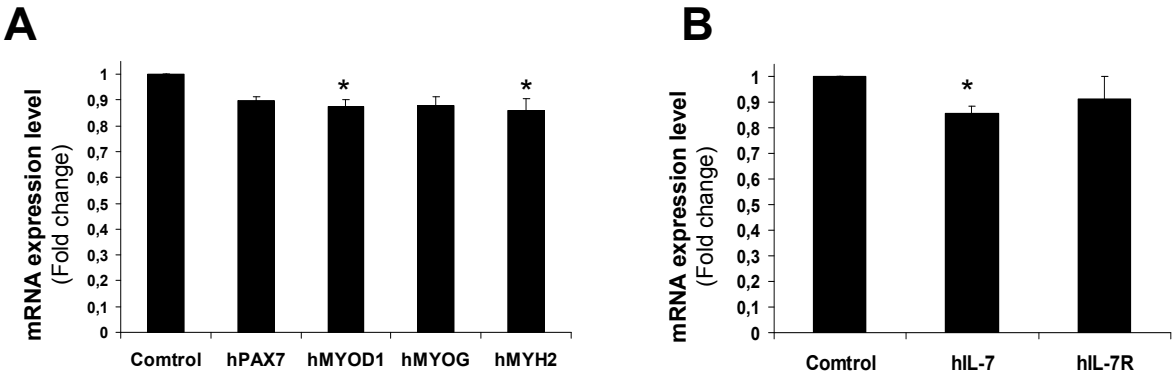

Figure 5. Effects of short term incubation with recombinant human IL-7 on satellite cell differentiation markers (A) and IL-7 (B). Satellite cells were harvested after 6 hours rhIL-7 incubation in M2 proliferation medium. Relative mRNA levels were determined by RT-PCR of the genes encoding; (A) paired box 7 (PAX7), myogenic differentiation 1 (MYOD1), myogenin (MYOG) and myosin heavy chain 2 (MYH2); (B) interleukin-7 (IL-7) and interleukin-7 receptor (IL-7R). Data are relative to RPLP0 and were normalized to control. Each point represents mean ± SEM from duplicates of 4 separate donors. *$P < 0.05$ using paired Student's t-tests.

3.4 Recombinant human IL-7 incubation does not influence satellite cell proliferation

The observed changes in myogenic differentiation markers during rhIL-7 incubation led us to hypothesise that IL-7 time-dependently could increase satellite cell proliferation rate. In a pre-trial, we assessed the effect of 50 ng/mL rhIL-7 in M2 proliferation medium by manually counting cells in a Bürcker counting chamber after 72 hour of incubation. The number of cells was increased 23 ± 8 % (mean ± SEM) compared to control ($P = 0.11$, $n = 3$). To assess the satellite cell proliferation more accurately we repeated the assay and used radioactively labeled thymidine [methyl-^{14}C] and monitored the cell division rate, time-dependently over 96 hours with a Liquid Scintillation Counter. Total protein was also monitored. No difference in proliferation was observed (Fig. 6A), both control and recombinant hIL-7 incubation showed a similar linear thymidine incorporation rate. However, recombinant hIL-7 incubation for 24-96 h increased cell lysate protein levels approximately 7-11 % above control (Fig. 6B). The difference was significant at 48 hours ($P = 0.001$). The thymidine incorporation rate per mg protein was also significantly lower in the hIL-7 incubation wells at 48 hours ($P = 0.0256$), (Fig. 6C). These results suggest that IL-7 does not affect proliferation of satellite cells, but might still have a slight positive effect on cell protein content.

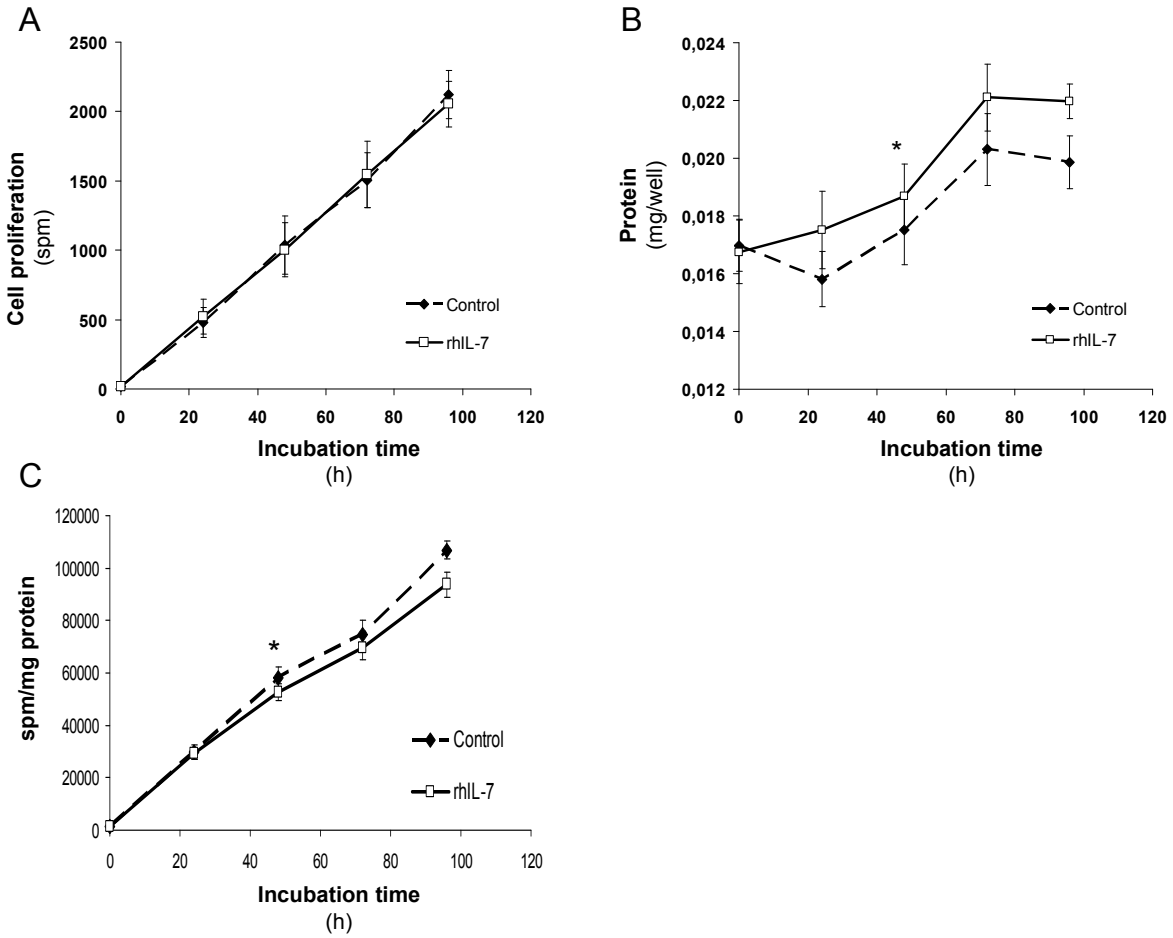

Figure 6. Effect of recombinant human IL-7 (rhIL-7) incubation on satellite cell proliferation rate (A) and protein content (B). Satellite cells were cultured in M2 proliferation medium ± rhIL-7 and radioactive labelled thymidine. Proliferation (A) was determined as cell-associated methyl-^{14}C - thymedine, scintillations per minute (spm). (B) Shows changes in cell lysate protein content per well. (C) Shows changes in thymidine incorporation rate per mg protein. Each point represents mean ± SEM from duplicates of 3 different cell donors. *P < 0.05 using paired Student's t-tests.

3.5 Effect of recombinant human IL-7 on satellite cell migration

In response to skeletal muscle injury it is essential that satellite cells are activated and migrates to the damage area. Several components (motogens) have been shown to influence satellite cell migration. Among them are cell tissue extract from crushed muscle tissue and HGF (30;31). HGF is also involved in other steps in the skeletal muscle regeneration (20), and it increases proliferation and inhibits differentiation.

This, together with the finding that IL-7 may influence satellite cell differentiation led us to hypothesize that IL-7 could influence satellite cell migration.

3.5.1 IL-7 may increase satellite cell migration

To investigate migration BD Falcon™ FluoroBlok™ Insert Systems was used and the cells were stained with green fluorescence. At 0 hours, the top insert compartment contained cells and DMEM with 1 % FCS. The bottom compartment contained DMEM with 10 % FCS (control) (Fig. 7A) added; 100 ng/mL rhIL-7 (Fig. 7B) or 10 ng/mL rhHGF (Fig. 7C). Relative to control there was significant increase, 40 ± 13 % (mean ± SEM), in migrations on insert membranes in + rhIL-7 compartments at 48 hours ($P = 0.026$) (Fig. 7D). This increase was not observed at 24 hours. HGF incubation, the positive control, raised the migration significantly at both 24 ($P = 0.014$) and 48 hours ($P = 0.003$). The increase was 50 ± 14 and 128 ± 26 % respectively. One donor was also tested with different rhIL-7 incubation concentrations (1, 10 and 100 ng/mL) to asses the optimal concentration. No significant difference in migration between the different rhIL-7 incubation concentrations was observed (data not shown). This finding indicates that IL-7 may increase satellite cell migration.

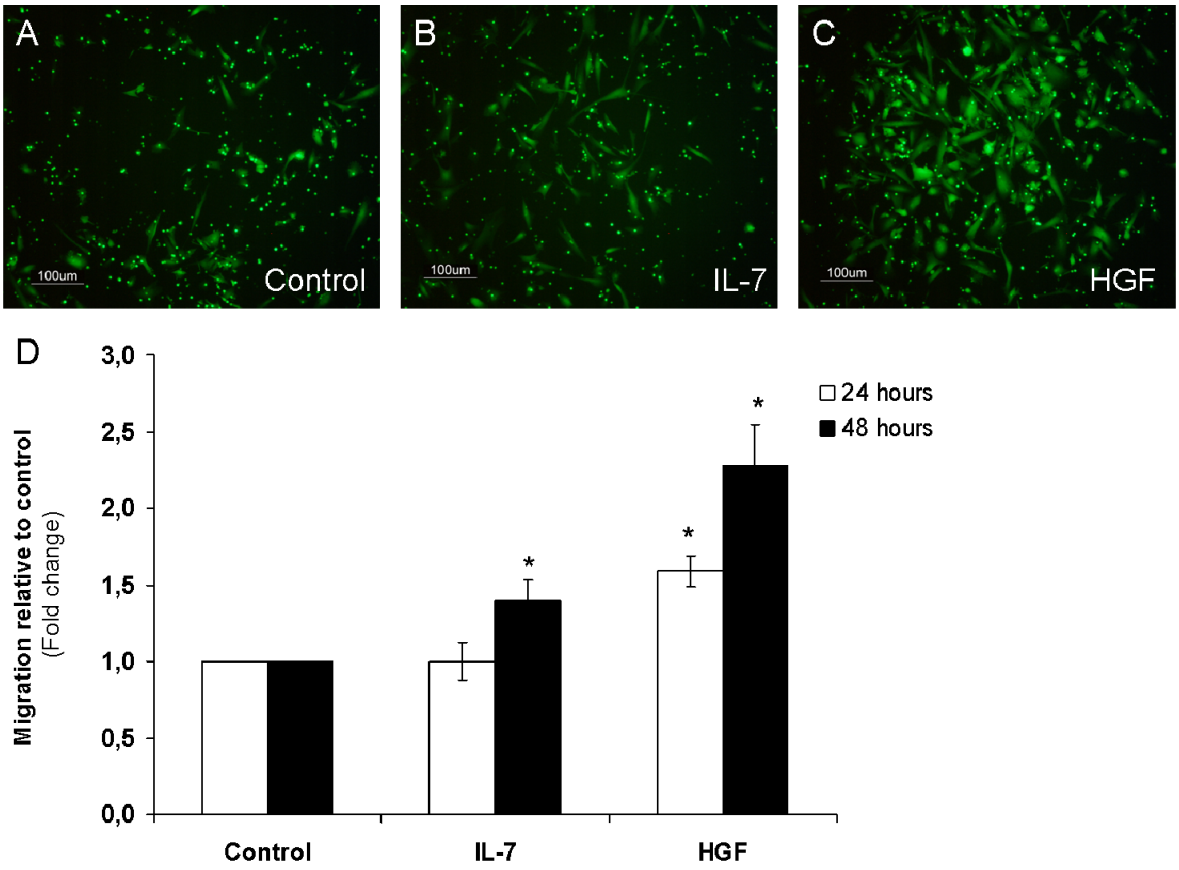

Figure 7. Effect of recombinant human IL-7 incubation on satellite cell migration. The photos show migrated satellite cells stained with green fluorescence on ECM coated fluorblok insert membranes in compartments containing DMEM with 10 % FCS (A) and; 100 ng/mL rhIL-7 (B) or 10 ng/mL rhHGF (C) (positive control). (D) Shows migration relative to control. Number of migrated cells was counted on one photo per insert by picture analyzing software or manual counting. Results were normalized to control. Each point represents mean ± SEM from triplets of 4 different cell donors. *P < 0.05 using non-paired Student's t-tests.

4. DISCUSSION

We demonstrate in this study that incubation with rhIL-7 may increase satellite cell migration and influence mRNA expression of satellite cell differentiation genes and IL-7. Our findings suggest that administration of rhIL-7 does not influence proliferation, glucose and oleic acid oxidation and basal glucose uptake in cultured human skeletal myoblasts. To our knowledge, this is the first time such effects of IL-7 have been reported.

Based on the use of the CO_2-trapping method (28), incubation of IL-7 with human skeletal myoblasts does not increase glucose and oleic acid oxidation and basal glucose uptake . Because my participation was limited in these experiments the data are not presented in this thesis.

During an 8 day differentiation study IL-7- and IL-7R mRNA expression level showed an inverse relationship. The data were not significant for IL-7 but it is consistent with earlier findings obtained in our research group. This finding may be seen in relation to the findings on T cells. It has been shown that IL-7 inhibit IL-7R protein, and mRNA expression in T cells (11;22) It is believed that IL-7 gives quiescent T cells a surviving signal, and that it is involved in their proliferation and differentiation (32). Due to these findings we hypothesized that IL-7 might influence human satellite cell differentiation.

The cell model system used in the differentiation study is well established (29), and the cells hold the characteristics for muscle cell markers described by Zammit et al. 2006 (21). In addition, we have observed striation and spontaneous contractions (33) at the end of the differentiation protocol.

Relative to control, a significant reduction was found for transcription of the muscle specific gene MYH2 after 7 days differentiation when rhIL-7 was incubated with cultured myoblasts. Myostatin (or GDF-8) was used as a positive control because it is associated with a strong reduction of the expression of differentiation markers (34).

The myoblast differentiation markers tested for (PAX7 and MYOG) showed the same mRNA expression trend during incubation with rhIL-7 and myostatin, and MYH2 was significantly reduced by both.

During the final stages of myoblast differentiation the expression of the structural muscle gene MYH2 increases (21). Our finding that IL-7 may down regulate expression of MYH2 mRNA suggests that IL-7 may influence the myoblast differentiation. These findings were supported by results in short-term incubation with rhIL-7 in a proliferation medium (Fig. 3C). We monitored the mRNA expression of four myogenic markers; PAX7, MYOD1, MYOG and MYH2 in proliferating satellite cells. All the markers were reduced in the presence of IL-7 as compared to control, but the changes were only significant for MYOD1 and MYH2. MYOD1 is an early marker for satellite cell activation (21), and the down regulation of it supports our findings from the differentiation experiments (Fig. 5A). These findings also suggest that IL-7 may influence human satellite cell differentiation. However, the finding is just limited to mRNA levels obtained *in vitro*. Thus, this result needs to be investigated on the protein level as well as in an in vivo model.

To further investigate IL-7 cytokine influence on satellite cell maturation we incubated proliferating satellite cells with radioactively labelled thymidine [methyl-^{14}C] with and without rhIL-7. The DNA replication was similar in control and rhIL-7 incubated cells, but there was a significant increase in protein content and reduced thymidine incorporation per mg protein at 48 hours. These results suggest that IL-7 does not affect proliferation of satellite cells, but may have a slight effect on their maturation. From this finding we hypothesized that cell survival or size was increased.

The use of BD Falcon FluoroBlok insert systems to monitor satellite cell migration is not well established, but the use of Boyden Chamber (35), a method that are based on the same principles, are reported used for this cell type (31). To validate the method we conducted several optimalisation experiments as outlined in attachment 3.

We observed that incubation of rhIL-7 with cultured myoblasts may increase satellite cell migration (Fig. 7D). Collection of data and calculations of the results were not blinded, which weakens the finding. The strength in this study as compared to other migration studies is that we have used four different cell donors instead of one or two commercial cell lines. The finding imply that IL-7 may influence cell chemokinesis (random movement), but it does not allow IL-7 to be defined as a chemo attractant. A checkerboard analysis (31) needs to bee conducted to distinguish chemokinesis capacity from chemotaxis (movement in the direction of a higher concentration of a chemical). The results need to be repeated in larger studies before further investigations are conducted.

In the differentiation assay, there was an inverse relationship between IL-7 and IL-7R mRNA expression. We therefore expected to see a down regulation of IL-7R mRNA expression by rhIL-7 incubation. However, both short- and long-term incubation with rhIL-7 failed to induce down regulation of IL-7R mRNA, although both short- and long-term incubations significantly decreased the expression of IL-7 mRNA. These findings suggest that IL-7 may reduce its own expression and that IL-7 expression might be controlled by local negative feedback. To some extent this is consistent with the findings in T cells (11), whereas the findings that IL-7 did not reduce mRNA expression of its own receptor, are not. It is important to keep in mind that mRNA expression levels not always correlates with protein levels. This, together with other limitations, makes it difficult to draw firm conclusions.

In response to skeletal muscle injury it is essential that satellite cells are activated and migrate to the damaged area, proliferate and fuse to heal the damage. Several components (motogens) have been shown to influence satellite cell migration. Among them are cell tissue extract from crushed muscle tissue, in addition to HGF (30;31) and IL-4, which may promote human satellite cell migration *in vitro* (36). Myostatin has been shown to decrease the number of migrated myoblasts from individual muscle fibers isolated from wild-type mice (37). In other studies HGF, IL-4 and myostatin have been reported to have other properties than regulating

migration. HGF may increase proliferation and inhibit maturation of satellite cells (38-40), whereas IL-4 promotes myoblast fusions (41) and myostatin may inhibit muscle precursor cell proliferation and myogenic differentiation (42). These findings show that one substance may have several functions in myogenesis. Thus, it is plausible that IL-7 may influence both satellite cell differentiation and migration, and we hypothesize that IL-7 may be involved in the restoration process after muscle cell injury.

Local factors seem to play an important role in satellite cell proliferation and migration (31). Of tissue extracts form liver, lung, heart, kidney and muscle, only muscle-derived extract resulted in significant growth of rat satellite cells (30), and it also increased migration. To our knowledge, there has not been a reported local change of IL-7 expression after muscle trauma in humans. In a recent study, were the authors monitored systemic and local cytokine kinetics after total hip replacement surgery, no change in local IL-7 levels was found in the wound area (43). However, recently we found a 5-fold increase in total mRNA in skeletal muscle biopsies from 10 male individuals after an 11 weeks strength training programme (Haugen et al, 2009, submitted for publication). It is not yet known, if this increase promotes a local increase in IL-7 protein secretion. But this, together with the migration findings, may suggest that skeletal muscle cell secretion of IL-7 is involved in muscle recovery after strength training (Fig. 8).

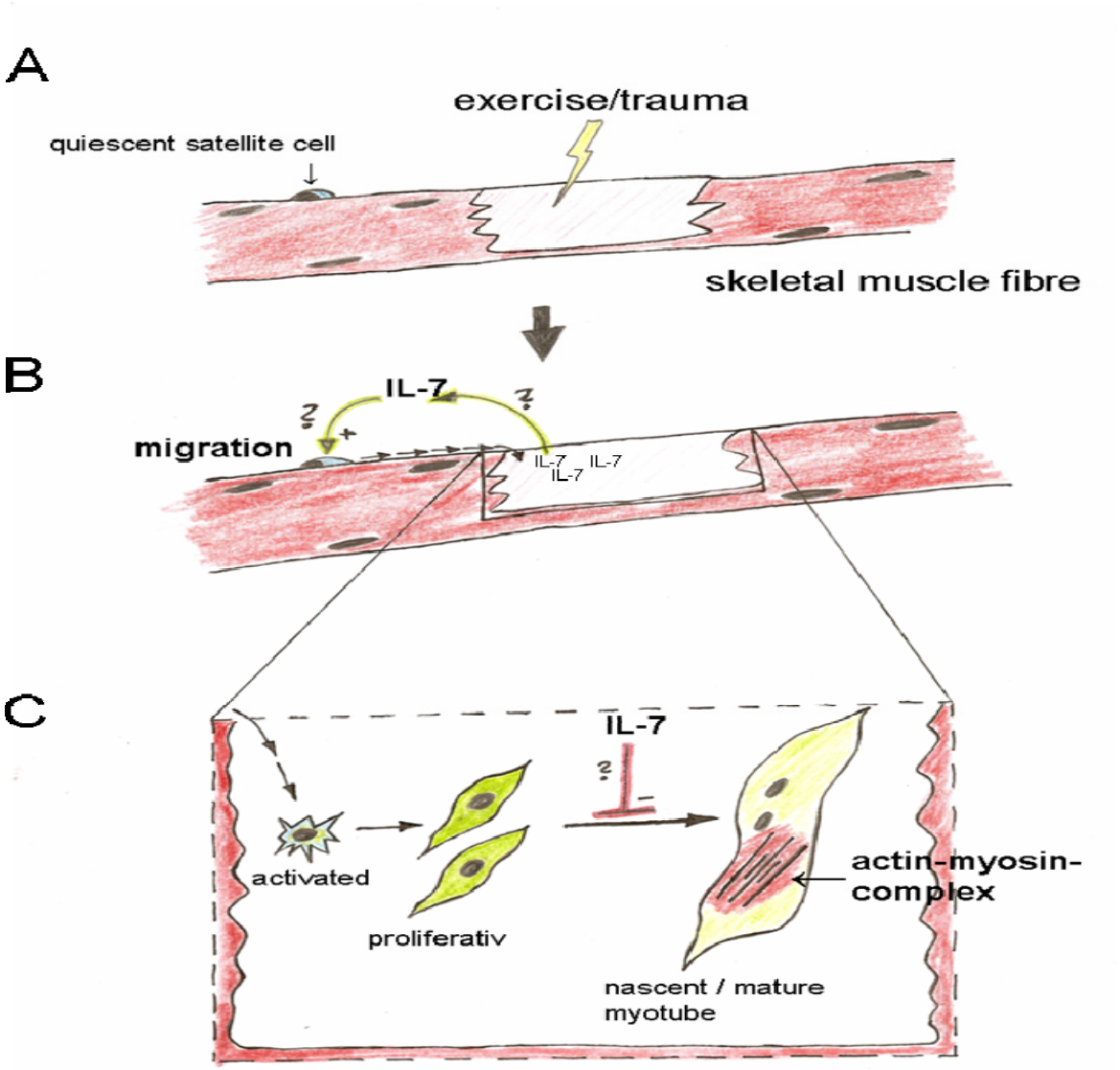

Figure 8. Potential local responses to contraction or skeletal muscle injury? A quiescent satellite cell is activated due to contraction or injury (A). IL-7 is secreted from the myofiber and stimulates the cell to migrate to the injury site (B). The IL-7 slows the differentiation process until enough satellite cells/mass have been produced to heal the damage or generate exercise-induced skeletal muscle hypertrophy.

In conclusion, the IL-7 mRNA expression findings are interesting, especially because the IL-7R signalling network work via the JAK/STAT-system in cells of the immune system, of which STAT3 and STAT5 are among the downstream signalling proteins that are phosphorylated in response to IL-7 (10). It seems like the JAK/STAT-system also might play a role in myogenesis. Two relative new studies have shown that:

JAK1-STAT1-STAT3 is a key pathway promoting proliferation and preventing premature differentiation of myoblasts (44), and that the JAK2/STAT2/STAT3 pathway is required for myogenic differentiation (45). Locally released IL-7 might play a role here.

It has also been shown that STAT3 is activated by several cytokines and play a central role in the regulation of growth, differentiation, and survival in many cell types (46). According to Yang et al the function of STAT3 in myogenesis still remains largely unknown, but in 2009 they showed that STAT3 could induce myogenic differentiation and that this effect might be mediated by interaction with MyoD (47). In addition to our findings that IL-7 may modulate satellite cell differentiation and increase migration raises many new questions. Can IL-7 influence myogenesis by downstream activation of STAT3 in myocytes, and is IL-7 involved in the repair process after exercise or muscle cell injury? And finally, is IL-7 released from skeletal muscle cells involved in a cross talk with the immune system?

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6. ATTACHMENTS

Attachment 1

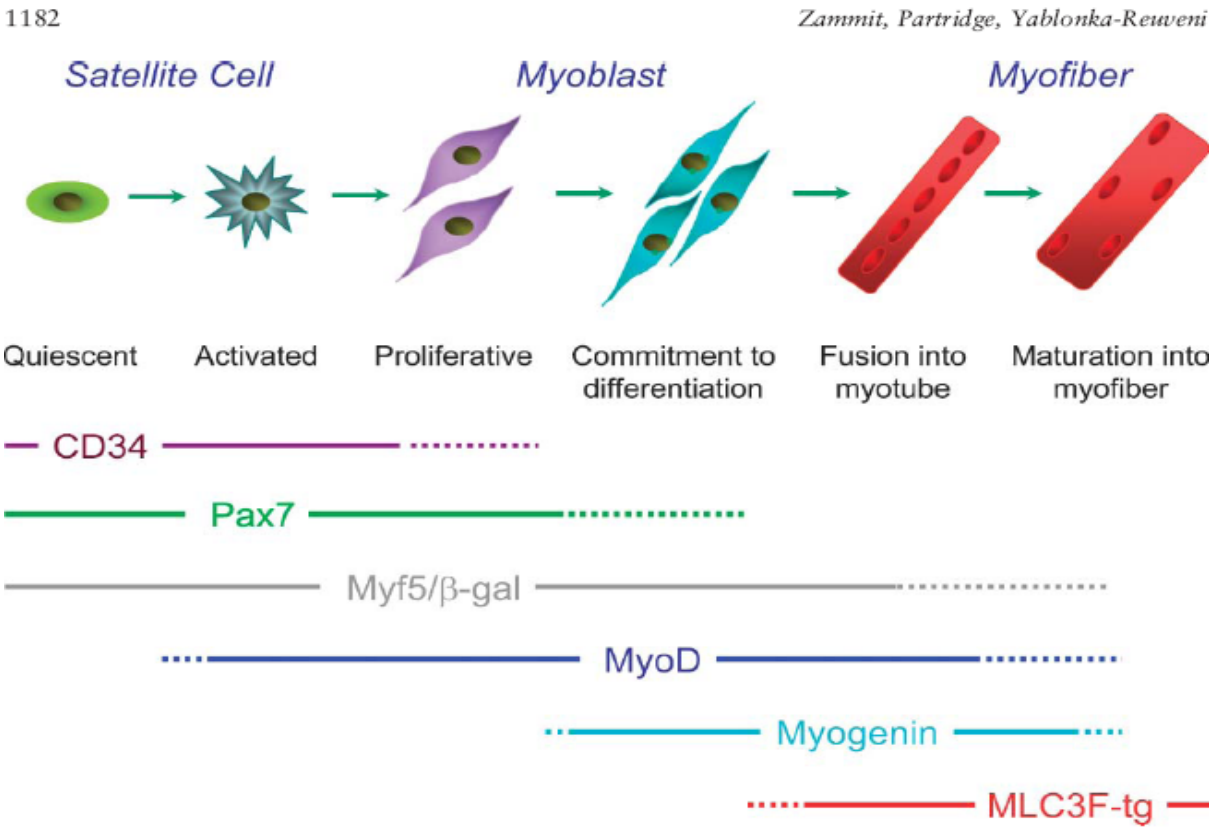
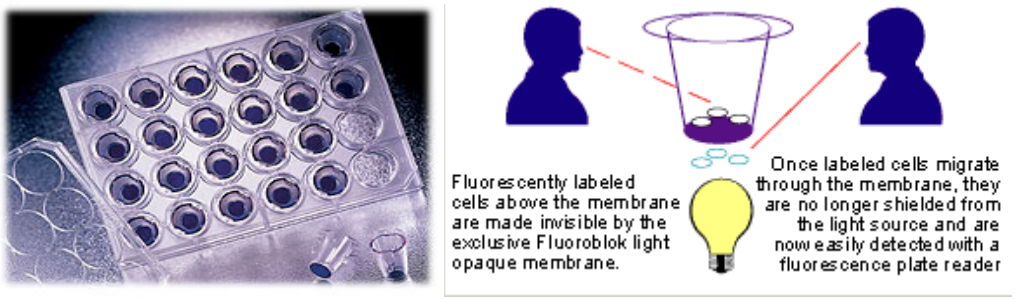


Figure 3 Schematic of satellite cell myogenesis and markers typical of each stage. Satellite cells are quiescent in normal adult muscle and can be activated by, for example, muscle damage. Once activated, satellite cells divide to produce satellite cell-derived myoblasts that further proliferate, before committing to differentiation and fusing to form myotubes, which then mature into myofibers (for clarity, satellite cell self-renewal is not included). CD34, Pax7, and Myf5/β-gal are expressed in quiescent satellite cells. Satellite cell activation is marked by the rapid onset of MyoD expression, whereas myogenin later marks the commitment to differentiation. The temporal expression pattern of MLC3F-tg is typical of many structural muscle genes such as skeletal muscle actin and MyHC, which mark sarcomeric assembly in the later stages of differentiation. Myf5/β-gal denotes the fusion protein product of the targeted allele of the *Myf5*$^{nlacZ+}$ mouse (Tajbakhsh et al. 1997), whereas MLC3F-tg is the product of the *3F-nlacZ-E* transgene (Kelly et al. 1995). (Adapted from Miller et al. 1999 with modifications by J. Beauchamp and the authors.)

The Skeletal Muscle Satellite Cell: The Stem Cell That Came in From the Cold. J Histochem Cytochem 54:1177–1191, 2006

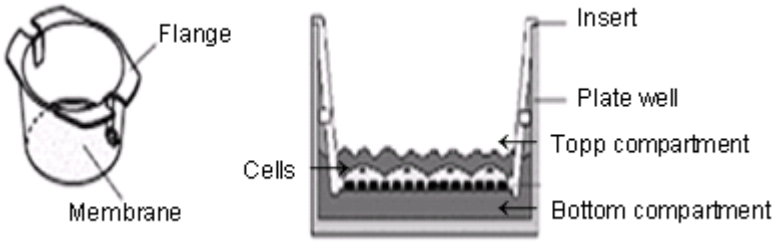
Attachment 2

1.



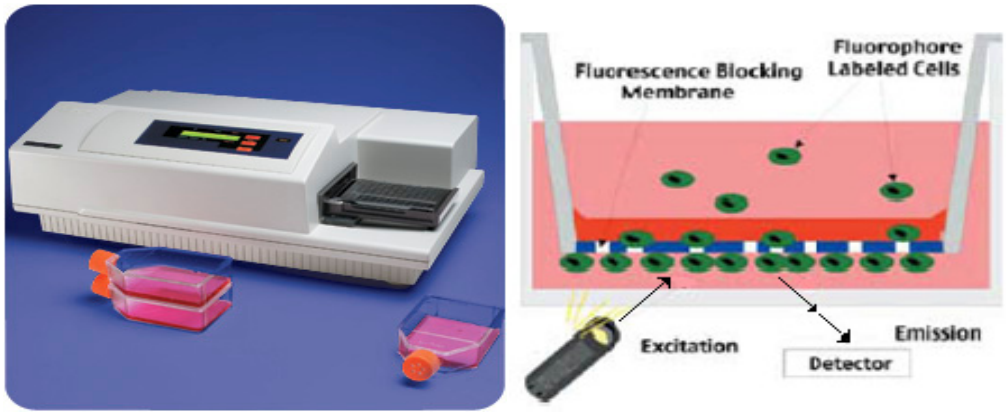
Picture of 24 well plate with BD Falcon™ FluoroBlok™ Insert Systems (BD Biosciences).

2.



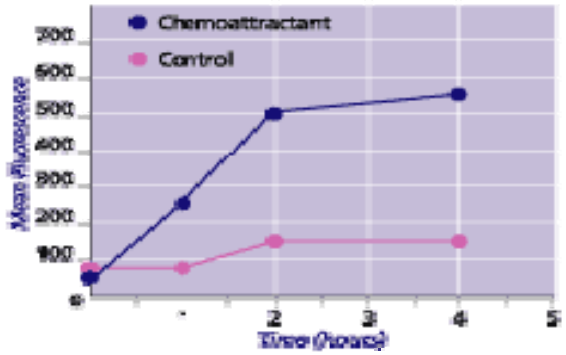
a. Cells are dyed with green fluorescence and seeded out on the membrane in the top compartment.
b. When cells have fixed, a chemo attractant/blocker is added in the bottom compartment.
c. Later, cell migration trough the membrane are measured from the underside of the membrane with a fluoresce plate reader or by counting cells (The picture is originally from BD Biosciences, but is modified).

3.

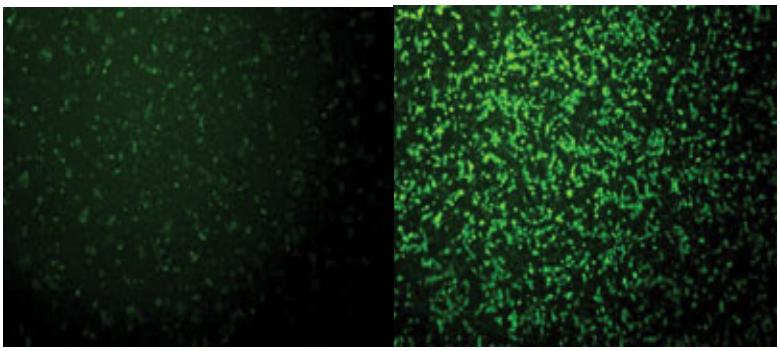


Fluoresce Plate Reader (SPECTRA MAX GEMINI EM) and simplified picture of how the readings are conducted (BD Biosciences). The fluorescence green dye was detected at 485 nm excitation and 530 nm emission.

4.



Results presented as increase in fluorescence (BD Biosciences).



Pictures taken before and after migration (BD Biosciences).

Attachment 3

6.1 Establishing a migration protocol

Our group had not worked with migration assays before; thus, we had to establish a method to conduct the assay. After considering the methods and hardware we possessed in our lab we decided to use of BD Falcon™ FluoroBlok™ Insert Systems (BD Biosciences, #351152) together with green fluorescence dye staining (CellTracker™ Green CMFDA, Invitrogen, #C7025). To measure the migration across the membrane we tested the following methods before including them into a final protocol: Fluoresce Plate Reader (SPECTRA MAX GEMINI EM); protein quantification; fluorescence microscopy observation and photography, and at last count the cells manually by use of a Bürcker counting chamber or count cells on pictures automatically by Kodak D1 3.6 software or manually. All the results from the migration protocol are presented later in attachment 3.

6.1.1 Migration methods

6.1.2 Establishing a satellite cell fluorescence staining protocol

First we tested the cell staining protocol provided by the manufacturer of the CellTracker™ Green CMFDA (Invitrogen, #C7025) to figure out a reasonable dye concentration. We also established an appropriate seeding density of satellite cells on the insert membrane. Prior to staining, the cells was seeded in four 25 cm^2 flasks coated with ECM and grown in SkGM until the desired confluence. At the day of staining the medium was removed and the cells washed 2 times with 5 mL pre-warmed PBS. Then the flasks were added 3 mL of a pre-warmed CellTracker™ dye working solutions (CellTracker™ dye dissolved in DMSO and diluted in low glucose DMEM with 1 % L-glut and 1 % P/S) in either 1 µM, 2.5 µM, 10 µM or 25 µM concentration. The cells were then put into an incubator (37°C, 5 % CO_2) for 30

minute incubation. The dye working solution was then replaced with fresh, pre-warmed DMEM with 1 % L-glut and 1 % P/S and incubated for another 30 minutes. During this time, the chloromethyl group (and for some probes, the acetate group) of the dye undergoes modification or are secreted from the cell. (It is not confirmed yet, but the fluorescent colouring agent is thought to react with thiols). Then the cells were washed once with PBS. Next, the cells were loosened from the flask by trypsin as previously described, counted and diluted to desired concentrations; 10000 per cm² (~ 3200 per insert) in DMEM with 0.1 % BSA. The cell suspensions were then distributed to empty wells in a 24 well plate or top compartment in wells with inserts (300 µl per insert). In the bottom compartment 700 µl of DMEM with 0.1 % BSA was added. Bubbles under the insert membrane, that could influence cell migration and fluorescence readings, were removed and the plate was placed in an incubator for 24 hours to let the cells attach. After 24 hours the media in the top and bottom compartment were replaced with 300 µl and 700 µl fresh DMEM with 0.1 % BSA respectively. Then the fluorescence was measured from the bottom of the plate in a fluorescence plate reader set at 485 nm excitation and 530 nm emission. Next the cells were investigated with a fluorescence microscope (Nikon Elippse TS 100 with C-SGH Mercury Illuminator and Epi-Fluorescence attachments) and pictures were taken with an Allied vision Thechnologies camera supported by Comet IV picture software.

6.1.3 Assessing insert coating and FCS concentration

We investigated if ECM coating and different FCS concentrations could influence the satellite cell migration. We tested single wells or duplicates with inserts. LD30, P7 satellite cells was seeded in upper compartment; 10 000 per cm² (~ 3200 per insert) in 300 µL low glucose DMEM with 0.1 % BSA, 1 % L-glut and 1 % P/S. The insert membrane was ± ECM coated on both sides and 700 µl low glucose DMEM with 1 % L-glut, 1 % P/S and; 0, 5 or 10 % FCS was loaded in the bottom compartment. Fluorescence readings and microscopy were done at 0, 24, 48, 72 and 96 hours.

6.1.4 Assessing cell seeding density and testing of a positive control

Living donor 30, P7 was stained and seeded in duplicates. The number off satellite cells experimented with was; 10 000- (~ 3200 per insert), 20 000- (~ 6400 per insert) and 30 000 per cm² (~ 9600 per insert). As positive control we used ± 10 ng/mL recombinant human hepatocyte growth factor (rhHGF) (R&D systems, #294-HG-005/CF) in 1 or 10 % FCS (vol/vol) in low glucose DMEM with 1 % L-glut and 1 % P/S. Prior to the experiment 5 µg rhHGF was dissolved in 50 µL 0.1 % BSA/PBS to a 100 µg/mL working solution. The cells were seeded in 1 % FCS and incubated for 4 hours. At 0 hours, the media was replaced in both top and bottom compartments with 1 or 10 % FCS ± rhHGF which was only added in bottom compartments. Fluorescence readings, microscopy and photography were planned to be done at 0, 24, 48 and 72 hours. At 48 and 72 hours the cells on the bottom side of the inserts membrane were harvested and counted. With some modifications, the trypsination- and counting protocols were conducted as previously mentioned.

6.1.5 Evaluating the cell counting protocol

Living donor 30, P7 was dyed and seeded in duplicates, 57 000 cells per cm² (~18 000 per insert). Prior to seeding the insert membrane was coated on the lower side only. The inserts were placed upside down on a sterile surface in an airflow chamber. Then 50 µL of ECM dilution was gently pipetted and distributed to cover the membrane. The ECM was left for 1 hour air drying. Then the cells were seeded. Four hours after seeding, the 1 % FCS media in the bottom compartments were discarded and replaced with fresh 1 or 10 % FCS with ± 10 ng/mL rhHGF.

Some comments about the migration methods

More test than mentioned above was conducted. In later assays the microscope camera and software was replaced to gain better resolution and colour. The Nikon digital camera DXM 1200 with ACT-1 software was replaced by a Allied vision Thechnologies camera and Comet IV picture software. Kodak D1 3.6 software was used to count cells on pictures. The picture counting of cells was also done manually. The pictures were taken with a 4x magnification which equals 2.66 mm² (~ 7.4 %) of the total 36 mm² membrane area.

A Lactate dehydrogenase test (Cytotoxicity Detection Kit, Roche Applied Science, #11 644 793 001) was also preformed to detect if the incubations resulted in cell-damaging or death. No significant damaging or death was found, data not shown.

The ordinary 24 well plates were replaced with plates designed to position the inserts in the middle of the wells (Notched TC Plate, BD Biosciences, #353504). This was done to improve the precision of fluorescence readings. Previously we had measured the total well area from the underside of the well. By doing this, we were able to extract the measurements from the membrane area only and remove the foil readings from the rest of the well, see attachment 4. However, in the end the precision of fluorescence readings was to low. Therefore this result is not presented in the final assay.

6.2 Migration results

6.2.1 Determination of optimal fluoresce dye concentration for staining satellite cells

The satellite cells were dyed with CellTracker™ Green CMFDA in either: 1-, 2.5-, 10- or 25 μM concentration. Microscopy, 2 hours after seeding, showed that fewer cells stained with 25 μM dye had attached to the well surface compared to the others.

Results form fluorescence readings at 48 hours of the wells with no inserts showed a linear increase with dye concentration (Fig. 9A). The fluorescence readings for 1-, 2.5-, 10- or 25 μM dye concentration in wells with out inserts was 57.4, 100.8, 336.4, and 820.9 respectively.

When the cells were studied under a microscope at 48 hours it was observed extensive cell death in the 25 μM well (Fig. 9B) and due to low BSA concentration 1-, 2.5 and 10 μM stained cells did not stretch out podocytes.

Fluorescence readings of the wells with inserts also showed a dye concentration related increase. It was 41.4, 41.9, 49.0 and 71.2 respectively. However when locked at in a microscope, no cells had migrated over the membrane at 48 hours. Therefore we suspected that the fluorescence readings were contaminated either by: light coming from the side walls of the insert; by excreted dye from living cells or dye-cell debris from dead cells that leaked from the top compartment to the bottom compartment. To investigate this we removed the inserts and conducted a new fluorescence reading. The new readings were almost identical to the readings with inserts (41.6, 42.8, 49.4 and 81.6) indicating that the first fluorescence results were based on excreted dye from living cells, or dye-cell debris from dead cells and not migrated cells. Based on these findings we decided to go on with 10 μM CellTracker™ Green CMFDA concentration because it gave the strongest fluorescence reading, and no obvious cell death was observed. Next we decided to test out different FCS concentrations and ECM coating to see if that could improve migration.

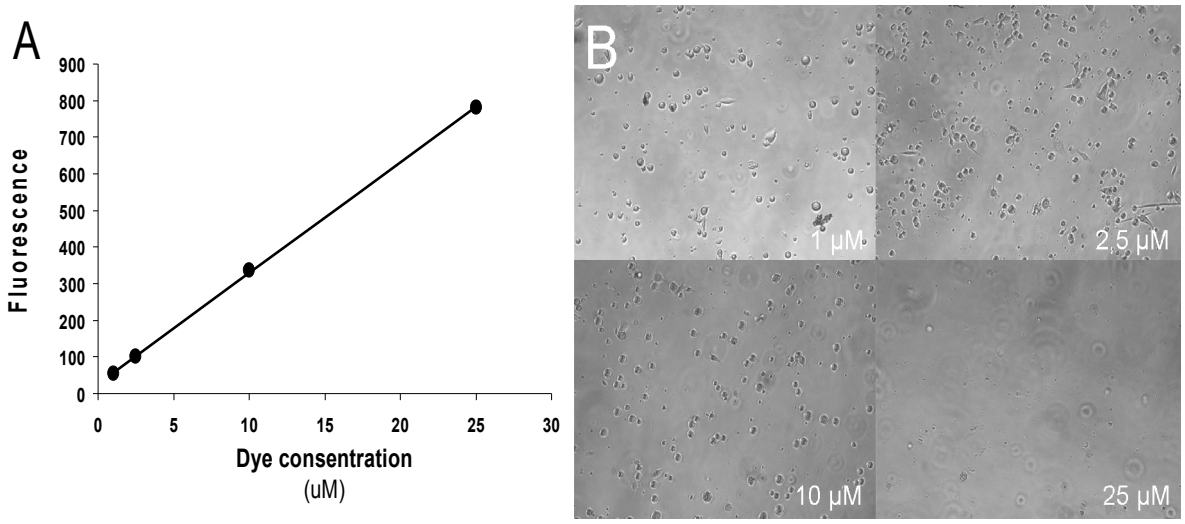


Figure 9. Effect of different fluoresce dye concentration on fluorescence readings (A) and cell death (B). Linear increase (A) in fluorescence form satellite cells stained with CellTracker Green CMFDA (1-, 2.5-, 10- and 25 µM) measured by fluorphotospectrometer. Due to low BSA consentration 1-, 2.5 and 10 µM stained cells did not stretch out podocytes (B). 25 µM dye concentration induced extensive cell death. Fluorescence measurements was conducted at 48 hours in a fluoresce plate reader. Cells were grown in low glucose DMEM with 0.1 % BSA. Pictures were taken at 48 hours in white-light at 10x magnification. Contrasts were sharpened in Adobe 6.0 Photoshop auto contrast.

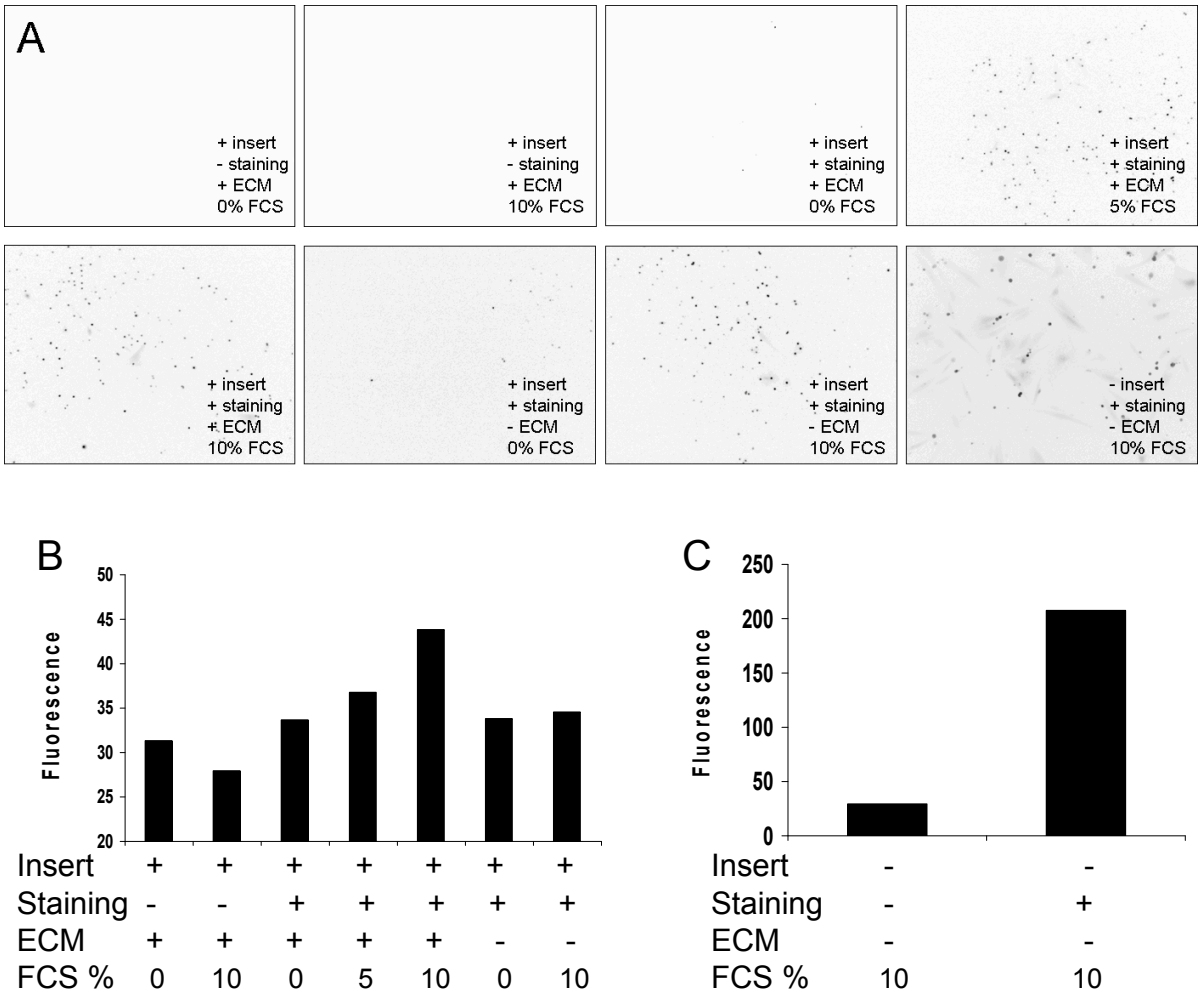
6.2.2 Effet of ECM insert coating and FCS in medium on cell migration

Microscope investigation showed that no cells had migrated over the membrane at 48 hours when no FCS was added in the bottom compartments. But on the 5 and 10 % FCS inserts there was extensive migration (Fig. 10A). There was observed more migrated cells in the 10 % FCS than in 5 %. It seemed to be more migrated cells on the +ECM- compared to -ECM coated membranes. Fluorescence readings at 48 hours did to some extent support these observations (Fig. 10B), but high background readings due to scanning of the whole well area instead of just the membrane did confound the results (attachment 4).

The fluorescence readings for the + staining + ECM + 0-, 5- or 10 % FCS was 33.6, 36.7 and 43.9 respectively.

In control wells without inserts the cells was ± fluorescence stained (Fig. 10C). The fluorescence readings were 29.3 and 207.6 respectively. This was done to show that the cell staining protocol did function.

These findings showed that FCS was necessary to achieve satellite cell migration. It also indicated that ECM improved migration and that 10 % FCS gave greater migration than 5 %.



Picture 10. Effect of ECM coating and FCS on satellite cell migration. Pictures in UV-light of satellite cells stained with 10 µM green fluorescence at 48 hours (10x magnification). Migration was photographed (A) and measured with a fluoresce plate reader (B and C) on ± ECM coated ± fluoroblok insert membranes. The bottom compartment in the wells contained 0, 5 or 10 % FCS in low glucose DMEM. A: Migration was greatest on + ECM + 10 % FCS insert membranes. B: Fluoresce plate readings showed that migration was greatest on + ECM + 10 % FCS insert membranes. C: Control wells without inserts. Pictures were run trough Adobe 6.0 Photoshop auto contrast and inverted.

6.2.3 Effect of recombinant human HGF on satellite cell migration

Next we tested different satellite cell seeding densities, ± recombinant human (rh)HGF in 1 or 10 % FCS. This was done to test the rhHGF as a positive control. Relative to control rhHGF incubation significantly increased satellite cell migration on insert membranes by 2.2 folds (P = 0.013) in the 1 % FCS compartments (Fig. 11). The results also indicated that; of the satellite cell seeding densities 10 000-, 20 000- and 30 000 per cm², the highest cell density gave highest migration count (data not shown). Summed up these findings showed that rhHGF may increase migration, and that higher cell seeding density gives more migration than lower seeding density.

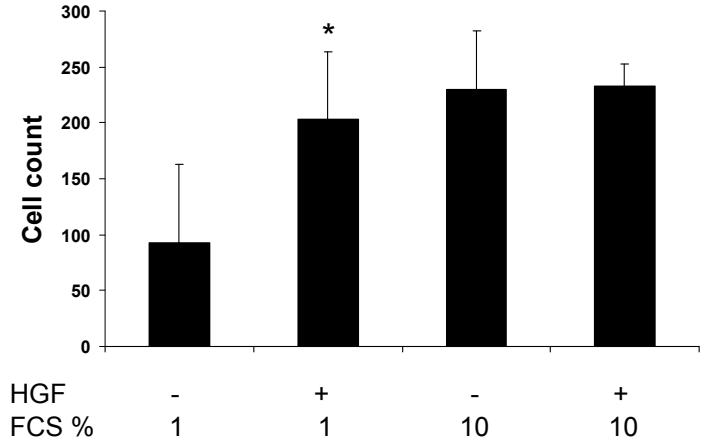


Figure 11. Effect of recombinant human hepatocyte growth factor (rhHGF) on satellite cell migration. Satellite cells grown in 1 or 10 % FCS in low glucose DMEM ± 10 ng/mL rhHGF. Average number of satellite cell migration on inserts membranes at 48 and 92 hours, manually counted in Bürker counting chamber. Each point represents mean ± SEM from 6 wells of one cell donor. *P < 0.05 using paired Student's t-tests.

6.2.4 Evaluating the cell counting protocol

Next we wanted to test if the manual counting protocol, by the use of Bürker counting chambers, was accurate enough to use in a final protocol. Duplicates of

satellite cells from one donor were tested in ± recombinant HGF in 1 or 10 % FCS. Microscopy observations showed that there were more satellite cells migrated in the 10 % FCS + rhHGF compartments compared to the other treatments. But relative to 0 hours readings there was not a significant difference in fluorescence readings on ± rhHGF inserts membranes at 48 hours (Fig. 12A).

Manual counting with Bürker counting chamber of cells trypsinated form the underside of the membrane showed the same trends as observed by fluorescence readings (Fig. 12B), but the results was not significant. The standard differentiation (SD) on raw data was high for manual counting with Bürker counting chamber (data not shown), and even after removing extreme values the SD was relative high in most of the compartments: 289 ± 0 cells (1 % FCS - HGF); 533 ± 314 cells, (1 % FCS + HGF); 667 ± 251 cells (10 % FCS - HGF); 1011 ± 361 cells (10 % FCS + HGF). Data are presented as means ± SD. Another problem experienced with this counting method was the risk of losing the whole cell pellet after trypsination and centrifugation. It was also a very time consuming method.

These observations indicate that use of Bürker counting chamber can work but that large number of parallels is needed to retrieve reliable data. Summed up, we found that manual counting of cells by the use of Bürker counting chambers are to inaccurate to use when few test parallels are used.

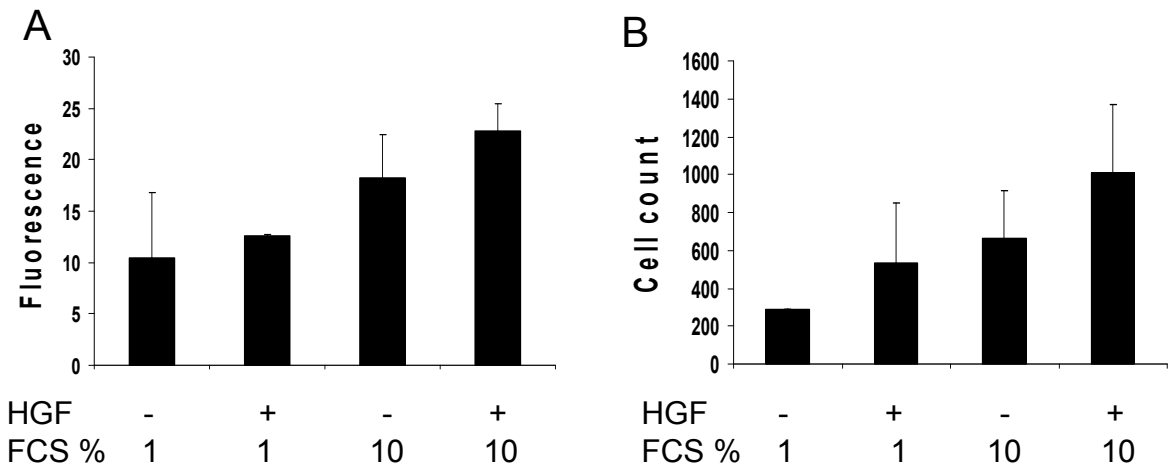


Figure 12. Comparing fluorphotospectrometer (A) versus manual counting using Bürker counting chamber (B). Satellite cells were dyed with green fluorescence and seeded in fluoroblok inserts. All the membranes were ECM coated. Cells was incubated with ± 10 ng/mL rhHGF and 1 or 10 % FCS in the bottom compartment. Fluorescence at 48 hours (A), relative to 0 hours, was measured in a fluoresce plate reader to assess migration. Mean migration counted (B) after 48 hours, assessed by the use of Bürker counting chamber. Each point represents mean ± SD from duplicates of one cell donor. *P < 0.05 using paired Student's t-tests.

6.2.5 Effect of recombinant human IL-7 or SPARC incubation on satellite cell migration

In this assay we tested Kodak D1 3.6 picture analysis software and compared it to the use of Bürker counting chambers to see if standard deviation and time consumption could be reduced. In this assay we also tested if recombinant secreted protein, acidic, cysteine-rich (rSPARC) could influence satellite cell migration. Why we tested SPARC, and the results, will not be discussed further here. The main focus in this section is validation of the methods. Triplicates of satellite cells from one donor were tested in low glucose DMEM with 1 % FCS (control) added either; recombinant HGF, recombinant SPARC or recombinant SPARC mixed into the ECM.

Fluorphotospectrometer readings did not reveal any significant difference in fluorescence readings between controls and any of the treatments at 48 hours (Fig. 13A). The fluorescence readings were not in accordance with microscopy observations.

Counting the cells both manually with Bürker counting chambers (Fig. 13B) and automatically with Kodak 1D (Fig. 13C) showed similar trends after removal of extreme values in the manual counting. These trends were in line with the microscopy observations.

The manual counts versus (VS) Kodak D1 3.6 picture analysis was for the different treatments, incubation in parenthesis: 500 ± 236 VS 1159 ± 211 (control); 795± 267 VS 1460 ± 224 (IL-7); 706 ± 114 VS 1199 ± 179 (SPARC); 332 ± 90 VS 577 ± 127 (SPARC in ECM). Data is presented as mean ± SD. These data shows that the Kodak D1 3.6 picture analysis software gives lower SD`s than Bürker counting chambers. However, these observations were not in accordance with the fluorphotospectrometer readings. This made us question fluorescence reading as a usable method in a final migration protocol.

The Kodak D1 3.6 picture analysis was in line with microscopy observations, but there was one problem. The analysed pictures were taken from the centre of the inserts membrane. Microscopy observations had revealed that the migration was most dense in the centre and along the outer lining of the membranes. Between this areas there tended to bee a circular area were there was less migration. This had a consequence. When the picture of centre only (covering ~ 7.4 % the total membrane area) was used to calculate total migration on the whole membrane, the results showed a higher migration than true migration (Fig. 13B versus 13C). However, when analysing statistics we used cell counts from the picture area only to eliminate this problem.

Pictures are true replicates of a an event, this together with narrower standard deviations by the use of Kodak D1 3.6 picture analysis software compared to use of Bürker counting chambers made us to decide to go on using Kodak D1. However, it is important to bee aware that there are some limitations using this software, the images have to be in focus and the migrated cells must not overlap.

Due to its described flaws, Bürker counting chambers was not used in further assays.

The fact that the fluorphotospectrometer readings was not in accordance with microscopy observations and counting data forced us to turn down this method for this use (the same observations was also done in later assays).

Summed up, as long images are in focus and the cell density is low the Kodak D1 3.6 picture analysis software can bee used to count fluorescence dyed cells.

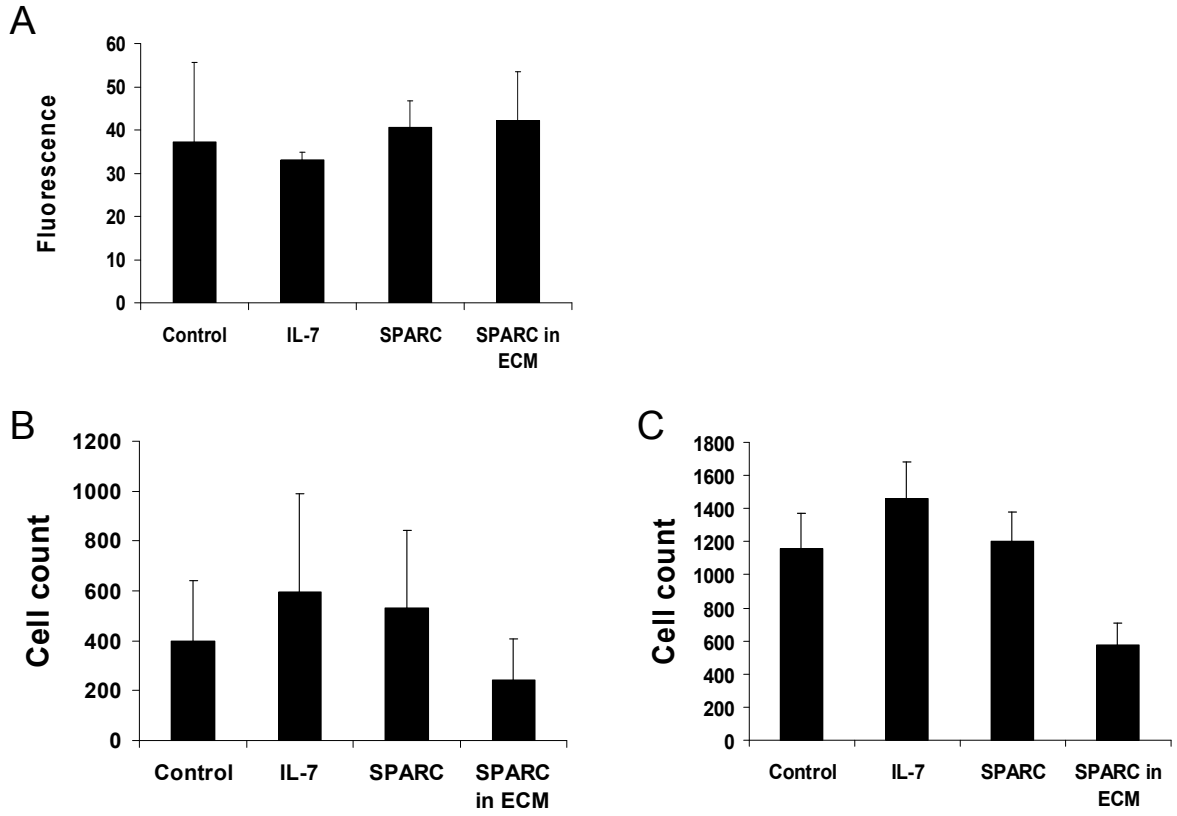
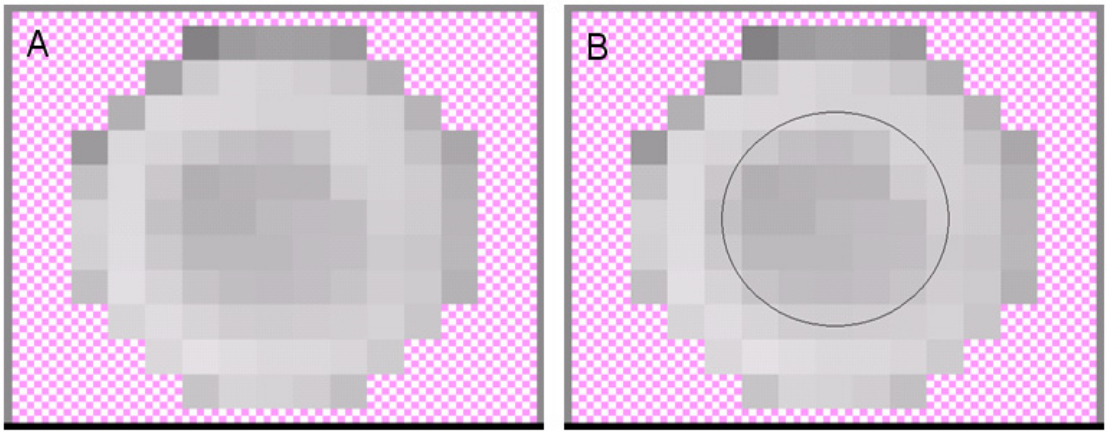


Figure 13. Effect of recombinant human IL-7 (rhIL-7) or secreted protein, acidic, cysteine-rich (SPARC) incubation on satellite cell migration. Satellite cells were dyed with green fluorescence and seeded in fluoroblok inserts. All the inserts membrane was ECM coated. Cell migration was measured at 48 hours with: 1 % FCS (control); + 100 ng/mL rhIL-7; + 2 µg/mL SPARC or + 1.4 µg SPARC in ECM in the bottom compartment. Migration measured with fluorphotospectrometer (A) (includes inserts membrane and the rest of the well bottom area). Trypsinated cells from the insert membrane underside were manually counted in Bürker counting chamber (B). Kodak D1 3.6 software was used to count cells on one picture per membrane (C). Data are relative to 0 hour readings. Each point represents mean ± SD from triplets of one cell donor. *$P < 0.05$ using paired Student's t-tests.

Attachment 4

Comments about the fluoresce plate readings. I was not able to use the fluoresce plate reader as intended in this assay. Therefore I have not put much emphasis on the results gained by this measuring method. But before that decision, I put mush time into this method trying to make it work.

One way or another, fluoresce readings was confounded or the plate reader was not sensitive enough to register the migration? I struggled to limit the fluorescence readings to the membrane surface only. In early trials the whole well surface was measured, after some improvisations I was able, to some extent, to restrict the readings to the area of interest, se figure below. But even then, the readings were not much of use. The zero hour readings resulted in intolerable standard errors and the 48 hour was not in line with microscopy observations of migration. Maybe leakage of the fluorescence dye from the cells during the 48 hours confounded the results? Maybe the leaked fluorescence reacted with the ECM coating on the membranes? Maybe the reader was not sensitive enough? Maybe the dye concentration was to low? I honestly don't know.



The figure shows graphics of the fluorescence readings. (A) Shows readings from the whole well bottom. The membrane can be seen as the darker area in the centre of the well. (B) shows the same reading but a ring is drawn to illustrate the borders of the membrane (the area of interest).