Differentiation of skeletal muscle cells in culture

Experimental conditions and expression of myokines

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Master thesis

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UNIVERSITY OF OSLO

May 2013

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Acknowledgments

This work was performed at the Department of Nutrition, University of Oslo, from June 2012 to May 2013, in the laboratory of professor Christian A. Drevon. I thank principal investigator Christian for kindly welcoming me to his research group, and for introducing me to the myokine research field, and the molecular nutrition and systems biology concepts.

I give thanks to my co-supervisor Anders Kielland, PhD student Marit Hjort and engineer Anne Randi Enget for patiently teaching me how to work in the lab, and for always being available. I especially thank Anders and Marit for guidance and discussions throughout this year. I also thank Anders for feedback during the development of this manuscript, and for critically evaluating the final product.

All assistance and enthusiastic discussions with members of our and other research groups have been much appreciated. Special thanks to Frode for sharing thoughts on PLAU and generously providing mRNA data from skeletal muscle biopsies. Special thanks also to Christian and Rikke for giving me valuable reagents and for help with western blotting procedures.

I would like to thank my fellow classmates for all coffee and lunch breaks, which have been much appreciated. I am also grateful for my friends and life outside Domus Medica for being a counterbalance to the all-consuming world of nutrition.

Finally, I owe a debt of gratitude to Kristin Torvik and my family for always being there, believing in me, pushing me, and for motivating and supporting me through hardship. I would not have been where I am without you.

Oslo, May 2013

Jacob Juel Christensen

Summary

Background: Physical activity can prevent or delay many obesity-associated diseases. Even though reduction in fat mass clearly is health promoting, beneficial effects of physical activity are also observed regardless of weight change. This suggests additional mechanisms linking physical activity and health status. The recent discovery that contracting muscles secrete peptides (named myokines) has put forward a hypothesis suggesting that working muscles regulate the systemic metabolism through humoral signaling. This may be an important mechanism involved in the beneficial health effects of physical activity. To investigating physical activity on molecular levels, *in vitro* models of exercise, such as cell cultures of skeletal muscles, is greatly beneficial.

Objectives: This thesis had two major objectives. The first objective was to optimize a protocol for differentiation of human skeletal muscle cells in culture by manipulating experimental conditions, and subsequently evaluate cell morphology and molecular aspects. I aimed to investigate which serum type (HS, FBS, UG) and concentrations that provided highest degree of differentiation, and also to investigate the effect of dexamethasone and insulin. Additionally, I aimed to characterize molecular aspects of cells differentiating in the most promising media. The second objective was to identify and characterize expression of myokines during *in vitro* myogenic differentiation. I aimed to investigate mRNA expression and secretion of both known and potential myokines.

Results: 1) Romanowsky staining was established and used to calculate a fusion index in order to determine the degree of differentiation in the muscle cell cultures. 2) HS at concentration of 0.5 and 1.0 % showed the best differentiation, but with no statistically significant difference from the other media. 3) Dexamethasone in higher doses inhibited differentiation, whereas increasing concentration of insulin in SFM promotes a tendency towards lower differentiation. 5) mRNA expression of PAX7 was reduced during differentiation of myoblasts in 1.0 and 2.0 % HS, while myogenin, myosin, alpha-actin, PGC1α and GLUT4 were increased. There was no differences between these concentrations of HS, but in SFM and the proliferation medium the gene profile was substantially different. Protein content of myosin and actin increased with differentiation, reaching the highest level on day 7 of differentiation. 6) mRNA expression of the myokines FNDC5, ANGPTL7 and PEDF strongly increased during differentiation, whereas PLAU decreased. Expression of IL-8

showed a minor change, and adiponectin was not detected. Secretion of PLAU and PEDF proteins in to the medium corresponded well with the mRNA expression pattern.

Conclusions: I have found that myoblasts differentiated for 7 days in 1.0 % HS result in well-differentiated muscle cell cultures, exhibiting at best 67 % of all nuclei inside of multinucleated myotubes. All serum-reduced media showed mRNA expression pattern corresponding to the expected changes during differentiation, but it is not possible to draw any conclusions regarding differences between 1.0 and 2.0 % HS. Furthermore, mRNA and protein expression kinetics of myosin and actin reached highest level on day 7, suggesting the cell culture to be most muscle-like at this stage. Finally, I observed distinct mRNA expression dynamics of several myokines during differentiation, and secretion of PLAU and PEDF in to the medium.

Abbreviations

ACTA1	Alpha actin 1
AMPK	AMP-activated protein kinase
ANGPTL	Angiopoietin-like protein
AUC	Area under the curve
BCA	Bicinchoninic acid
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CKI	CDK inhibitor
DMEM	Dulbecco’s modified eagle medium
(h)EGF	(human) Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPS	Electric pulse stimulation
F12	Ham’s F12 nutrient mix
FBS	Fetal bovine serum
(hb)FGF	(human basic) Fibroblast growth factor
FNDC5	Fibronectin type III domain containing 5
GLUT4	Glucose transporter 4
HS	Horse serum
IGF	Insulin-like growth factor
IL	Interleukin
LDH	Lactate dehydrogenase
LIF	Leukemia inhibitory factor
MAPK	Mitogen-activated protein kinase

MGB	Minor groove binder
MRF	Myogenic regulatory factor
MYH	Myosin heavy chain
MYOG	Myogenin
PAGE	Polyacrylamide gel electrophoresis
PAX	Paired box
PEDF	Pigment epithelium-derived factor (also known as SERPINF1)
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1α
PLAU	Plasminogen activator urokinase (also known as uPA)
pRb	Retinoblastoma protein
PVDF	Polyvinylidine fluoride
RPLP0	Human large ribosomal protein P0
SDS	Sodium dodecyl sulfate
SERPINF1	Serpin peptidase inhibitor F1 (also known as PEDF)
SFM	Serum-free medium
SIX1/4	Homeobox protein SIX1/4
SLC2A4	Solute carrier family 2 member 4 (GLUT4)
SPARC	Secreted protein, acidic and rich in cysteine
TBS	Tris-buffered saline
TCA	Tricarboxylic acid
TF	Transcription factor
UG	Ultroser G
uPA	Urokinase-type plasminogen activator (also known as PLAU)

Contents

1 Introduction

1.1 Health in the modern society

The global increase in overweight (BMI ≥ 25 kg/m^2) and obesity (BMI ≥ 30 kg/m^2) is one of the main health related problems of modern societies (1). As infections were the main causes of death in the year 1900, heart disease and cancer rise to be the main causes of death in the 21st century, partly because of great developments in treating infectious diseases, and partly because of the overweight and obesity epidemic (2). Weight gain due to expansion of fat mass is a result of energy intake in excess of energy expenditure, and abdominal as well as subcutaneous adipose depots seem to enhance in response to energy surplus (3). Many environmental factors affect the risk of developing overweight and obesity, such as availability of tasty and energy dense foods, and low physical activity due to mechanization and digitalization of modern life (4-7). Knowledge of body function, health in relation to risk factors, and education and financial background also affects this risk (8). Excess body fat increases the risk of having metabolic syndrome, type 2 diabetes, hypertension, cardiovascular disease, different types of cancer, respiratory problems, gall bladder disease, musculoskeletal disorders, gout, infertility, complications during labor and surgery, non-alcoholic fatty liver disease, depression and other mental disorders, as well as enhanced total mortality (9, 10). Obesity possibly mediates these effects via adipose tissue-derived factors (i.e. increased plasma levels of intracellular adhesion molecule (ICAM)-1, leptin, interleukin (IL)-6, tumor necrosis factor (TNF)-α and resistin, and lower levels of adiponectin) (11). These signaling molecules may influence body weight homeostasis as well as insulin resistance, lipid levels, blood pressure, coagulation, fibrinolysis and endothelial function (11).

There are no updated obesity prevalence data in Norway available, but it is assumed that around 15-20 % of all adults in Norway classify as being obese, with an increasing trend (8). However, the incidence of coronary heart disease in Norway has declined by > 70 % the past 40 years. This suggests a change in other risk factors, like dietary fatty acid pattern and smoking (12). Similar developments are observed in other countries as well (13, 14).

1.2 Health effects of physical activity: the myokine concept

Physical activity is any form of body movement caused by muscle work, and that results in increased energy expenditure (15). Physical activity can metabolically be devided into exercise and non-exercise activity thermogenesis (16). The latter includes common activity such as walking, sitting and labor, and constitutes between 10 and 50 % of the total energy expenditure, while exercise can constitute between 0 and 75 % of the total energy expenditure (16, 17). Reduced level of physical activity without a complementary reduction in energy intake has been suggested to be the major cause of the progressive obesity epidemic the last 15-20 years (18). Modern objective methods (accelerometer) of measuring physical activity level have shown that approximately 20 % of the adult population in Norway meets the recommended 30 minutes daily moderate physical activity, with a clear socioeconomic trend (8, 19). Moreover, there is a steep decline in the level of physical activity from 9 to 15 years old children, which in future perspective potentially will exacerbate the obesity prevalence in Norway (8, 20).

Physical activity can offer one of the most extreme situations the organism ever has to cope with (21), and there are major physiological changes occurring upon initiating activity, such as a 10-25 times increase in oxygen consumption within minutes, reflecting severe metabolic adjustments in the body (15). Depending on intensity and duration, physical activity will affect most organ systems in the body (15). For this adaptation to occur, many organ systems have to adjust to increasing demands as intensity progresses. The metabolic and hormone-secreting tissues are central coordinators in this matter. For example, there are vast changes in hormone concentrations in blood, suggesting a coordinated involvement of all the classical and non-classical endocrine organs (15, 22-24). Importantly, the catecholamines are well-characterized with respect to roles during physical activity, as they contribute to most of the described physiological changes (21, 24, 25). This includes circulatory and metabolic alterations, mediated by intensity-related secretion from the adrenal glands, and tissues-specific receptor distribution (α_1, α_2, β_1, β_2 and β_3 receptors). Catecholamines increase the hearts contraction force and rate of beating, and regulate the peripheral vasoconstriction. In liver, catecholamines stimulate glycogenolysis, whereas this requires simultaneous contraction (increased [Ca^{2+}] and [P_i]) in muscle. Catecholamines stimulate lipolysis in

adipose tissue, and both inhibit and stimulate glucose uptake and utilization in skeletal muscle (21, 24, 25).

Importantly, physical activity can prevent or delay many obesity-associated diseases, regardless of weight status (15, 26-32). There are numerous potential reasons for this, including improvements in heart function, muscle function, lipid profile, blood pressure, blood glucose control, coagulation regulation, bone remodeling, cartilage and connective tissue synthesis, lung function, and gastrointestinal and liver function (15). However, the precise molecular basis for these improvements is not clarified, but there are several hypotheses. Firstly, a reduction of fat mass may be one explanation, with subsequent changes in circulating cytokines and adipokines. Secondly, regularly occuring physical activity-related known endocrine changes, such as catecholamine spikes, may also explain a vast amount of the health effects. Thirdly, as the brain operates in packages, an increase in physical activity may also increase the motivation and willingness to eat healthy. Fourthly, the increased flux of metabolites through the organism, such as periodically depletion of local glycogen and fat stores in muscle and liver, may explain some of the health effects. The latter may have several spillover effects, such as AMP-activated protein kinase (AMPK) activation, improved insulin signaling, better glucose tolerance, and better regulation of energy storage. Importantly, as muscle tissue is responsible for about 70-90 % of insulin-stimulated glucose uptake (33), a slight increase in insulin sensitivity has profound effects on blood glucose regulation. Finally, secreted factors from muscle tissue may be of importance for the observed health improvements. Muscle is not a specialized (classical) endocrine organ, but similar to adipose tissue, the liver, the gastrointestinal tract, the heart, and the kidneys, skeletal muscle is a potentially major source of blood-circulating substances (34). Skeletal muscle is a highly dynamic tissue, and comprises approximately 30-50 % of the adult human body mass (34, 35). Physical activity and inactivity may result in muscle hypertrophy and atrophy, respectively, which might be followed by an acute or chronic change in circulating muscle-derived signals. Signaling peptides secreted from muscle cells are known as myokines (36). Myokines may act in an endocrine, autocrine or paracrine manner (36). In view of the previous discussion, myokines probably complement and supplement the well-known physical activity-related humoral factors, such as adrenaline, cortisol and others. Moreover, acute and chronic physical activity probably alters the secretion of myokines to meet the changing demands for power and support, and also for muscle cells to maintain their fine-tuned balance with other cells and organs. The observed health effects may therefore be a side

effect in this matter. For example, if the muscle experiences an energy crisis (i.e. increasing intensity and duration of the activity), it will signal to surrounding tissues that it craves more substrates for ATP regeneration. The general signal will therefore be: *reduce your metabolic activity and/or direct all available energy towards the working muscle*. If this signal were to be transferred efficiently, it would have to circulate the blood stream, exposing all cells and tissues with receptor expression to the same (or somewhat modified, depending on other circulating substances) signal. Such a signal could potentially inhibit energy- and mitogen-dependent proliferation elsewere in the organism, such as in the colon. Such a signal could also potentially remove accumulated glucose and fatty acids (and toxic derivatives) from non-storage organs and tissues, such as the liver. Hence, the inhibition of colon tumorigenesis, and increase in insulin sensitivity and glucose tolerance may be side effects of energy redirection during physical activity.

Whereas myostatin was the first protein fulfilling the criteria as a myokine, IL-6 is most extensively studied (36). Myostatin inhibits muscle hypertrophy, and its expression is inhibited by hepatic follistatin during exercise (36). Thus, myostatin is important for inhibiting skeletal muscle to become very big compared to other tissues. IL-6 is important for inflammation, lipid and glucose metabolism, muscle hypertrophy, and for the compensatory increase in insulin secretion during insulin resistance (36). Leukemia inhibitory factor (LIF), IL-4, IL-7 and IL-15 are also potentially important for muscle hypertrophy, whereas brain derived neutrotrophic factor (BDNF) and myonectin (CTRP15) are important for energy metabolism (36, 37). Insulin-like growth factor (IGF)-1 and fibroblast growth factor (FGF)-2 contribute to bone remodelling, and follistatin-like protein (FSTL)-1 promotes endothelial revascularization (36). Irisin promotes a shift from white to *brown* adipose tissue in mice (36). Secreted protein, acidic and rich in cysteine (SPARC) is important for extracellular matrix remodelling, and possibly for inhibition of tumorigenesis (38-40). Matrix proteins such as lumican, collagen and metalloproteinases have also been identified as myokines (41). In addition, other potential myokines include FGF-21, IL-8, chemokine (C-X-C motif) ligand-1 (CXCL1) and pigment epithelium-derived factor (PEDF) (36). The changes in secretion of myokines may explain health effects of physical exercise, either through improvements in known physiological systems, or yet undiscovered pathways.

1.3 Muscle tissue

To fully appreciate the complexity of muscle physiology, it is important to have detailed knowledge of the embryonical development of muscle tissue. Skeletal muscle tissue is of mesodermal origin (42, 43). Mesoderm buds off repeated structures called somites alongside the neural tube. The somites form a number of structures, such as the sclerotome, the syndetome, and the dermomyotome. The dermomyotome segregates to give rise to the dermatome and myotome, and the latter is the embryonic region where muscle differentiation first occurs. The transcription factors (TFs) Paired box (PAX) 3 and PAX7 are the first known markers that label myogenic precursors, and the expression is regulated by signals from surrounding tissues. Other TFs are also important in early myogenesis, such as Dachshund homolog (DACH) 2, homeobox protein SIX (SIX) 1 and Eyes absent homolog (EYA) 2 proteins. PAX3 and 7 are regulators of muscle development and are upstream of myogenic genes in somites, limb muscles and satellite cells. PAX3 and 7 control the expression of myogenic regulatory factors (MRFs), which are TFs that support the proliferation and survival of myoblasts before differentiation. Cells migrating from the myotome are the main source of proliferating myoblasts, important for myogenesis in different anatomical regions of the body. Differentiation starts when cells exit the cell cycle and start to express muscle-specific markers, while the PAX genes are down-regulated. Myogenic factor (MYF) 5 is the earliest known marker of determined muscle cells, closely followed by Myogenic differentiation 1 (MYOD). The major adult muscle stem cells are called satellite cells. They are derived from migrating cells from the central dermomyotome and reside between the basal lamina and sarcolemma in adult skeletal muscle. The various anatomical regions of vertebrate muscle tissue develop slightly differently. This is to large extent controlled by signals from the surrounding tissue, such as the neural tube, notochord, overlaying ectoderm and lateral mesoderm. Important examples include wingless ints (WNTs), sonic hedgehog (SHH), bone morphogenetic proteins (BMPs) and Noggin. MYF5 and MYOD, together with MRF4 and myogenin, are the core network of TFs important for activating myogenesis. When experimentally overexpressed in non-muscle cells, these MRFs can override the existing cellular program, determining the cells for a myogenic fate. There is also a pronounced redundancy between the MRFs, shown by knock-out models. Moreover, the MRFs can activate the expression of each other. The exception to this is MYF5, which seems to act on top of the transcription cascade. Regarding redundancy, myogenin appear to be the only MRF absolutely required for myogenesis (42, 43). MRFs heterodimize with members of the

myocyte enhancer factor (MEF)-2-family of TFs and bind DNA motifs called E boxes in the genome (43). Chromatin immunprecipitation combined with sequencing analyses (ChIP-seq) have revealed that MyoD bind lots of these E boxes (43). Muscle-specific promoters often contain both MRF and SIX1/4 complex-binding motifs, which has led to the hypothesis that a cooperation between these modules may be required for transcriptional activation of muscle-specific genes (43, 44). Furthermore, evidence is accumulating showing the existence of a higher order regulation involving miRNA (43, 45-48). For example, it has been showed that miRNA associate with and degrades mRNA for PAX7 (45, 47). This is an important feature of miRNAs, as transcripts important in previous cell cycle phase may be abundant in the cell, and potentially delay the transition to G0 phase if not degraded. Thus, this mechanism results in an efficient switch from a proliferative state to differentiation (45). In summary, a small number of TFs (the MRFs) are important for muscle development, and diverse embryonic starting point converge on these TFs to activate the myogenic apparatus.

Although muscle fibers only develop until last period of fetal life, satellite cells in adult skeletal muscle are important in muscle regeneration and hypertrophy (42, 49). These quiescent cells reside beneath the basal lamina (42). Satellite cells *in vivo* are under strickt control by the stem cell niche, which can be thought of as *a specific location in a tissue where stem cells can reside for an indefinite period of time and produce progeny cells while self-renewing* (50, 51). Factors influencing satellite cell activity in their niche have been investigated, and probably comprise location (between sarcolemma and basal lamina, i.e. sub-laminarly), polarization (basal and apical side), partner cells (myocytes or other stem cell populations), vasculature (endothelial cells and endocrine factors), ECM (and factors bound to ECM components), neural activity, and autocrine factors. Hence, the activity of satellite cells is probably determined by both direct cell-cell contacts (i.e. integrin α7β1-laminin and M-cadherin-myofiber contacts) and secreted factors from cells. Important examples of regulators are nitric oxide (NO), IGFs, epidermal growth factors (EGFs), hepatocyte growth factors (HGFs), FGFs, transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), endothelial-derived growth factor (EDGF), members of the IL-6 family (i.e. IL-6, LIF), and testosterone (42, 51). Interestingly, 30 % of all sub-laminar nuclei in murine muscle at birth are satellite cell nuclei, whereas only 3-7 % of all sub-laminar nuclei in murine adult skeletal muscle are satellite cells (42, 52). In adult skeletal muscle, other cell types also contribute to myogenesis, including mesoangioblasts (vessel-associated stem cells), the poorly defined muscle side population

stem cells, muscle-derived stem cells, pericytes (microvasculature sub-laminar localization), and $CD133^+$ cells (circulating the blood stream) (51, 53). However, satellite cells are considered to be the most important cell population in muscle regeneration, which upon stimuli are activated and differentiate to fuse with existing fibers.

In adult skeletal muscle tissue, the muscle fiber is regarded as the functional unit (also named muscle cell or myocyte) (49). The muscle fibers are arranged in bundles (called fascicles), and whole muscles are comprised of numerous such bundles (fig. 1.1). In a muscle, connective tissues called epimysium, perimysium and endomysium surround the whole muscle, the fascicles, and the fibers, respectively. The intertwined connective tissue is tightly bound to the skeleton via tendons. Each muscle fiber contains large amounts of structures called myofibrils. They are 1-2 μm thick and stretch longitudinally in the muscle fiber. The myofibrils are again composed of repeating sections of contractile units called sarcomeres. These are comprised mostly of actin and myosin molecules, and are responsible for the dynamic movement. Additional proteins building up the sarcomeres are troponin C, I and T, tropomyosin, titin filaments, desmin and alpha-actinin. The sarcomeres are attached to the sarcolemma (plasma membrane of the muscle fiber) via dystrophin. Thin perpendicular invaginations of sarcolemma form the T-tubuli, which are in close connection with the sarcoplasmic reticulum (sarco-tubuli). Upon contraction, action potentials propagate to the motor end plate where they induce release of acetylcholine in to the synaptic cleft, which binds to the nicotinic acetylcholine receptor on the muscle fiber. A post-synaptic action potential is generated, which flows along the sarcolemma and down the T-tubuli. This causes a conformational change in the T-tubuli-bound dihydroxypyridin receptor, which by interaction with the sarcoplasmic reticulum-bound ryanodine receptor cause calcium ion efflux from the sarcoplasmic reticulum to cytosol. Calcium ions bind to troponin C inducing changes in the troponin tropomyosin complex exposing myosin's binding site on actin, initiating the contraction cycle (49). Contraction of these functional units results in shortening of the muscle, and because tendons of most muscles attach on either side of at least one joint, this causes bodily movement.

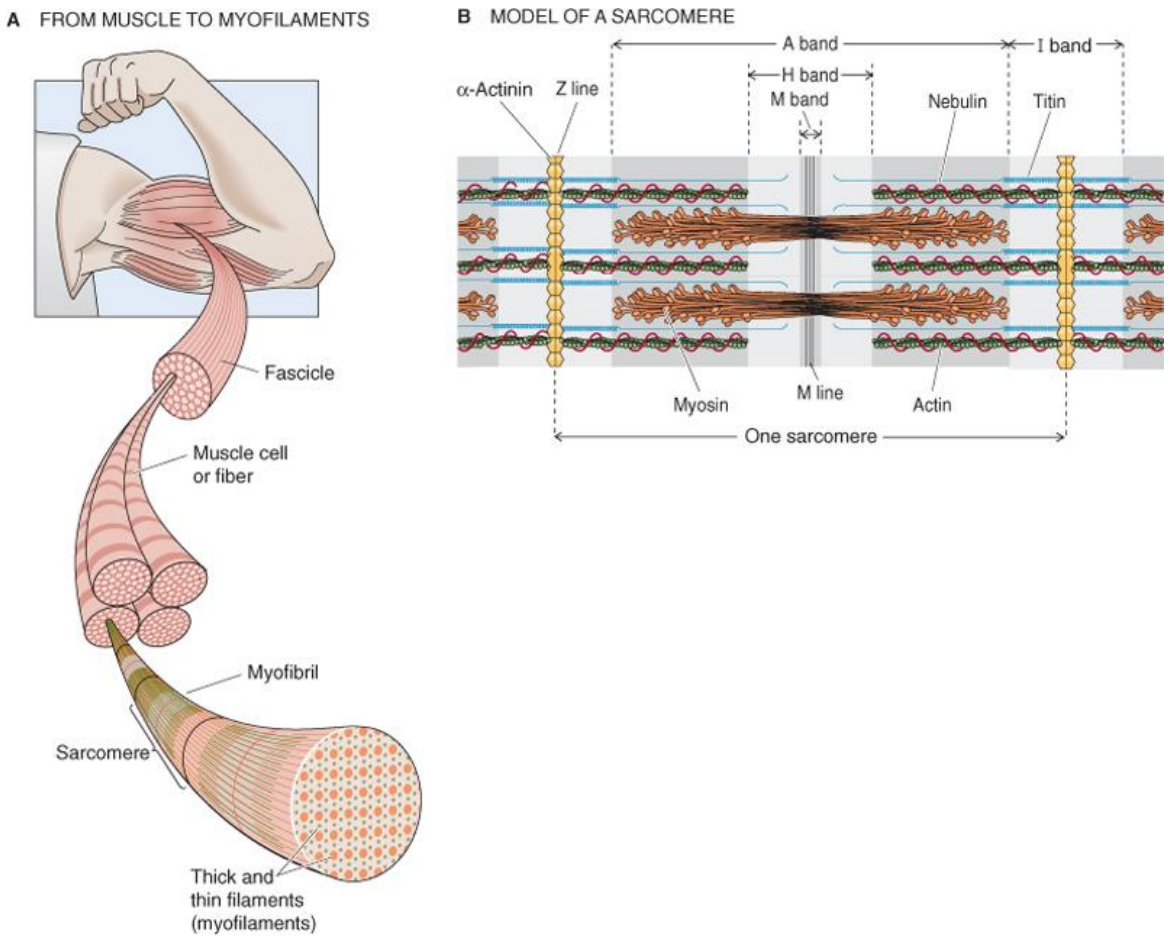


Figure 1.1. Structure of skeletal muscle. Illustration taken with permission from (54).

1.3.1 Muscle cells in culture

A cell culture model can be an important tool in investigating muscle physiology on a molecular level. Cell culture research has been the reason for several major scientific breakthroughs in history, suggesting its potential for future discoveries. Cell culture can be used in basic studies of molecular biology, biochemistry, genomics and proteomics (55). There are many types of cell cultures, but the term *cell culture* is here used to describe disaggregated tissue that forms monolayers at solid-liquid interphase, or as suspension in the culture medium (55). When initiating a cell culture, the *primary culture* is the first in a serie of passages, and from the second passage, the culture is known as a *cell line* (56). Both animal and human cells can be cultured, and for the former several immortal (cancerous) cell lines exists, such as murine C2C12 and SOL8 cells, and rat L6 cells. These cell lines have avoided senescence, which normally occurs after a time in culture (weeks, months), and given rise to

continuous cell lines. Genetic instability is a trademark of continuous cell lines, as the transformation to immortalization is dependent on genetic variation, which can be seen as chromosome abberations. Primary cultures and cell lines can be more difficult to culture, possibly because they are not as well characterized as continuous cell lines, and hence introduce more variation (56).

To establish a primary muscle cell culture satellite cells are isolated from a skeletal muscle biopsy (57). Each muscle biopsy has a relatively low output of satellite cells, which needs to be amplified for efficient utilization. Satellite cells are seeded on plastic or glass plates and the first culture is termed passage 0. The dividing cells are now defined as myoblasts. Before the cells reach confluency, trypzination and subculturing can be performed, yielding passage 1 myoblast cultures. Pre-coating the solid surface with extracellular matrix proteins, such as collagen, fibronectin and/or laminin, is done to help cells attach to the surface, but also to mimic the *in vivo* microenvironment supporting a myogenic nature (58). Furthermore, the first period of muscle cell culture is called *the proliferation period*, in which myoblast numbers increase. During proliferation, growth curves often show an exponential phase, a linear phase, and a saturation phase. The next period is called *the differentiation period*, in which confluent myoblasts fuse to form multinucleated syncytial myotubes. However, cells in culture are often said to be in a state of balance between proliferation and differentiation (56). The position of this balance depends on culture conditions. Some culture conditions will favor cell proliferation, while others will promote differentiation. Dedifferentiation of cells back to a multipotent state, or stem cell state, can be forced by various factors, such as growth factors or cytokines (i.e. by inducing a more proliferative phenotype) (58). Woods et al. have elegantly illustrated proliferation and differentiation as two independent but intertwined periods (59). They show proliferation of myoblasts, quantified by higher content of DNA, and differentiation, quantified by higher content of myogenic markers (such as creatine kinase) (59). After (myogenic) differentiation is achieved, over-differentiation and senescence may result. Myotubes may spontaneously contract, which in combination with improper attachment and other factors, may result in detachment from the well surface and innevitable cell culture death. Both during the proliferation and differentiation periods cell cultures require specific media. There is no definition of what these are, but high and low serum content for proliferation and differentiation, respectively, is standard.

2 Objectives

This thesis has two major objectives:

1. Optimize a protocol for differentiation of human skeletal muscle cells in culture.

    - Establish a method to evaluate morphologically by nuclei count the degree of differentiation in a muscle cell culture.

    - Investigate effects of different concentrations of HS, FBS and UG on morphological degree of differentiation.

    - Investigate effects of dexamethasone and insulin on morphological degree of differentiation.

    - Evaluate the myogenic development of the differentiating cells by dynamically characterizing mRNA and protein expression.

2. Characterize mRNA expression and protein secretion of known and potential myokines during myogenic differentiation.

3 Materials

Chemicals

Azure B (Prod no A4043)	Sigma-Aldrich	St Louis, MO, USA
Eosin Y (Prod no E4382)	Sigma-Aldrich	St Louis, MO, USA
DMSO (Prod no D4540)	Sigma-Aldrich	St Louis, MO, USA
HEPES (Prod no H4034)	Sigma-Aldrich	St Louis, MO, USA
TaqMan® Gene Expression Master Mix (Part No 4369016)	Applied Biosystems	Foster City, CA, USA
RNase AWAY™ Reagent (Cat no 10328011)	Applied Biosystems	Foster City, CA, USA
Methanol (Prod no 1070189026)	Merck	Darmstadt, Germany
Acetone (Prod no 1000142500)	Merck	Darmstadt, Germany
BSA standard (Prod no UP36859A)	Uptima, Interchim	Montluçon, France
XT MOPS Running Buffer 20X (Cat no 161-0788)	Bio-Rad	Hercules, CA, USA
XT Sample Buffer 4X (Cat no 161-0791)	Bio-Rad	Hercules, CA, USA
XT Reducing Agent 20X (Cat no 161-0792)	Bio-Rad	Hercules, CA, USA
Restore Plus Western Blot Stripping Buffer (Cat no 46430)	Thermo Scientific	Waltham, MA, USA
Amersham ECL Plus Western Blotting Detection Reagents (Prod no RPN2132)	GE Healthcare Biosciences	Pittsburgh, PA, USA
100 % Ethanol (Prod no 601557)	Kemetyl	Vestby, Norway
Skim Milk Powder (Prod no 70166)	Sigma-Aldrich	St Louis, MO, USA
Trizma Base (Prod no T6066)	Sigma-Aldrich	St Louis, MO, USA
Glycine (Prod no G7126)	Sigma-Aldrich	St Louis, MO, USA
SDS (Prod no L5750)	Sigma-Aldrich	St Louis, MO, USA
Trizma HCl (Prod no T5941)	Sigma-Aldrich	St Louis, MO, USA
NaOH (Cat no 106498)	Merck	Darmstadt, Germany
NaCl (Cat no 106404)	Merck	Darmstadt, Germany

Cell culture reagents and supplements

PBS (Prod no D8537)	Sigma-Aldrich	St Louis, MO, USA
Collagen Coating Solution (Prod no C3867)	Sigma-Aldrich	St Louis, MO, USA
Ham's F12 Nutrient Mixture w/ $NaHCO_3$, w/o glutamine (Prod no N4888)	Sigma-Aldrich	St Louis, MO, USA
Penicillin/Streptomycin (Prod no P4458)	Sigma-Aldrich	St Louis, MO, USA
FBS (Prod no F7524)	Sigma-Aldrich	St Louis, MO, USA
HS (Prod no P5552)	Sigma-Aldrich	St Louis, MO, USA
Insulin (Cat no CC-4025N), part of Clonetics SkGM BulletKit (Cat no CC-3160)	Lonza Walkersville, Inc.	Walkersville, MD, USA
hEGF (Prod no E9644)	Sigma-Aldrich	St Louis, MO, USA
hbFGF (Prod no F5392)	Sigma-Aldrich	St Louis, MO, USA
Dexamethasone (Prod no D2915)	Sigma-Aldrich	St Louis, MO, USA
GlutaMAX (Cat no 35050038)	Gibco	Renfrew, Paisley, UK
DMEM w/o glucose, w/o glutamine (Prod no MED-252)	Seralab	Haywards Heath, West Sussex, UK

Primers and antibodies

Monoclonal Anti-Myosin (Skeletal, Fast) Mouse Antibody (Cat no M1570)	Sigma-Aldrich	St Louis, MO, USA
Polyclonal Anti-Actin (Skeletal muscle specific, alpha and beta actin) Rabbit Antibody (Cat no A2103)	Sigma-Aldrich	St Louis, MO, USA
Monoclonal Anti-PLAU Mouse Antibody (Prod no MON U-6-02)	Thermo Scientific	Waltham, MA, USA
Monoclonal Anti-alpha Tubulin Mouse Antibody (Cat no T5168)	Sigma-Aldrich	St Louis, MO, USA

Monoclonal goat anti-mouse IgG (H+L)-HRP conjugated antibody (Cat no 1031-05)	Southern Biotech	Birmingham, AL, USA
Monoclonal goat anti-rabbit IgG (H+L)-HRP conjugated antibody (Cat no 4050-05)	Southern Biotech	Birmingham, AL, USA

Equipment

MicroAmp® 96-Well Reaction Plate (Cat no N8010560)	Applied Biosystems	Foster City, CA, USA
MicroAmp® Optical Adhesive Film (Cat no 4311971)	Applied Biosystems	Foster City, CA, USA
Criterion™ Blotter system	Bio-Rad	Hercules, CA, USA
Immobilon-P PVDF membrane (Cat no IPVH00010)	Millipore	Billerica, MA, USA
Pipet tips w/ and wo/ filter	Biotix	San Diego, CA, USA
Serological pipet tips	Becton Dickinson Biosciences	Franklin Lakes, NJ, USA
Culture tissue plate 6 wells (Cat no 087721B)	Becton Dickinson Biosciences	Franklin Lakes, NJ, USA
Culture tissue plate 12 wells (Cat no 0877229)	Becton Dickinson Biosciences	Franklin Lakes, NJ, USA
15 mL Conical Centrifuge Tubes (Cat no 1495949B)	Becton Dickinson Biosciences	Franklin Lakes, NJ, USA
50 mL Conical Centrifuge Tubes (Cat no 1443223)	Becton Dickinson Biosciences	Franklin Lakes, NJ, USA
Cell Scraper (Cat no 087711B)	Becton Dickinson Biosciences	Franklin Lakes, NJ, USA
Micro pipets	Thermo Scientific	Waltham, MA, USA
PCR strips (Cat no AB-0266)	Thermo Scientific	Waltham, MA, USA
Finnpipette Novus Single-Channel Pipetters (1-10 µl: Part no 46200100; 10-100 µl: Part no 46200400)	Thermo Scientific	Waltham, MA, USA

Finnpipette Novus Multichannel Pipetter (Part no 46300000)	Thermo Scientific	Waltham, MA, USA
Stands/racks	Nalgene	Rochester, NY, USA
Pipetboy Acu (serological pipet)	IBS Integrated Biosciences	Lewisberry, PA, USA
Countess™ cell counting chamber slides (Cat no C10283)	Invitrogen	Carlsbad, CA, USA
Lens cleaning paper (Best no 1019)	Assistant	Bavaria, Germany
Small vortex (Lab dancer) (Art no 444-0004)	VWR International LLC	PA, USA
Spectrafuge™ Mini Centrifuge (Prod no C1301)	Labnet International inc.	NJ, USA
Microtubes (Prod no MCT 150-A)	Axygen	CA, USA

Instruments and larger equipment

Forme Steri-Cycle CO2 incubator	Thermo Scientific	Waltham, MA, USA
Heraens Pico 17 Microcentrifuge (Part no 75002410)	Thermo Scientific	Waltham, MA, USA
Heraeus Multifuge 3S Plus (Part no 75004361)	Thermo Scientific	Waltham, MA, USA
ABI Prism 7900HT SDS machine (Serial no 279001365)	Thermo Scientific	Waltham, MA, USA
Fume Hood	Kojair	Vilppula, Finland
Titertek Multiscan® PLUS (Multiscan MS v4.0) (Cat no 1506260)	Labsystems	Helsinki, Finland
Inverted microscope	Leica DMIL	Lysaker, Oslo, Norway
ColorView III Soft Imaging System (CCD Camera)	Olympus Soft Imaging Solutions GmbH	Münster, Germany
Countess™ automated cell counter (Cat no C10227)	Invitrogen	Carlsbad, CA, USA
LS6000 Nitrogen Tank (Serial no 762-004-U1)	Taylor-Wharton® Lab Systems	Mildstedt, Germany
NanoDrop (ND)-1000	Saveen & Werner	Malmö, Sweden

Spectrophotometer		
Vortex Mixer (Prod no L46)	Labinco	DG Breda, The Netherlands
Gel Logic 2200 Imaging System	Carestream Health, Inc	New Haven, CT, USA
Fridge, 4 °C	Zanussi	Økern, Oslo, Norway
Freezer, -20 °C	Electrolux	Økern, Oslo, Norway
Freezer, -86 °C (Forma 900 series)	Thermo Scientific	Waltham, MA, USA

Kits

RNeasy Mini Kit (Cat no 74104)	Qiagen	Hilden, Germany
High Capacity cDNA Reverse Transcription Kit (Prod no 4368814)	Applied Biosystems	Foster City, CA, USA
High sensitivity PEDF ELISA kit (Cat no PED613)	BioProductsMD, LLC	Middletown, MD, USA
Cytotoxicity detection kit (Cat no 11644793001)	Roche Diagnostics	Mannheim, Germany
Bicinchoninic Acid (BCA) Protein Assay Kit (Cat no UP40840A)	Uptima, Interchim	Montluçon, France

Software

SDS software, v2.3	Applied Biosystems	Foster City, CA, USA
RQ Manager, v1.2	Applied Biosystems	Foster City, CA, USA
ImageJ (Fiji), v1.46r (v1.45b) The program and plugin is free for download at the NIH website: http://rsbweb.nih.gov/ij/	National Institutes of Health (NIH)	MD, USA
Cell counter	National Institutes of Health	MD, USA
NanoDrop (ND)-1000 software, v3.8.1	Thermo Scientific	Waltham, MA, USA
Cell^A	Olympus Soft Imaging Solutions GmbH	Münster, Germany
Carestream Molecular Imaging	Carestream Health, Inc	New Haven, CT, USA

Software

SPSS Statistics v19.0	IBM	Armonk, NY, USA

4 Methods

4.1 Growth and differentiation of human skeletal muscles in culture

Myoblast stocks were a kind gift from professor Arild Rustan at Department of Pharmaceutical Biosciences (at the School of Pharmacy, University of Oslo). I have grown myoblasts at 2-4000 cells/cm^2 in culture wells or T75 flasks with 1:1 DMEM (added GlutaMax and penicillin/streptomycin, without glucose) and Ham's F-12 (added GlutaMax and penicillin/streptomycin, contains 10,0 mmol/L glucose). In addition to DMEM/F12, the proliferation medium contained 20 % FBS, 1 µg/mL insulin, 10 ng/mL hEGF, 2 ng/mL hbFGF, and 0.4 µg/mL dexamethasone. Every 2^{nd}- 3^{rd} day during proliferation, the media were renewed. When cells were 80-90 % confluent, around day 3-7 depending on cell growth, differentiation was initiated by reducing the serum concentration. For most experiments, differentiation media were renewed daily to every 3^{rd} day. All cell cultures were incubated at 37°C and 5 % CO_2. All experiments were performed using human muscle cells from two donors: EO2 (male, aged 46 years, BMI 40.0 kg/m^2, blood pressure 142/85 mmHg, fasting blood glucose and lipids within normal range) and LD30 (male, aged 36 years, BMI 26.2 kg/m^2, fasting blood glucose, lipids and blood pressure within normal range). All experiments were performed at least three times.

4.2 Romanowsky staining

To investigate degree of differentiation, I used morphological (cytological) evaluation. The cells were proliferated until 80-90 % confluency and differentiation was initiated by reducing serum concentration in the medium. We differentiated the cultures for 7 days. The design of each experiment is explained in detail in the results section. The main outline is visualized in supplementary figure S1*A*.

On day 7 of differentiation, cultures were fixed and stained (60). Cultures were washed twice in PBS, fixed in methanol for 5 min, and air-dried. The Romanowsky solution was prepared by dissolving 0.3 g azure B in 40 mL of DMSO, and 0.1 g of Eosin Y in 60 mL of methanol at 37 °C. The dyes were dissolved, and slowly the azure B-DMSO and the Eosin Y-methanol

were mixed to produce the stock solution. 50 mL/L DMSO in 10 mmol/L HEPES buffer in distilled water were mixed with stock solution at a ratio of 1:15 to produce the working solution (pH 6.8). Fixed cultures were rinsed briefly in distilled water, air-dried, stained in Romanowsky working solution for 30 min, rinsed in distilled water, and air-dried. The Romanowsky stain promotes a broad range of colours, from purple nuclei, blue RNA rich cytoplasm, and light blue nucleoli.

4.3 Determining the fusion index

Myotubes were defined as cells with 3 or more nuclei. Degree of differentiation in a culture was defined as the ratio between total nuclei in myotubes and total nuclei in the culture (fusion index). To this, 6 representative areas in each well were selected, each covering 0.57 mm^2, a total of 3.44 mm^2 (fig. 5.1). A standardized protocol was used to select areas. Macroscopically, 6 marks were made on a straight line from left to right in each well, 5 mm apart (red marks). These marks were localised, and the areas just above the marks (green-turquoise area) were photographed (blue area) using a Leica inverted microscope (20X lens) and CellA software, and uploaded in ImageJ for counting of nuclei. The fusion index was used to evaluate degree of differentiation between different media. Marks were made directly on the picture on the computer screen. When in doubt if a nucleus was part of a myotube or myoblast, the nucleus in question was localized and thoroughly observed using the 20X and 40X lenses. To maintain a cautious and conservative approach, if still in doubt, the nucleus was defined as part of a myoblast.

4.4 Electrical pulse stimulation (EPS)

EPS is a potentially good *in vitro* model of exercise (61, 62). Our research group has invested heavily in developing and validating an EPS protocol for human primary cell cultures. There has been several difficulties with this process, including toxicity of the EPS electrodes. Hence, I have not been able to use EPS in my thesis. However, much of the work performed, including the use of 6 well plates and simultaneous isolation of RNA and protein, have been performed with the intention of performing subsequent EPS experiments.

4.5 RNA and protein analysis

4.5.1 Isolation of RNA and protein

Only 6 samples can be harvested per EPS experiment. For maximal efficiency, I purified mRNA and protein simultaneously from each sample and eluated separate fractions. I used the RNeasy Mini Kit, which selectively isolates RNA molecules longer than 200 nucleotides, according to manufacturers instructions (63). Cells were washed twice with PBS, and 350 µL Buffer RLT, containing guanidine thiocyanate, which inhibits nucleases and proteases, was added to lyse cells and stabilize RNA. After homogenization, 350 µL 70 % ethanol was added to promote binding to the RNeasy membrane. The mixture was transferred to the RNeasy Mini spin column and centrifuged 31 seconds at 13 000 rpm. The eluate fractions were transferred to a protein low bind Eppendorf tube. Three successive washing steps, one with 700 µL RW1 and number two and three with 500 µL RPE, were conducted, and fractions were collected in the first two. Fractions from identical samples were pooled as the washing steps were conducted. Protein tubes were immediately frozen at -20 °C to precipitate salts and preserve proteins. 40 µL RNase free (destilled) water was applied directly to the spin column membrane, and RNA was eluated by centrifuging 1 min at 13 000 rpm and stored at -86 °C (63).

4.5.2 Quantification of mRNA by real-time RT-PCR

RNA concentration and quality were determined with the NanoDrop-1000 Spectrophotometer and NanoDrop software. 550 ng RNA was reversely transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit and a Gene Amp PCR 9700 thermal cycler. Further, 12,5 ng cDNA was used in the RT-PCR reaction in a 96 well plate using a 7900HT Fast instrument and the SDS 2.3 software, according to manufacturers protocols (41, 64, 65). Predeveloped primers and probe sets were used to analyze mRNA levels of the following target genes (official gene symbol in parentheses): Paired box 7 (*PAX7*), Hs00242962_m1; myogenin (*MYOG*), Hs00231167_m1; myosin heavy chain 1 (*MYH1*), Hs00428600; alpha actin 1 (*ACTA1*), Hs00559403_m1; peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC1α (*PPARGC1A*), Hs00173304_m1; solute carrier family 2 member 4 (*SLC2A4*, *GLUT4*), Hs00168966_m1; interleukin-6 (*IL6*), Hs00985639_m1; interleukin-8 (*IL8*), Hs00174103_m1; pigment epithelium-derived factor (*SERPINF1*, *PEDF*),

Hs01106934_m1; plasminogen activator urokinase (*PLAU*), Hs01547054_m1. All genes were normalized to the endogenous control human large ribosomal protein P0 (RPLP0), Hs99999902_m1, to control for RNA loading and reverse transcription efficiency. Ct values were set to 40 for mRNA level 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 (66). All *in vitro* mRNA data are presented as median (min-max) fold change in relation to a set time point (41).

4.5.3 Protein isolation and western blotting

Conditioned medium from cultured myocytes (myoblasts and myotubes) was collected for protein analysis. Briefly, the medium was carefully mixed, 800 µL was transferred to an Eppendorf tube and centrifuged for 10 min at 13 000 rpm at 4 °C to remove cell debris. The supernatant was transferred to a new Eppendorf tube and stored at -20 °C.

Intracellular proteins and RNA were isolated simultaneously as decribed above. Three successive fractions from the RNeasy column were collected and immediately precipitated and stored at -20 °C. Before SDS-PAGE separation, protein samples were prepared according to published reports (67, 68), with slight modifications. Shortly, guanidine thiocyanate salts were removed by three successive washing steps, first in 500 µL acetone, then 500 µL ethanol, then 500 µL acetone, interspersed by 15 min cooling at -20 °C and 10 min centrifugations at 13 000 rpm and 4 °C. Supernatants were carefully removed by pipetting. The dry protein pellet was dissolved in 55 µL buffer containing 200 mM Tris and 4 % Sodium dodecyl sulfate (SDS) (pH 8). To ensure complete dissolution, samples were heated 5 min at 95 °C on heat block. Protein concentration was measured using the bicinchoninic acid (BCA) Protein Assay Kit, following the manufacturer's instructions (69, 70). For extracellular proteins, the above-mentioned procedure was not performed. Equal amounts of total protein (20 µg) or 5 µl conditioned medium were mixed with 20X XT Reducing Agent and 4X XT Sample Buffer, heated for 5 min at 95 °C, and separated by SDS-PAGE using Criteron XT Bis-tris 10 % gel and XT MOPS running buffer. No positive control was added. A Dual colour ladder was loaded as a molecular weight standard. After separation, proteins were electrotransferred to a methanol-activated 0,45 µm Immobilon-P polyvinylidene fluoride (PVDF) membrane. The 10X transfer buffer consisted of 30.3 g Tris base, 144 g Glycine and 1 L distilled and deionized water. The final working buffer was diluted 10X and methanol

was added to 10 % of the final volume. Tris-buffered saline (TBS) contained 24.23 g Trizma HCl, 80.06 g NaCl and 1 L distilled deionized water (pH 7.6), and was 10 times diluted to TBS-T by adding distilled water and Tween-20 (1-2 ml). Membranes were incubated 1 hour at room temperature in blocking buffer (TBS-T with 5 % skimmed milk powder), washed 6 times in TBS-T (totally 30 min), and incubated either 1 hour at room temperature or overnight at 4 °C with primary murine or rabbit anti-human IgG antibody diluted in blocking buffer. Membranes were washed 6 times in TBS-T, incubated 1 hour at room temperature with secondary goat anti-mouse or anti-rabbit IgG (H+L)-horseradish peroxidase (HRP) conjugated antibody diluted in TBS-T, and finally washed 6 times. The non-radioactive method of ECL Plus enhanced chemiluminescence and Gel Logic 2200 Imaging System were used to visualize the proteins.

4.5.4 Protein quantification

The concentration of PEDF was determined in conditioned medium using a commercially available ELISA kit according to the manufacturer's protocol (71), described here. Samples were not pre-treated with urea. Samples of conditioned medium were diluted 1000 times and pipetted on 96 well plates in duplicate, along with the predetermined standard curve (100 µL per well, in duplicate). Plate was incubated 1 hour at 37 °C (w/ plate sealer), followed by 5 consecutive wash steps in a pre-programmed ELISA wash machine, using provided Plate Wash Buffer. 100 µL PEDF Detector Antibody Working Solution was added, and plate was incubated 1 hour at 37 °C (w/ plate sealer), followed by 5 similar consecutive wash steps. 100 µL Streptavidin-Peroxidase Working Solution was added, plate was incubated 30 min at 37 °C (w/ plate sealer), followed by 5 similar consecutive wash steps. Wells were completely dried after each wash. 100 µL TMB Substrate was added, and plate was incubated 20 min at room temperature (w/o plate sealer). 100 µL Stop Solution was added, and optical density measured spectrophotometrically at 450 nm. Concentrations were determined using Microsoft Office Excel software. A pre-run was performed to titrate concentrations to fit within the predetermined standard curve. Selected samples of conditioned medium (lowest and highest expression according to mRNA data, and start and end of differentiation period) were diluted 10, 100 and 1000 times, and 100 µL from each original and diluted sample pipetted on 96 well plate in singlicate, along with the standard curve (100 µL in duplicates). The intra-assay coefficients of variation (CV) were between 0.4 and 10.2 %, except for one sample with 21.0 % (71).

4.6 LDH measurements

To determine integrity of the cell cultures, release of lactate dehydrogenase (LDH) was measured using an LDH kit. Medium was centrifuged 10 min at 13000 rpm and 4 °C to remove cell debris, and the supernatant was stored at 4 °C a maximum of 4 days. From each experimental medium sample collected, two reference samples were also collected (high and background controls). Cells were lysed using Triton X-100, promoting 100 % cell death, yielding high controls. Background controls were obtained from containers of fresh medium (serum free medium: *SFM*; medium containing 1.0 and 2.0 % HS: *1.0 % HS, 2.0 % HS*; or proliferation medium: *proliferation medium*). All samples were pipetted in duplicate on 96 well plates (25 µL), and water was added to all wells (25 µL). Reagent A and B was mixed (per 100 wells on plate: 250 µL of reagent A and 11.25 mL of reagent B) and added to all wells (100 µL per well). Plate was covered with aluminium foil, incubated 30 min at room temperature and optical density was measured spectrophotometrically at 492 nm. If the OD was above 1.6-1.8, the actual samples were diluted 10X, and measurements were repeated. Results from each sample are presented as percentages of these references, according to this equation:

$$Cytotoxicity\ (\%) = \frac{experimental\ value - background}{high\ control - background}\ x\ 100$$

4.7 mRNA expression in skeletal muscle biopsies

As described in (41), our research group has conducted a strength training intervention, briefly rendered here. Note: I did not do any of the work related to this study, I only present the results from qRT-PCR measuring plasminogen activator urokinase (PLAU) mRNA expression before and after the strength training intervention (see discussion). Volunteers participated in a strength-training program three times weekly for 11 weeks, and muscle biopsies were taken before and after the intervention. Each workout included 1–3 sets of leg press, leg extension, leg curl, seated chest press, seated rowing, latissimus dorsi pull-down, biceps curl, and shoulder press. Elaborating information of methods and procedures can be found in (41).

4.8 Statistical analysis

Effect measures for fusion indices are reported as means ± SEM, and for LDH measurements, RNA data and ELISA measurements as means ± SD. Exceptions to these are specified in the text. Statistical differences were tested with IBM SPSS Statistics 19.0, and $P < 0.05$ were considered significant. Differences between incubation media evaluated morphologically were tested with one-way ANOVA. Correlations are calculated based on a linear regression model. Area under the curve (AUC) is calculated in Microsoft Excel based on the Trapezoid method: Area = (average height)*Width. Differences between incubation media at different time point during differentiation (RNA data and ELISA), AUC, and fold changes in mRNA expression, were tested with Kruskal-Wallis test, and significant differences were explored *post hoc* with pairwise Mann-Whitney tests and corresponding manual Bonferroni correction. It should be noted that because of low n (n=3-7) and mostly inconclusive Kolmogorov-Smirnov tests, all significant tests were performed with both parametric and non-parametric alternatives.

5 Results

5.1 Establishing a method to evaluate the degree of differentiation in muscle cell cultures

I wanted to examine the degree of differentiation by counting nuclei inside myotubes and myoblasts because theoretically, the more nuclei found inside multinucleated myotubes, the better differentiation of the culture. However, there existed no method of counting nuclei in our lab. Hence, I established a protocol for staining and photographing muscle cells in culture, followed by software-based cell-specific counting of nuclei. The final protocol is described below.

PROTOCOL

1. Proliferate and differentiate muscle cell cultures according to defined protocols. I applied the protocol presented in fig. S1*A* to a 6 well format.

2. Wash cultures twice in PBS, fix in methanol for 5 min, and air-dry in room temperature. Make sure cultures are completely dry before next step. Cultures can also be stored in room temperature for later staining.

3. Prepare the Romanowsky solution as described in methods. Briefly rinse fixed cultures in distilled water and air dry. Stain with Romanowsky solution for 30 min, rinse in distilled water, and air dry. Cultures can be stored for several months.

4. Underneath the culture plate, mark 6 spots on a straight line with an inc pen, 5 mm apart, in each culture well (red marks in fig. 5.1). Using an inverted microscope with a camera that is connected to a computer, locate the areas directly above the marks (green-turquoise area). 20X lenses provide 0.57 mm^2 per visual field (per image).

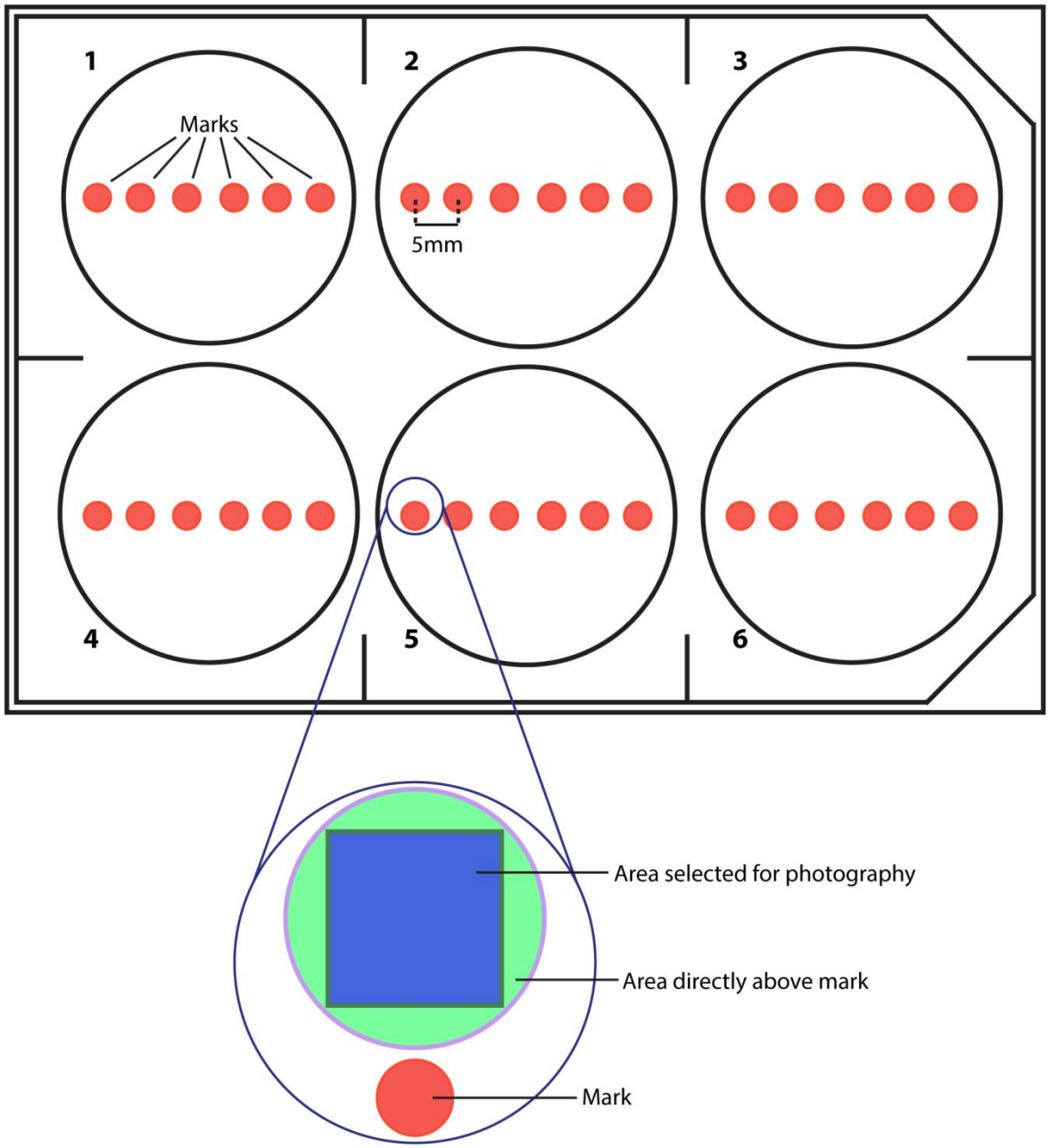


Figure 5.1. Outline of how areas in 6 well plates were selected for evaluation. Cells were seeded on 6 well plates, proliferated until near confluency, and differentiated for 7 days in serum reduced medium. The cell cultures were fixed and stained with Romanowsky stain. The area marked blue was evaluated with respect to nuclei content.

5. Camera settings: Adjust for full light transparency and no phase contrast. Open Cell^A software. Adjust color settings to the following: red = 2.25, green = 1.69, blue = 5.92, gamma = 0.99, saturation = 0.07. Choose manual exposure (20 ms), and select *Video* to turn camera on.

6. Adjust focus on the microscope (to maximum sharpness on the computer screen) and photograph (2576x1932 pixles) the selected areas (blue area).

7. Upload images in ImageJ, and open the CellCounter plugin. Click *Initialize* to start counting, and maximize the picture for best nucleus recognition. Tick all nuclei that meet the pre-defined criteria as myotube or myoblast nuclei. Mark the box *Type 1* or *Type 2* in advance to distinguish and quantify the two different nuclei types (fig. 5.2).

8. Definition of myotube in our cultures: Cells with 3 or more nuclei. All other nuclei were considered non-myotube nuclei. When in doubt if a nucleus is part of a myotube, the nucleus in question can be localized and thoroughly observed using the 20X and 40X lenses. If still in doubt, the nucleus should be defined non-myotube.

9. Calculate fusion index (degree of differentiation) according to this equation:

$$Fusion\ index = \frac{Myotube\ nuclei\ count}{Total\ nuclei\ count}$$

It is of utmost importance to follow the universal lab safety rules when working with stained cultures and software equipment. The Eosin Y and Azure B dyes are toxic (irritates mucosal surfaces), and gloves should therefore be worn when handling stained cultures. Always remove gloves before handling door handles, mobile phones, computers or other equipment, and wash hands before intake of food.

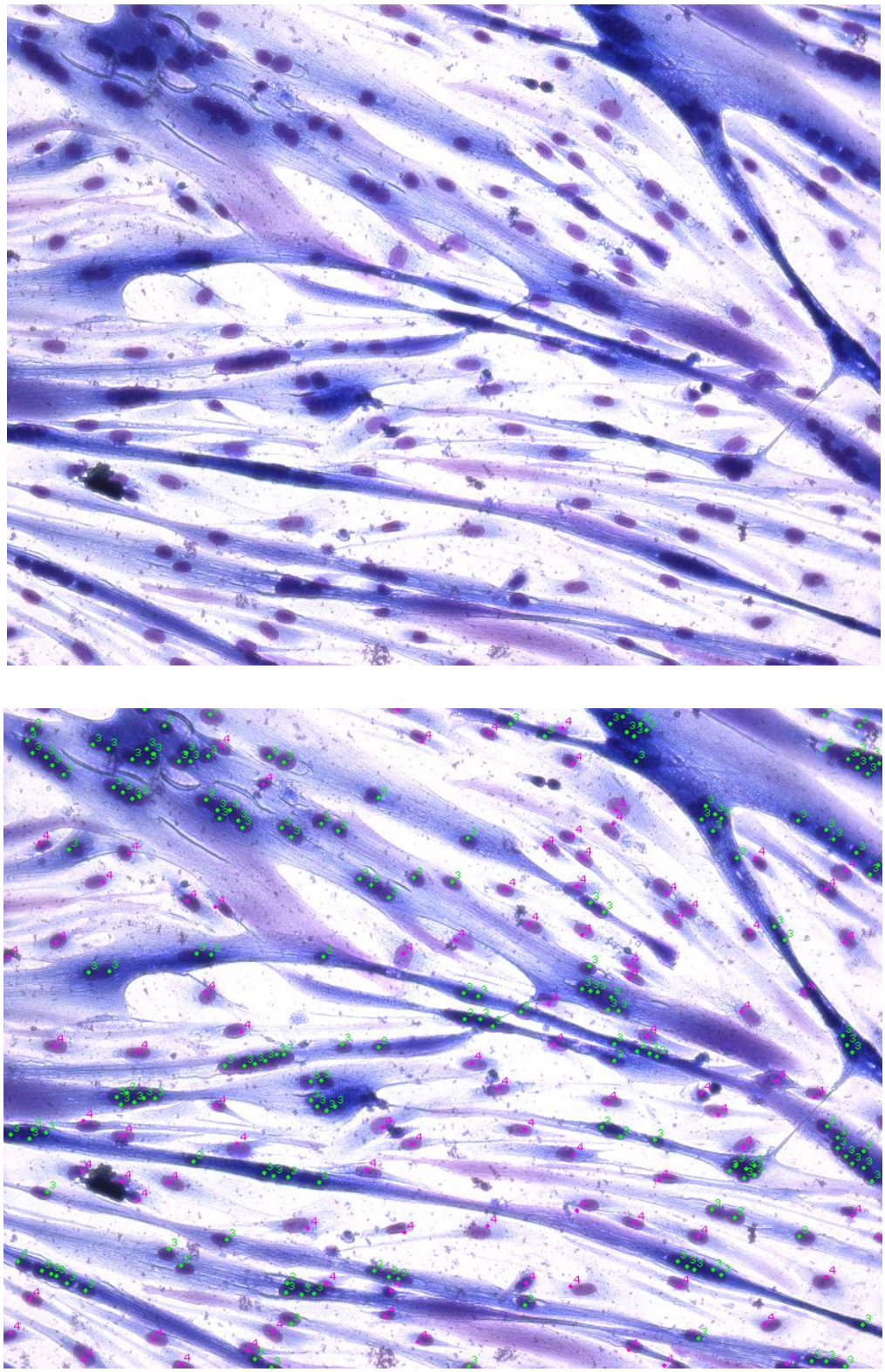


Figure 5.2. Computer screenshots. Pictures are computer screenshots from the counting process, presenting nuclei in myotubes (green marks) and myoblasts (pink marks). The Cell Counter plugin for ImageJ was used. The program and plugin is free for download at the NIH website: http://rsbweb.nih.gov/ij/.

5.2 Optimizing differentiation of myoblasts into myotubes in culture

5.2.1 Effect of serum type on differentiation

Muscle cell culture differentiation is initiated by reducing serum concentration in the medium. I wanted to investigate if different types of serum influenced differentiation with various degree. Both HS and FBS, and also the serum substitue Ultroser G (UG), have been used by others (72, 73). Hence, I differentiated cultures in different concentrations of HS, FBS and UG (fig. 5.3*A*). I found no statistically significant difference between incubation groups (P = 0.313), although there was a clear tendency for lower differentiation with FBS. Highest fusion index was seen with 1.0 % HS and 0.2 % UG, both with means of 0.35 (SEM 0.06 and 0.08, respectively). We chose to exclude FBS and UG from further experiments, and the reason for excluding the latter is briefly this. UG has unknown composition but, according to manufacturer, contains rather large (non-physiological) quantities of insulin. In addition, UG is expensive and, at best, provide similar degree of differentiation as HS, at least according to these experiments. On the other hand, UG would possibly provide more reproducable results, as batches of HS may introduce variability. In summary, I conclude that 0.5 and 1.0 % HS potentially provide the best differentiation after 7 days, but clearly more experiments are necessary to prove this.

5.2.2 Effect of concentration of HS on differentiation

Next, I wanted to further elucidate which concentration of HS that provided the best differentiation. This was performed using media with concentrations of HS ranging from 0.2 % till 2.0 % (fig. 5.3*B*). Also, medium starting at a low concentration (0.2 % HS) and switching to higher (1.0 % HS), and opposite, was investigated. Again I did not find any statistically significant differences between any of the incubation media (P = 0.766), although a concentration of 1.0 % HS provided the highest fusion index with mean of 0.44 (SEM 0.08). I conclude that a medium containing 1.0 % HS is most promising, and warrants further investigations.

5.2.3 Effect of dexamethasone on differentiation

Incubation with low-concentration of dexamethasone (0.4 µg/L) is described to promote differentiation of myoblasts via increased expression of myogenic mRNAs and proteins (74). Additionally, in view of the well-known muscle atrophy-inducing effects of glucocorticoids (75), I wanted to examine the addition of dexamethasone in the differentiation medium. I tested various concentrations (0-1000 µg/L) of dexamethasone in 1.0 % HS (fig. 5.3*C*). I observed a clear reduced differentiation with increasing concentration of dexamethasone (*P* for linear regression trend < 0.001). The maximum and minimum fusion index were 0.55 (SEM 0.03) and 0.33 (SEM 0.05) for 1.6 and 1000 µg/L, respectively (*P* = 0.027). The linear regression coefficient was 0.696 (r^2 = 0.484), and the change in fusion index was -0.044 for each increment in dexamethasone (95 % CI = -0.064, -0.023). My findings suggest that dexamethasone may inhibit differentiation of myoblasts at higher concentrations. This is especially evident between 1.6 and 8 µg/L. Hence, I conclude that dexamethasone can be added to the differentiation medium at low-dose, but that high doses can have a detrimental effect on differentiation.

5.2.4 Effect of insulin on differentiation

Dexamethasone is shown to counteract the molecular effects of insulin signaling (75). Also, IGFs are well-known mediators of both proliferation and differentiation of muscle cells (76). Hence, I therefore investigated the effect of adding insulin to the differentiation medium, and I hypothesized that this would increase the fusion index. Moreover, I investigated the effect of insulin with or without serum in the medium. Serum free medium would potentially increase the fusion index, aided by a more rapid cell cycle exit. Again, I did not find any statistically significant differences between incubation media (*P* = 0.186), but a tendency of insulin to lower the fusion index (fig. 5.3*D*). The maximum and minimum mean value was 0.67 (SEM 0.03) and 0.53 (SEM 0.07) for 1.0 % HS and SFM with 1000 µg/L insulin, respectively. I conclude that increasing concentration of insulin in SFM promotes a tendency towards lower differentiation, and that high-concentration insulin should be omitted from our differentiation medium. However, elucidation of insulin effects at lower dose can be focus for future research.

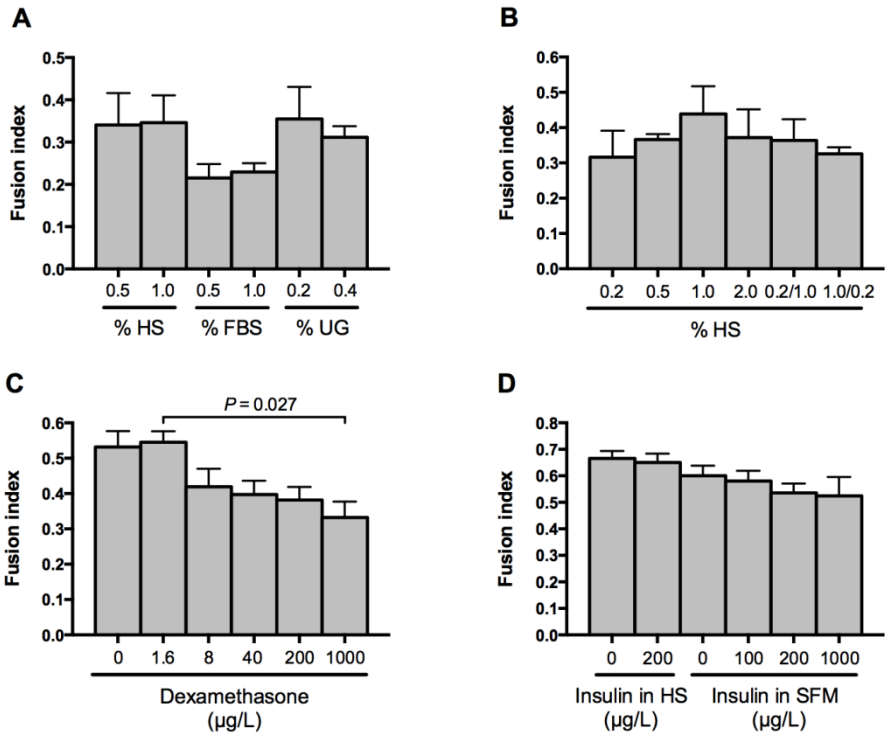


Figure 5.3. Effect of type and concentration of serum and supplements on myoblast fusion. *A,* confluent myoblast cultures were differentiated for 7 days in various concentrations of HS, FBS and UG (n=4); *B,* in different concentrations of HS (n=3); *C,* in 1.0 % HS and different concentrations of dexamethasone (n=4); *D,* in 1.0 % HS, SFM and different concentrations of insulin (n=3). All experiments were performed using two donors (donors were not pooled). For all experiments, proliferation, fixation, Romanowsky staining and counting of nuclei were performed as described in Methods. Error bars are SEM.

5.2.5 Serum concentration in the medium influence gene transcription

In view of the initial findings, we decided to further characterize the differentiation of human skeletal muscle cells in HS. Published reports use higher concentrations of serum, most often 2.0 % HS, when differentiating muscle cells, whereas I find that 1.0 % presumably promotes better differentiation (59). Hence, I investigated the differentiation transcriptome kinetics of 1.0 and 2.0 % HS. Importantly, I chose to include two experimental controls, SFM and proliferation medium (20 % FBS), as these were considered extremes. SFM had been shown to promote differentiation previously, but I hypothesized that the proliferation medium would show low levels of differentiation. Important muscle specific genes were investigated using RT-qPCR. Genes (primers) were selected on basis of a role in myogenesis, *PAX7* and *MYOG*, importance in muscle contraction, *MYH1* and *ACTA1*, or importance in the muscle metabolic

phenotype, *PPARGC1A* (PPARγ coactivator 1α, PGC1α) and *SLC2A4* (Glucose transporter 4, GLUT4).

PAX7 mRNA levels decreased throughout myogenesis for all incubation groups, as expected (fig. 5.4*A*). Serum-reduced media promoted gradual reductions in expression from first day of differentiation, with a maximum reduction after 7 days. The median (min-max) fold changes for 1.0 % HS and 2.0 % HS on day 7 of differentiation were 0.20 (0.20-0.29) and 0.20 (0.09-0.21), respectively. In addition, there were statistically significant differences between the groups at almost all time points, but *post hoc* tests did not reveal specifically which groups that differed. *MYOG* mRNA levels increased in all serum reduced incubation groups (fig. 5.4*B*), already from day 1. SFM and 1.0 % HS showed highest mRNA expression of myogenin. The peak of the mRNA expression level was on day 5 for SFM and 1.0 % HS, and on day 4 for 2.0 % HS. The highest median (min-max) fold change level for 1.0 % HS and 2.0 % HS were 67 (19-3934) and 67 (32-1569) related to day 0 of differentiation, respectively.

MYH1 mRNA levels increased in all incubation media (fig. 5.4*C*). *MYH1* expression was similar between the different conditions, except for 2.0 % HS. Elaborating figures for comparison between incubation groups with regard to *MYH1* expression are presented in supplemental materials (fig. S2*A*-*D*). The peak of the mRNA expression level was on day 7 for SFM and 2.0 % HS, and on day 3 for 1.0 % HS. The median (min-max) fold change level for 1.0 % HS and 2.0 % HS were 190 (41-548) and 52 (35-6503) related to day 0 of differentiation, respectively. *ACTA1* mRNA expression levels showed increases in all incubation groups, especially in 1.0 % HS (fig. 5.4*D*). Elaborating figures for comparison of *ACTA1* mRNA expression levels between donors are presented in supplemental materials (fig. S3*A*-*D*). The peak of the mRNA expression level was on day 6 for SFM and 1.0 % HS, and on day 7 for 2.0 %. The median (min-max) fold change level for 1.0 % HS and 2.0 % HS were 1273 (218-48449) and 138 (91-23038) related to day 0 of differentiation, respectively.

PPARGC1A mRNA expression level increased almost identically for all incubation groups (fig. 5.4*E*). The peak mRNA expression level was on day 5 for SFM and on day 4 for 1.0 and 2.0 % HS. The median (min-max) fold change level for 1.0 % HS and 2.0 % HS were 20 (11-172) and 18 (10-255) related to day 0 of differentiation, respectively. *SLC2A4* (GLUT4) exhibited a drastic increase during differentiation, especially for SFM, which had a peak expression level on day 5 (fig. 5.4*F*). 1.0 % and 2.0 % HS had their peaks on day 4. The median (min-max) fold change level for SFM, 1.0 % HS and 2.0 % HS were 322 (110-515),

85 (47-108) and 40 (18-162) related to day 0 of differentiation, respectively. There were statistically significant differences between the groups at almost all time points, but *post hoc* tests did not reveal specifically which groups that differed.

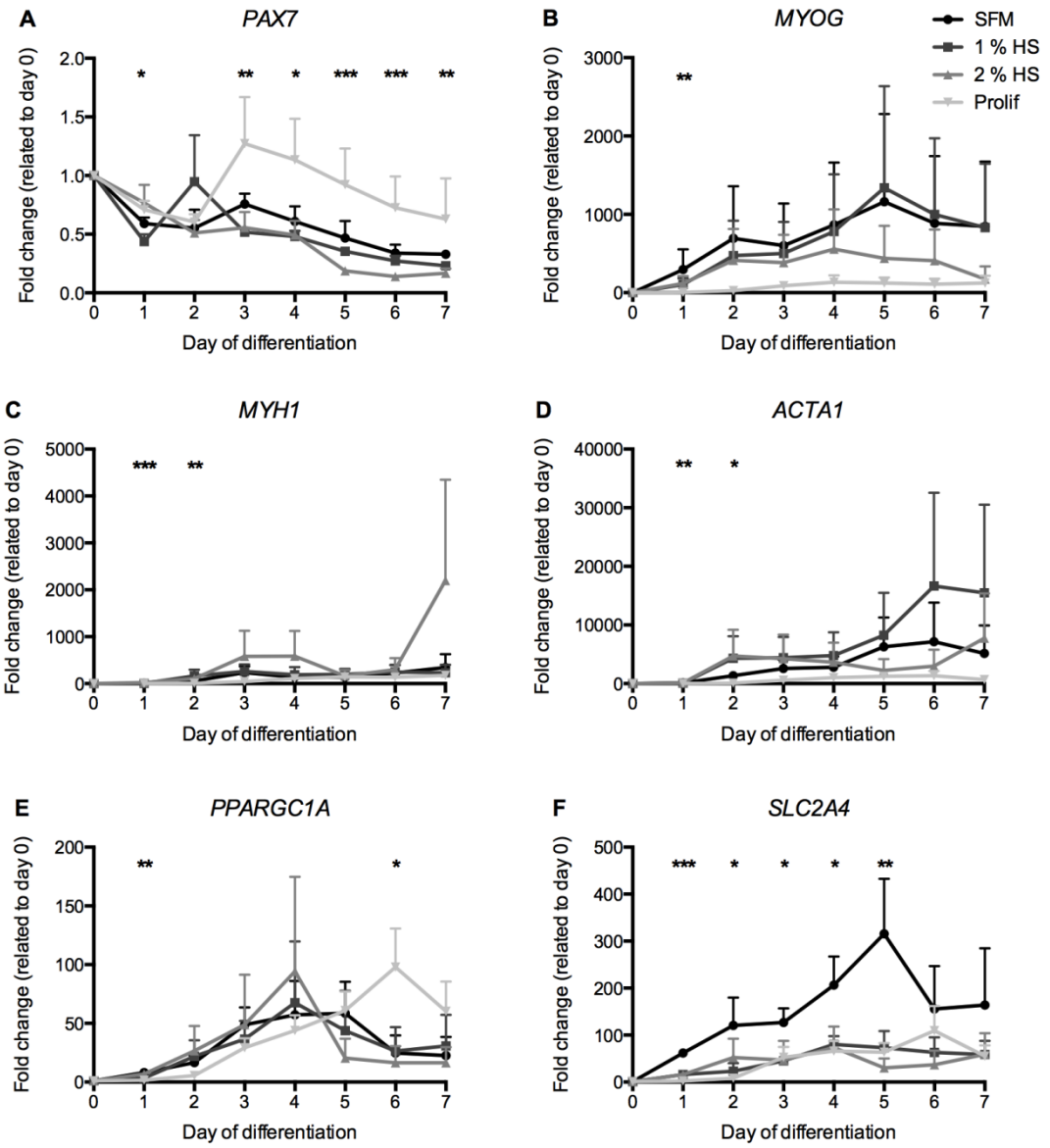


Figure 5.4. Changes in mRNA expression during myogenesis. Confluent cultures were differentiated for 7 days in either serum free medium or medium containing either 1.0 % HS, 2.0 % HS or 20.0 % FBS + insulin, EGF, FGF and dexamethasone (see Methods). The experiments were performed three times, once with the LD30 cells and twice with EO2 cells. All experiments were performed using cells from passage 5. Error bars represent SD. Statistical testing have been performed by Kruskal-Wallis test between all incubation groups at each time point during differentiation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

To unreveal any cumulative differences in mRNA expression that may occur, I calculated AUC for all expressions throughout differentiation and compared between incubation groups. Only AUC for *PAX7* and *SLC2A4* mRNA expression was statistically significant between groups (P = 0.001 and P = 0.009, respectively). Pairwise comparisons did not reveal any differences between 1.0 and 2.0 % HS (P = 0.400 and P = 0.700 for *PAX7* and *GLUT4*, respectively).

Taken together, these data indicate that all serum-reduced media provide mRNA changes that contribute to the fusion and differentiation of myoblasts to myotubes, whilst promoting expression of muscle-specific genes. It is impossible to draw conclusions regarding differences between 1.0 % HS and 2.0 % HS, as this would require increased strength, either by repeating the experiments more times, including more donor cell types, or preferably both.

5.2.6 Protein expression of myosin and muscle-specific actin

According to the fusion index and mRNA data above, the muscle cell cultures differentiated well. However, as function is related to proteins, not mRNA, this needed to be verified with appropriate protein measurements. Also, establishing terminal differentiation of the myotube cultures is of importance, especially for timing the subsequent EPS experiments.

Nuclear proteins (PAX, PGC1α and MRFs) were excluded from protein measurements, as they possibly would require fractioning of cellular contents, which is beyond the scope of this Master Thesis. For the same reason, GLUT4 was not investigated, as membrane-bound proteins possibly require specialized isolation methods. Hence, I decided to perform western blotting of the cytosolic proteins myosin and muscle-specific actin. α-Tubulin was chosen as reference, as it has shown applicable in similar experiments previously (77). Myosin exhibited increased protein level from day 1, but possibly the highest protein level on day 7 of myogenesis (fig. 5.5). The interpretation of myosin protein data must be done with caution, because of the presence of backgroud even on day 0. Muscle-specific actin shows increased protein level from day 2 of myogenesis, and increased steadily till day 7. Aside from day 0 of myogenesis, α-tubulin shows insignificant changes, suggesting that it is a good loading control. I conclude that protein levels of myosin and muscle-specific actin increase in parallel with mRNA, and that terminal differentiation is not before day 7 of differentiation.

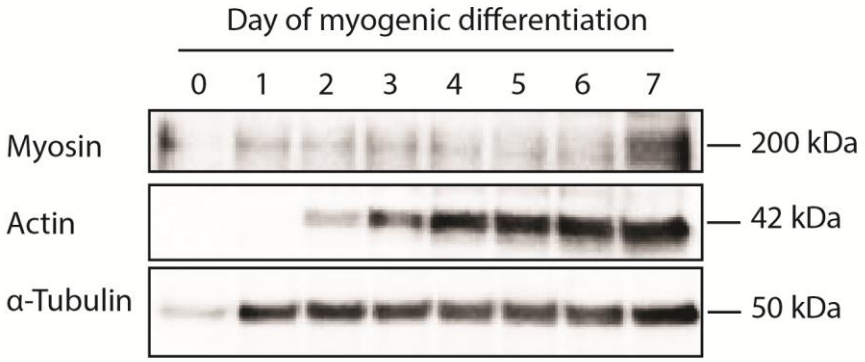


Figure 5.5. Changes in protein level of intracellular structural proteins during differentiation. Confluent cultures were differentiated for 7 days in medium containing 2.0 % HS. Equal amounts (20 µg) of protein were separated by SDS-PAGE, transferred to an activated PVDF membrane, and visualized. Anti-myosin antibodies were incubated in block buffer overnight at 4 °C, while anti-actin and anti-tubulin antibodies were incubated in block buffer at room temperature for 1 hour. Similar results was observed in a second western blot (separate donor and experiment).

5.3 Myokine expression kinetics during myogenesis

5.3.1 Myokine mRNA expression

Myokines may be important in paracrine communication between precursor myoblasts and differentiated myotubes and myofibers, and in autocrine communication. This communication may be especially important when there are great changes in the myoblast-myotube populations, such as during the differentiation process. To evaluate the transcription of known and potential myokines during differentiation in 1.0 % HS, RT-qPCR was performed. Genes (primers) were selected on basis of work published by other groups, such as IL6, IL8 and Fibronectin type III domain containing 5 (FNDC5), or work done by our research group, such as Angiopoietin-like protein 7 (ANGPTL7), PEDF and PLAU.

IL6 mRNA expression shows a time-dependent increase from day 4, and reaches max expression level on day 7 of myogenic differentiation (fig. 5.6*A*). Median (min-max) fold change of *IL6* on day 7 was 8 (1.6-14) compared to day 0 of differentiation. I found no particular pattern of mRNA expression for *IL8* (fig. 5.6*B*). The highest value of expression is on day 7, with a fold change of 1.8 (1.0-3.3) compared to day 0 of differentiation. Moreover, *FNDC5* shows a rapid fold increase in mRNA expression of 5.0 (4.6-10.0) from day 0 to day

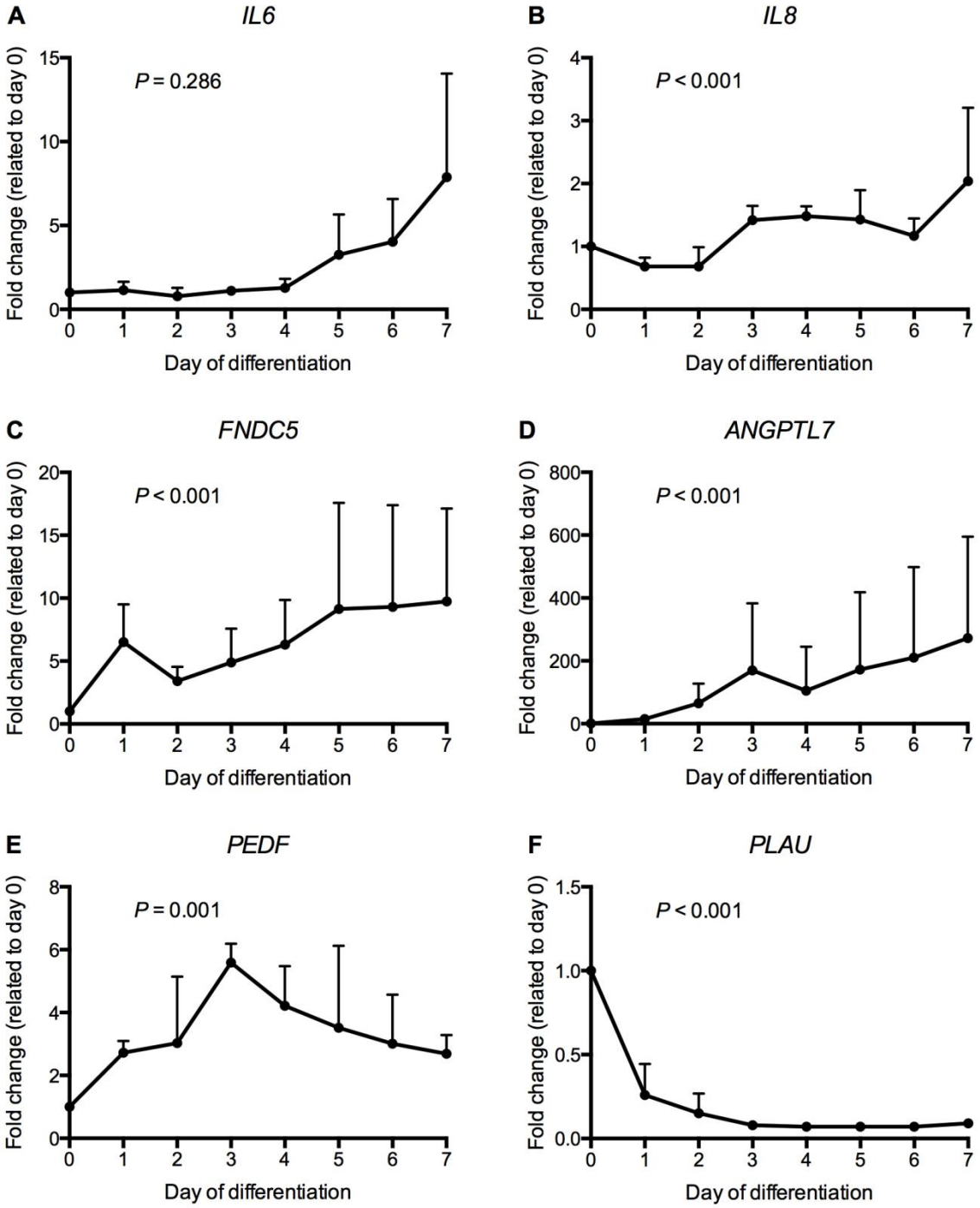


Figure 5.6. Changes in mRNA expression of known and potential myokines during myogenesis. Confluent cultures were differentiated for 7 days in medium containing 1.0 % HS. *A*, *IL6*; *B*, *IL8*; *C*, *FNDC5*; *D*, *ANGPTL7*; *E*, *PEDF*; *F*, *PLAU*. The experiments were performed three times, once with LD30 cells and twice with EO2 cells. All experiments are performed using cells from passage 5. Error bars represent SDs. Statistical testing has been performed by Kruskal-Wallis test between each time point during differentiation.

1 of differentiation, declines till day 2, and slowly increases and stabilises at 5.5 (3.1-18.8)-fold on day 5, compared to day 0 of differentiation (fig. 5.6*C*). I also found that *PPARGC1A* and *FNDC5* mRNA expression levels have a correlation coefficient of 0.383 (r^2 = 0.147) during differentiation, which is a rather weak but significant (P = 0.007) correlation (data not shown). Furthermore, I found that *ANGPTL7* steadily increase in mRNA expression level (fig. 5.6*D*). There is a 134 (41-641)-fold increase in mRNA expression on day 7 of differentiation, compared to day 0. Moreover, I found that *PEDF* shows a 5.0 (5.1-6.3)-fold increase in mRNA expression by day 3 of differentiation, then decreases and stabilises at a 2.5 (2.2-3.3)-fold increase compared to day 0 of differentiation (fig. 5.6*E*). Finally, *PLAU* shows a decrease in mRNA expression already at day 1 of differentiation, and this continues to a 0.07 (0.07-0.10)-fold decrease (7 % of day 0), where it stabilises (fig. 5.6*F*).

I conclude that well-known and potential myokines are transcribed during myogenesis. My experiments show that *FNDC5* (P < 0.001), *ANGPTL7* (P < 0.001) and *PEDF* (P = 0.001) all increase during differentiation, whereas *PLAU* decrease (P < 0.001). *IL6* appeared to increase, but did not change expression statistically significantly (P = 0.286), and *IL8* showed no particular pattern, but increased expression statistically significantly (P < 0.001). I also tested adiponectin expression, but no transcripts were detected throughout differentiation.

5.3.2 Secretion of PLAU and PEDF from differentiating muscle cell cultures

I found the mRNA expression kinetics of *PEDF* and *PLAU* to be especially interesting. Hence, I chose to analyze the potential secretion of PEDF and PLAU. SDS-PAGE separation and immunoblotting was performed on equal amounts of medium (fig. 5.7). PLAU is clearly detected in medium from the undifferentiated culture (day 0), but not in the consecutive days of differentiation.

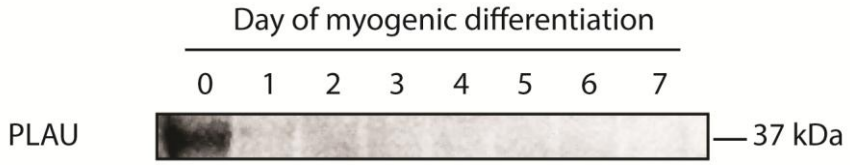


Figure 5.7. Changes in protein content of PLAU in the medium during differentiation. Confluent cultures were differentiated for 7 days in medium containing 2.0 % HS. Equal amounts (5 µl) of conditioned medium

were separated by SDS-PAGE, transferred to a PVDF membrane, followed by incubation with antibodies against PLAU. The experiment was repeated once using a separate donor, with similar results.

To quantify the amount of PEDF in the medium from cultured myoblasts and myotubes, ELISA for PEDF was performed (fig. 5.8). Means (SD) PEDF concentrations in conditioned medium were 135 (74) and 83 (36) ng/mL on day 0 and day 1, respectively. The concentration increased steadily until day 5 of differentiation, reaching max at 293 (4) ng/mL. PEDF concentration declined at the end of differentiation, with 217 (69) ng/mL on day 7. There is a significant difference between all timepoint during differentiation ($P = 0.008$), and *post hoc* calculations show a significant difference between day 1 and day 5 of differentiation ($P = 0.020$).

Taken together, these data indicate that both PLAU and PEDF are expressed on mRNA and protein level during differentiation. PLAU seems to be secreted mainly when there is an abundance of myoblasts (i.e. early in differentiation), whereas PEDF secretion seems to increase until late mid-differentiation.

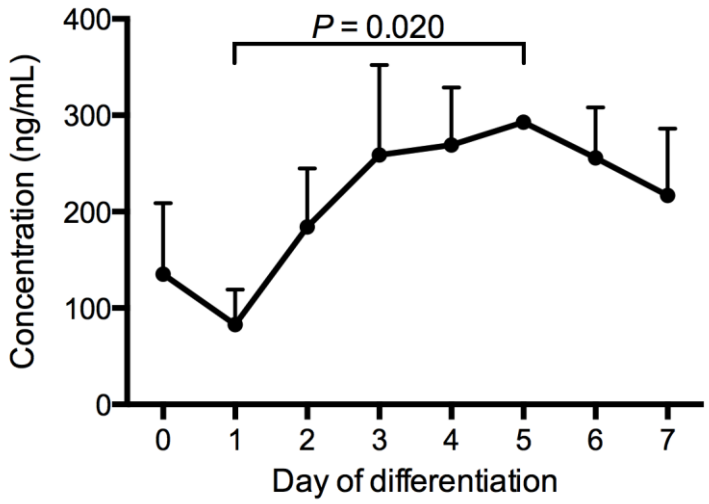


Figure 5.8. Concentration of PEDF in medium during differentiation. Confluent cultures were differentiated for 7 days in medium containing 1.0 % HS. Conditioned medium was collected each day, as described in methods. Each day 50 % of medium in all wells were substituted for fresh medium. The experiment was performed three times, once with LD30 and twice with EO2. All experiments are performed using passage 5. Error bars represent SD. Statistical testing has been performed using one-way ANOVA test between each time point during differentiation, with *post hoc* Bonferroni comparisons. *, $P < 0.05$.

5.4 LDH release

To evaluate integrity of the cell cultures used in experiments, LDH was measured in conditioned medium. All values of LDH are related to values of 100 % cell lysis within one culture well. On day 1 of myogenic differentiation, all serum-reduced media had almost no LDH accumulation (fig. 5.9), whereas the proliferation medium exhibited higher LDH accumulation ($P < 0.001$). On day 4 of differentiation all groups were almost identical with respect to LDH accumulation, albeit with statistical significant differences ($P < 0.05$). On day 7 of differentiation SFM had 9.1 (SD 5.8) % LDH accumulation, indicating an increase in cell death, whereas the other media remained at a low percentage ($P = 0.013$).

In conclusion, these data suggest that *serum-reduced* media, not *serum-free* medium, serve as healthy differentiation media with respect to cell culture integrity, at least when differentiating 7 days.

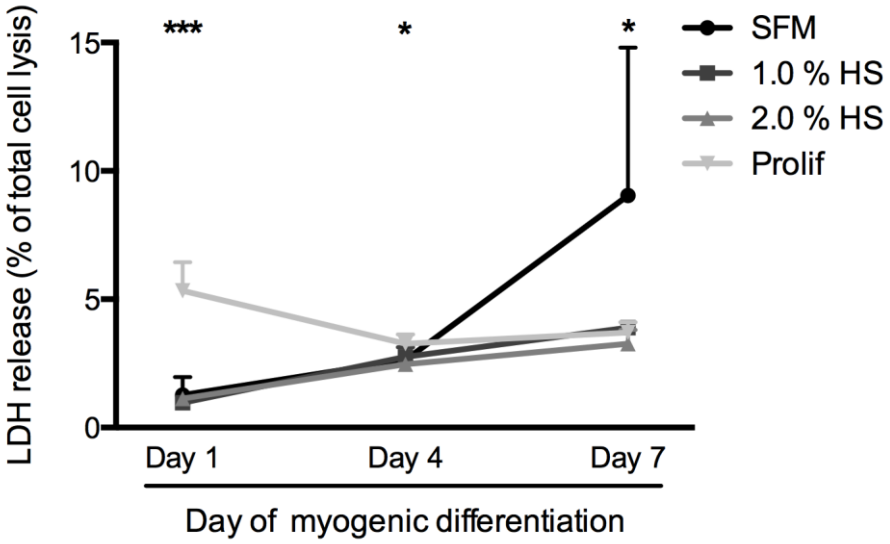


Figure 5.9. LDH release at day 1, 4 and 7 of differentiation in different media. Confluent cultures were differentiated for 7 days in either serum free medium or media containing 1.0 % HS, 2 % HS or 20.0 % FBS + insulin, EGF, FGF and dexamethasone (see Methods). The experiments were performed four times, twice with LD30 and twice with EO2 donor cells. All experiments were performed using passage 5. Error bars represent SD. Significance stars are between groups at each time point. *, $P < 0.05$; ***, $P < 0.001$.

6 Discussion

6.1 Methodology

The following sections discuss methodological issues relevant to this thesis. I start by discussing the use of cell cultures in biomedical research, and compare this with *in vivo* physiology. Next, I discuss Romanowsky staining and mechanisms of action, and the use of nuclei count as a method of evaluating degree of differentiation. Next, I discuss RT-qPCR for mRNA quantification, western blotting and ELISA for protein visualization and quatification, respectively, and finally LDH measurements for culture integrity.

6.1.1 The cell culture model system

Cell and tissue culture have, as a vital part of biological and medical research, gone through a massive evolution the last century (55). Since Harrisons frog embryo nerve fiber outgrowth *in vitro* in 1907 (78), major developments in cell and tissue culture research have occurred, such as trypsinization and subculture (79), the use of antibiotics (80), the realization of mycoplasma infections (81, 82), the development of laminar flow cabinets (83), the use of growth factors (84), the confirmation of cross-contamination of many cell lines (85), and many other major breakthroughs (55). As the human genome has been sequenced (86, 87), cell culture research opens for genomic, proteomic and metabolomic understanding of cells, tissues, organ systems, and whole organisms. Recently, reprogramming of somatic cells to (induced) pluripotent stem (iPS) cells has been topical (88, 89), also emphasized in 2012, when sir John B. Gurdon and Shinya Yamanaka shared The Noble Prize in Physiology or Medicine for their research in stem cell biology in tadpoles and mice, respectively, even though their work were done 40 years apart (90, 91). Yamanaka's group has since the revolutionary discoveries in 2006, replicated the main findings in mammalian cells as well (92). Central to their research methods are cell culture, combined with advanced experimental procedures, such as plasmid construction and transfection, virus production and infection, immunocytochemistry, immunohistochemistry, classical staining techniques, sequencing, luciferase assays, and DNA, RNA and protein measurements (92). Cell culture can be used to investigate intracellular signaling and flux, and genomics, proteomics and metabolomics, and cell-cell interactions. This basic knowledge of cell functionality is important for applied

science, such as biotechnology, immunology, pharmacology, tissue engineering and toxicology (55). I believe this emphasizes the usability, importance and potentially scientifically propulsive force of cell culture research.

The term *cell culture* comprises many scientific methods, including organ culture, explant culture, cell culture and organotypic culture (55). The different methods possess different advantages and limitations. Organ culture, with the main architecture characteristic of the tissue retained, may be used for histological studies of embryonic organs and adult tissue fragments. In primary explant culture, cells migrate from fragments of tissue, and are used for cytology. Organotypic cultures are recreations of *in vivo* organs in an *in vitro* setting, with different cells cocultured with or without matrix. These cultures have a wide variety of applications, and are very future oriented. Cell cultures, as I work with in this thesis, are disaggregated tissue that forms monolayers at solid-liquid interphase. Some cell types can be cultured in suspension in the culture medium. It is relatively easy to perform, compared to organ culture, explant culture and organotypic culture, and there are many molecular techniques available for characterization (55).

Cell and tissue culture have many advantages in research (55). One can easily control the physiochemical environment, including pH, temperature, osmotic pressure and CO_2 and O_2 tension. This is of utmost importance when working with live cells, which responds to even the slightest shift in surroundings. Hormone, growth factor and nutrient concentrations can also be controlled, especially if using the same batch of serum (horse serum, fetal bovine serum) in all experiments. This allows for control of concentration (dose) and time exposure of individual molecules and substances. One can also, to some extent, control the microenvironment, including cell-cell and cell-matrix interactions. Further, homogeneity of cultures can be achieved and contamination can be prevented by selective flow cytometry and selective growth conditions. All of these advantages contribute to the low variability seen in cell culture research, compared to animal research. Further, the cell cultures can easily be characterized, for example by cytology, DNA profiling or immunostaining. Importantly, the stocks can be preserved for long periods if stored in liquid nitrogen, and the experiments are relatively cheap considering reagents, scale, time saving and reduction in animal use (55).

Just as any other scientific method, cell and tissue culture has limitations (55). The need for expertise and environmental control is high. This includes strict sterile technique, avoidance and detection of chemical and microbial contamination, and awareness and detection of

misidentification. Also, automated routines for isolation and cleanliness of the workplace, incubation and pH control, containment and disposal of biohazards are of utmost importance. As continous cell lines are cancerous, changes in chromosome numbers frequently occur. This may introduce both instability, dedifferentiation, adaptation and selective overgrowth. This may especially occur when serum concentrations are high (possibly because of high growth factor content). Also, these genetic changes may introduce undesirable heterogeneity and variability, confirming the definite needs for frequent chromosome analyses in work with continous cell lines. This is not a problem when working with primary cell cultures (55).

6.1.2 Differences between *in vitro* and *in vivo*

It is favorable that any *in vitro* measurement can be interpreted in terms of the *in vivo* response of the same or similar cells, or at least that the differences that exist between *in vitro* and *in vivo* measurements are clearly understood (55). Firstly, the tissue specific three dimentional structure is not present in cell cultures. This means that the ECM-cell and cell-cell signaling, both input and output, is lost. Even though we are attempting to achieve homogenous cultures, this comes at the prize of loss of paracrine signaling between different cell types. Further, hormonal and nervous input are also lost. Scientists have met this problem by adding hormones to their cultures, a trend that continues to evolve. As there is no haemoglobin (or RBC's for that matter) present in the cultures, oxygen concentration may be different from *in vivo*. This means that preferred substrates for oxidation may be different than *in vivo* (55).

Generally, results from *in vitro* studies must be interpreted with caution, and should preferably be verified by other kind of studies, such as animal and human experiments. To illustrate the importance of maintaining a cautious approach when interpreting results, discussing the use of cultured satellite cells is appropriate. In adult skeletal muscle, quiescent satellite cells occupy a sublaminar position, squeezed between existing myofibers (42). The transcriptional machinery of satellite cells is also quiescent, as high proportions of heterochromatin is a key morphological feature of this cell population. Upon injury (such as chemical or mechanical damaging, strength training, myopathy or pathological dystrophy), these cells become activated, increasing their cytoplasmic content, proliferating, differentiating and migrating for myofiber fusion. The myofiber is then regenerated with hypertropic potential. However, adult skeletal muscle is not the sole proprietor of muscle-

forming precursor cells. Primary muscle fibers are derived from embryonic myoblasts, whereas secondary muscle fibers are derived from fetal myoblasts. Hence, three distinct muscle-cell precursors were until rather recently known to contribute to muscle tissue development and maintenance: embryonic and fetal myoblasts, and satellite cells (42). Today, additional cell populations have been identified with potential for contributing to muscle generation, such as the side population cells (SP cells), mesoangioblasts (vessel-associated stem cells), muscle-derived stem cells, pericytes and $CD133^+$ stem cells (51). The contributions of these cell populations is yet not fully mapped, but they are, however, very interesting for their potential in transplantation and basal medical science research. However, based on this knowledge, it is tempting to ask: *What do I (and others) actually study using satellite cells from adult skeletal muscle in muscle differentiation experiments?* Organogenesis of muscle tissue? Development of primary or secondary myofibers? Or do we study the fusion of precursor cells as they would behave in adult skeletal muscle? Are the precursor cells still satellite cells, or do they possess characteristics of post-natal myoblasts? Interestingly, studies show that asymmetric mitosis gives rise to two types of satellite cell populations in adult muscle: the self-renewing (satellite stem cells) and the muscle progenitor satellite cells (myoblasts) (93). Bareja and Billin suggests that the satellite cells harvested from biopsies and cultured in the laboratory begin to exit the quiescent state soon after seeding, producing mostly myogenic progenitors and rarely dividing to self-renew (93). To summarize, in view of the discussion above, the myoblasts in culture may be very different from the satellite cells from which they originated. Hence, depending on application, it is of utmost importance to characterize the cell cultures. The process of characterizing muscle cell cultures is a vast part of this master thesis, and I review this in detail later.

6.1.3 Romanowsky staining

The state of differentiation in the muscle cell cultures was evaluated by nuclei content of the cells. To be able to distinguish between various parts of the cell cultures, such as nuclei, cytoplasm, different cells, and overlapping cells, a cytological stain was applied – the Romanowsky stain. Even though Ernst Malachowski deserved to be recongnized as the co-founder of this stain, the current name stands (94). The Romanowsky stain has a long and impressive history, as its components were originally used for cytopathological stain of blood smears, important for detecting *Mycobacterium tuberculosis* and malaria parasites (94). Its components, methylene blue, azure B and eosin, are the predecessors of Giemsa-type and

Hematoxylin and Eosin (H+E)-type stains, which are important stain methods in modern cytology and cytopathology (94). To date, Romanowsky stain allow better estimation of relative cell and nuclear sizes, and superior visualization of cytoplasmic details, smear background elements and intercellular matrix components than other stains (95). A combination of the less-invasive fine needle aspirates and cytological evaluation provide a good, in some cases the best, if not *the only*, method of diagnosing certain diseases (95). Common practice dictates that more than one type of stain should be used to evaluate cytopathological specimens (95), but this is not really necessary in the case of my cell cultures. Romanowsky stain is especially valuable in case of detecting microorganisms and diagnosis of hematolymphoid neoplasms, and with the Diff-Quik stain, Romanowsky stain is commercially available in a rapid on-site diagnosis format (95). Limitations to Romanowsky stain include the loss of nucleoli identification and distict nuclear outlines, and inability to stain keratinized or uneavenly distributed cells (95).

I have used the Romanowsky stain as described by the Expert Panel for Stains and Staining Method, established by the International Committee for Standardization in Haematology (ICSH) in 1976 (60). The procedure is based on a two-dye mixture of azure B plus eosin Y. Mechanistically, the azure B and eosin Y partly replace the mobile inorganic cations and anions that surround nucleic acids and proteins, respectively (96). This explaines some of the effects seen with the stain, including affinity and selectivity, and impact of pH changes (96). Still, this does not fully explain three potentially importance biological phenomenon. First, there may be dye-biopolymer attractive forces such as those occuring between the polarizable aromatic rings of dyes and of the bases of nucleic acids. Second, the hydrophobic effects that arise in aqueous milieu when both dye and biopolymer have hydrophobic regions can come in contact. Third, the dye-biopolymer effect may be modified by dye-dye complex formation.

Biologically, the color purple should appear at chromatin, granules of neutrophils and platelets in blood smears and tissue sections, in the bands of G/banded chromosomes, and in collagenous connective tissue and mucus granules of tissue sections (96). Further, eosin Y is almost three times the size of azure B, indicating that penetrating tissue may require more time. Hence, the first step involves basic dyeing of DNA by azure B. Next, the blue staining is coverted to purple as an azure B/eosin complex is formed. This color is then intensified by a template effect, suggested to happen when azure B and eosin Y salts precipitate and deposit near the biopolymer (96).

My main motivation for using Romanowsky stain was to be able to distinguish between nuclei and cytoplasm, and to identify distict membranes. This was accomplished without significant difficulties (fig. 5.2). Nuclei were consistently purple-pink (deep indigo), whilst cytoplasm was blue and pink. The nucleic color is satisfactory, according to published reports (96). The variation in cytoplasmic color is probably caused by variation in cytoplasmic content of ribosomes, RNA and protein.

6.1.4 Nuclei counting as a method of evaluating degree of differentiation

Following Romanowsky staining of muscle cell cultures, sections were selected and photographed, and an evaluation of differentiation was performed by counting nuclei inside myotubes and myoblasts. The ratio (fusion index) was used as a measure of differentiation. To standardize the selection of areas to be evaluated, 6 photographs were taken on a straight line (5 mm apart) across the culture wells (fig. 5.1). Other groups have used similar but slightly different approaches. Ozawa and Kohama drew a coordinate system underneath culture wells, and numbers were allocated distinct points (97). Next, they used a random number table to select 20 areas in the wells which were to be evaluated (97). Ogihara et al. wanted to overcome the possibility of biased cells located centrally and peripherally, and chose to randomly select 5 areas between the center and edges to be evaluated (98). Although they used endothelial cells, the same bias could be discussed in muscle cell cultures. Improper seeding technique can result in myoblasts attaching to the collagen coating centrally rather than evenly distributed. Moreover, I chose areas covering 0.57 mm^2, comprising a total of 3.44 mm^2 per well. This is in line with a report by Hausman and Poulos, who selected 6-8 areas in each well, comprising a total of 3.5 mm^2 (99).

In my initial experiments, the serum experiments (fig. 5.3*A* and *B*), differentiation was initiated too late. This resulted in overgrown cultures, as evidenced by nuclei count of 9000-12000 (data not shown). In the dexamethasone and insulin experiments (fig. 5.3*C* and *D*), the nuclei count was lower, about 3000-5000 nuclei (data not shown). Other researchers have chosen to randomly select up to 10 areas, and to count 400-1000, and more than 1000 nuclei per experiment (59, 100, 101). During my counting, myotubes were classified as a syncytial fiber-like cells with 3 or more nuclei. Cells with one or two nuclei were classified as myoblasts. This is in line with many published reports (59, 101, 102). Some researchers

choose to sub-classify myotubes as myotubes with 2, 3 or 4 and more nuclei (74). Summarized, my evaluation method of choice is a conservative approach, limiting the possibility of false positives. Furthermore, I calculated a fusion index based on nuclei inside myotubes and total amount of nuclei (mt-nuclei:total-nuclei), which is also a much used method in the literature (59, 100, 101). Alternative fusion indices are applicable, such as nuclei inside myotubes and inside myoblasts (mt-nuclei:mb-nuclei), or amount of myoblast nuclei per 100 myotube nuclei (103). My findings show there are large differences between mt/t nuclei ratio of 1.0 % HS from different experiments (fig. 5.3*A*-*D*): 0.35, 0.44, 0.53 and 0.67. This probably results from many things, of which the following reasons are most important. First, as the absolute number of nuclei increase, more cells overlap in the collagen-coated plastic wells, resulting in measurement errors and misclassificating of myotube nuclei as myoblast nuclei. The total number of nuclei is related to overgrowth and over-proliferation, and late-onset differentiation. Secondly, as the counting progressed, I developed a skill for better distinguishing myotube and myoblast nuclei from each other. This is presented in supplemental material, illustrated with experiments with HS, FBS and UG (fig. S4*A* and *B*). Experiment number 4 appeared to have greatest impact on the final result (even if not statistically significantly different). Fig. S4*A* shows fusion indices for the separate experiments, of which experiment 4 stands out. Fig. S4*B* shows the absolute number of nuclei for the serparate experiments. Here, experiment 1-3 present decreasing nuclei count, but no appearant differences between groups. Also, experiment 4 presents as decisive for the final result (fig. 5.3*A*).

In retrospect, I believe the subjective part of this evaluation would have been overcome if staining of cultures had been done with immunohistochemistry. Many researchers use antibodies to myosin or desmin for muscle cell visualization, and often also Hoechst, DAPI or Bromodeoxyuridine (BrdU) for DNA (nuclei) visualization (74, 104). This way, specificity could be achieved, and results from subsequent quantification (counting) would have been more reliable. Alternative methods to evaluate fusion also exists. Laarse and coworkers quantified muscle cell numbers by measuring content of two LDH isoenzymes and DNA (105). They concluded that the ratio of LDH to DNA content can be used to assess cellular composition (105). A similar approach was used by Ruas et al., in which the ratio of protein:DNA content of cell cultures was used to evaluate cellular hypertrophy, a phenotypic trait of differentiated muscle cells (106).

6.1.5 RNA isolation and quantification

RNA and protein was isolated simultaneously using the RNeasy kit, cDNA synthesized with the High Capacity cDNA Reverse Transcription Kit, and mRNA levels were quantified by RT-qPCR using the TaqMan MGB system, as described in Methods.

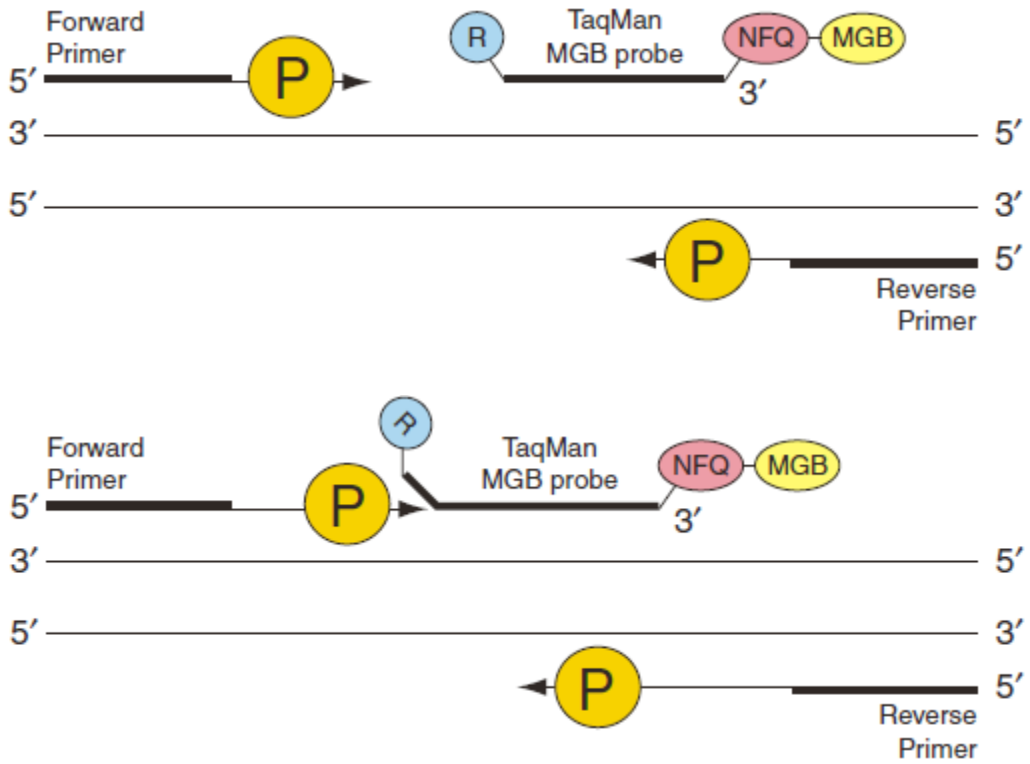
The RNeasy Mini kit procedure is a well-established easy-to-follow technology that selectively isolates RNA molecules longer than 200 nucleotides (63). It is based on a combination of selective binding properties of a silica-based membrane and microspin technology. The biological samples are lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in RNase-free water. All bind, wash, and elution steps are performed by centrifugation in a microcentrifuge. This procedure is an enrichment of mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15-20 % of total RNA) are selectively excluded (63). Phenol-chloroform and ethanol precipitation is an alternative technique to isolate DNA, RNA and protein (107). Albiet more comprehensive, this technique often provides isolation of higher quality (107). One must always remember that RNA isolation is somewhat difficult to evaluate. Differences in isolation yield between native mRNA molecules may arise from differences in mRNA length, folding, localization in the cell, complexation to proteins and other factors (108). Still, as long as protocol for isolation is similar between samples and experiments, reproducibility should be maintained.

The High Capacity cDNA Reverse Transcription Kit is a well-established four-step technology for generating single-stranded cDNA from all RNA molecules present in a sample (64). First, the sample is heated to 25 °C for 10 min. Next, the sample is heated to 37 °C for 120 min to conduct RT-mediated cDNA synthesizing using deoxyribonucleotides (dNTPs). Next, the sample is heated to 85 °C for 5 min to inactivate the reverse transcriptase (RT) enzyme, and finally cooled to 4 °C for temporary storage (maximum 24 hours). All steps are performed in an aqueous buffer solution containing RNase inhibitor. cDNA not immediately used in the RT-qPCR reaction is stored at -20 °C (64). Importantly, because numerous random oligonucleotide primers anneal to one single RNA molecule, the resulting cDNA is correspondingly fragmented (109). An alternative primer, the oligo-dT primers, selectively

reverse transcribes RNA with intact polyadenylation (poly-A tail) (108). This is especially important for cloning of cDNA. Also, polyadenines do not fold into higher order structures, increasing the efficiency of the reverse transcription. Still, because the transcription may not reach its PCR target sequence, oligo-dT primers may introduce bias for amplicons located near the poly-A regions. Also, RNA lacking the poly-A tail are not transcribed and therefore not possible to detect in the downstream PCR reaction. Another alternative primer is the gene specific primers for reverse transcribing specific gene sequences in which a limited number of RNAs shall be analyzed. The advantage to this is that the two-step procedure of cDNA synthesis and RT-qPCR may be reduced to a single reaction, albeit with lower sensitivity (108). Still, to maintain the diversity and frequency distribution of all RNA in a complex population when reverse transcribed to cDNA, random primers are used instead of oligo-dT primers and gene specific primers. This is also important since the RT enzyme tends to detach from the RNA template, possibly resulting in an incomplete reverse transcription of RNA sequences (109). Because the random primers are hexamers of random deoxyribonucleotides (adenine, A; guanine, G; cytosine, C; thymine, T), the total number of different primers/oligos are (4^6 =) 4096.

The RT-qPCR using TaqMan MGB system is a highly reputable and sensitive system for detecting and quantifying specific cDNA molecules present in a sample (65). The RT-qPCR method was developed in 1992, and is a refined technique of the original polymerase chain reaction (PCR) developed in the 80's (108, 110, 111). The RT-qPCR reaction is performed by temperature cycling. First, the temperature is raised to approximately 95 °C to separate the strands of the cDNA, then temperature is lowered to about 50 °C to let primers anneal to the template, and finally the temperature is set to around 60-72 °C,which is optimum for the DNA polymerase that extends the primers by incorporating the dNTPs. The first cycle produces double-stranded DNA from cDNA, and as of the second cycle new DNA molecules are being synthesized (108). The TaqMan MGB probe system allows real-time monitoring of product accumulation, and contain a reporter dye (FAM) at the 5´ end, and a minor groove binder (MGB) and a nonfluorescent quencher (NFQ) at the 3´ end (65). The TaqMan MGB probes anneal specifically to a complementary sequence between the forward and reverse primer sites (fig. 6.1, 1st image). When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence (fig. 6.1, 1st and 2nd image). The DNA polymerase cleaves only probes that are hybridized to the target. Cleavage separates the reporter dye from the quencher dye, promoting increased fluorescence by the

reporter (fig. 6.1, 3rd image). Polymerization of the strand continues, but because the 3´ end of the probe is blocked, no extension of the probe occurs during PCR (fig. 6.1, 4th image) (65). During the initial cycles the signal is weak and cannot be distinguished from the background (108). As the amount of product accumulates a signal develops that initially increases exponentially. Thereafter the signal levels off and saturates due to the reaction running out of critical components, such as primers, the reporter, the dNTPs or the polymerase. The response curves are separated in the growth phase of the reaction, reflecting the difference in their initial amounts of template molecules. The difference is quantified by comparing the number of amplification cycles required for the samples' response curves to reach a particular threshold fluorescence signal level. The number of cycles required to reach threshold is called the certain threshold (Ct) value. Threshold can be set manually, but computer software can also do it automatically. For example, threshold can be automatically set by algorithms calculating the average exponential level, i.e. the level with an average perfect doubling of fluorescence. Because of the arbitrariness of this threshold, affected by instrument software and settings, one should avoid comparing individual Ct values between experiments (108).



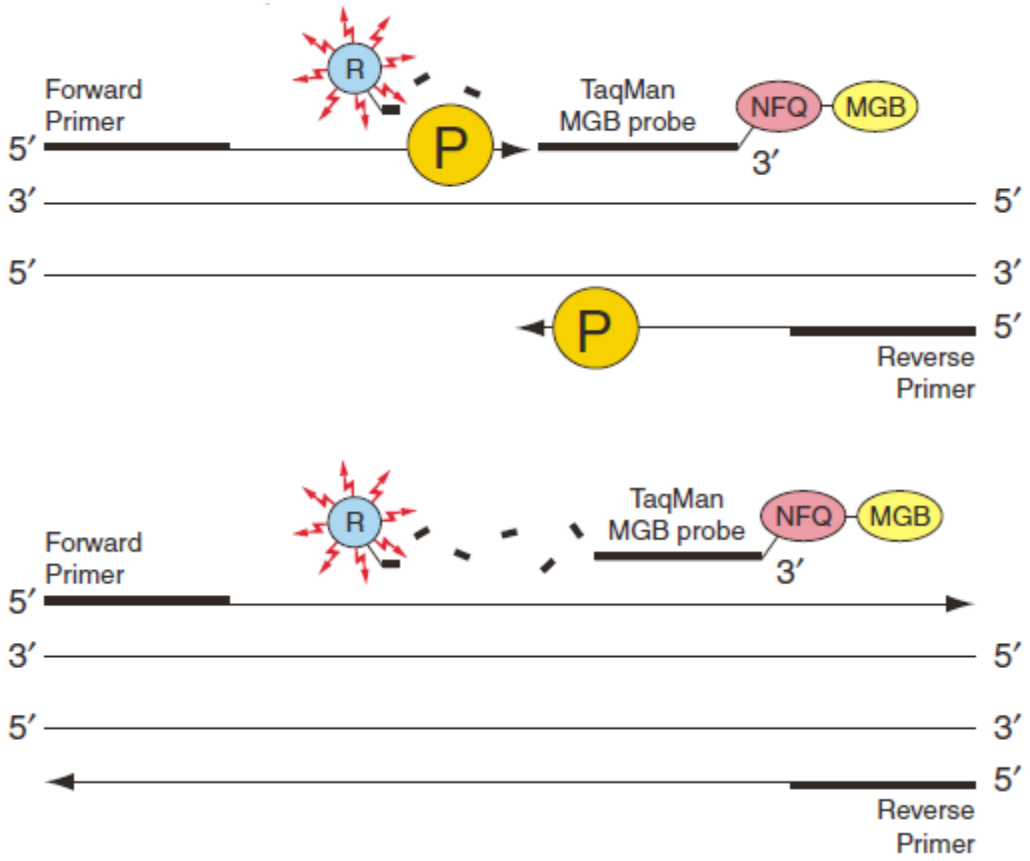


Figure 6.1. The TaqMan MGB probe system. Illustration taken with permission from (65).

PCR efficiency is assumed to be 100 %, even though this is rarely the case (108). PCR efficiency should be estimated by serial dilution of a standard (purified PCR product or target sequence-containing plasmid), a biological sample, or addition of standards. Determing PCR efficiency is important for quantifying the relative expression pattern of genes more accurately. Furthermore, the TaqMan MGB probes used by our groups is only one of many fluorescence reporters used in RT-qPCR. Other reporters use similar technology, such as quench and release of light before or after binding the target sequence. Sequence non-specific dyes, such as SYBR Green, hybridize with double stranded DNA, and emits light only when bound. This may introduce measurement errors, as samples may contain double stranded primer-dimer products. In fact, primer-dimer products should be adjusted for by performing a melting curve. Also probe-based PCRs may be affected by primer-dimer products, and high Ct values should be suspected as possible results of primer-dimer product presence. The BOXTO dye has been developed to directly measure presence of competing primer-dimers,

used in combination with standard probes for simultaneous target sequence amplification (108).

Quantification of mRNA is an extremely sensitive technique, possibly distinguishing a single mRNA molecule difference between two samples. Still, this requires high level of laboratory expertise as most steps from isolation to quantification can introduce measurement errors. In the case of my samples, I believe differences between samples may have been affected largely by variation in the RT step. Complete cDNA synthesis is an absolute prerequisite for accurate measurements, as this reflects the presence of mRNA initially. Comparing the technical reproducibilities of the RT reaction and the PCR it was found that the RT reaction contributes with most of the variation to the experimental determination of mRNA quantities (108). It was concluded that substantially higher experimental accuracy is obtained when performing replicates starting with the RT reaction compared to splitting the samples after the RT step to perform real-time PCR replicates only (108).

Two different methods of presenting quantitative gene expression exist: absolute and relative gene expression (66). Absolute quantification calculates the copy number of the gene usually by relating the PCR signal to a standard curve. Relative gene expression presents the data of the gene of interest relative to some calibrator or internal control gene. A widely used method to present relative gene expression is the comparative Ct method also referred to as the $2^{-\Delta\Delta Ct}$ method. Absolute quantification is required when a precise quantity of amplicon is desired. The disadvantage of absolute quantification includes the increased effort to generate standard curves. Furthermore, it is often unnecessary to present data as absolute copy number and relative expression will suffice (66). The importance of estimating the PCR efficiency for correct measurements is discussed above. All mRNA data are calculated according to these equations:

$$\text{Fold change} = 2^{-\Delta\Delta Ct} = (2^{-\Delta Ct}\text{sample A}) / (2^{-\Delta Ct}\text{sample B})$$

$$\Delta Ct_{\text{sample X}} = (\text{Ct gene of interest} - \text{Ct internal control})$$

$$\Delta\Delta Ct = (\text{Ct gene of interest} - \text{Ct internal control})_{\text{sample A}} - (\text{Ct gene of interest} - \text{Ct internal control})_{\text{sample B}}$$

6.1.6 Western blotting

High quality protein isolation and sample preparation is critical for correct protein measurements. I used the combined RNA and protein isolation method for intracellular proteins, which has been validated by other groups (67, 68). Generally, different proteins prefer different isolation methods, so there is no guarantee for good isolation of all proteins present. The RNeasy protocol uses a guanidine thiocyanate-containing buffer for lysis and RNase inhibition. No specific protease inhibitors are added to the lysis solution, but since the mechanism of action of guanidine thiocyanate is protein denaturation, this buffer also potentially inhibits proteases, DNases, lipases, phospholipases and other catabolic enzymes (112). As described in Methods, proteins were thoroughly washed and dried prior to dissolving in the SDS-Tris buffer. We chose to use this SDS-Tris buffer for protein solubilization due to an appearantly better Coomassie staining after SDS-PAGE separation, as shown in fig. S5 in supplemental materials. To completely dissolve the proteins heating was required, and we chose to standardize this heating to 5 min at 95 °C. Immediately after heating, protein concentration was measured using the BCA method. This method is based on a two-step reaction, in which Cu^{2+} is first reduced to Cu^{1+} forming a complex with protein amide bonds (Biuret reaction). Secondly, BCA forms a purple complex with Cu^{1+} in a 1:2 ratio which is detectable at 562 nm (69). The BCA method is a fast, sensitive and accurate way of measuring protein concentration. Alternatives to the BCA method is the Bradford method, absorbance at 280 nm, Biuret method and the Lowry method (107). The Bradford method utilizes the dye Coomassie blue G-250, which is red-brown at a pH below 1 but turns blue when binding to protein, causing a shift in the pKa of the bound dye. This is a fast, sensitive, accurate and time-independent method of measuring protein concentration. Absorbance at 280 nm is a fast measure of presence of the aromatic amino acids, especially tryptophan, as they absorb light strongly at 280 nm, but is not very accurate. The Biuret method rapidly measures peptide bonds, but is not very accurate for low protein concentrations. The Lowry method works similarly to BCA and need even less material, but depends on the presence of tyrosine in the sample. The presence of detergents will interfere with most of these methods including the BCA method, and the standard curve was therefore prepared with equal amounts of SDS-Tris buffer for the protein concentration measurements (107).

Before applying samples to the gel, they were mixed with reducing agent and loading buffer and diluted. The reducing agent break the disulfide bonds, promoting complete unfolding of protein primary structure. The loading buffer contains an anionic detergent and Tris base for optimized conditions for the SDS-PAGE separation of proteins. Also, the loading buffer contains glycerol and a blue dye (bromophenol blue) for increased density and visualizing of the samples, facilitating accurate loading and monitoring of the migration front during SDS-PAGE. We used commercially available reducing agent and sample buffer, but could have used alternatives. Most western blotting protocols include the use of either β-mercaptoethanol (β-ME) or dithiothreitol (DTT) as reducing agent, whereas the commercial variant we used probably contains the relatively odorless TCEP-HCl. The Laemmli buffer is probably most widely used for the purpose of SDS-PAGE separation of proteins (113, 114).

The SDS-PAGE was performed using commercially available gel and running buffer at 200 V (constant) for approximately 1 hour. Proteins solubilised in SDS bind the detergent uniformly along their length to a level of 1.4 g SDS/g protein. This creates a mass/charge ratio which is consistent between proteins. For this reason, separation on a polyacrylamide gel in the presence of SDS occurs by mass alone, and protein molecular weight can be determined within 5-10 % accuracy. I wanted to visualize proteins with broad range of molecular weight, and decided to use 10 % gels for all SDS-PAGE separations. Other gel percentages could have been used as well, but 10 % separated 42 (actin) and 50 (α-tubulin) kDa fairly distinct, as well as 200 (myosin) and 37 (PLAU) kDa. PLAU was run on a separate gel which also was 10 %. I used pre-cooled MOPS running buffer for the SDS-PAGE, but could just as well have used a standard Running buffer. To verify the presence of proteins after separation, over night Coomassie staining of a dedicated gel was performed, showing protein bands similar to the two columns at far right in fig. S5 in supplemental materials. The separation of proteins on SDS-PAGE allows for downstream analyses, such as proteomics, but I only applied the method for further transfer of proteins to membrane (western blotting) (114).

Electro transfer of proteins to the methanol-activated PVDF membrane (positively-charged nylon) was performed at 0.6 A (constant) for about 1 hour. The 10X Transfer buffer was prepared manually. The working buffer was prepared at least a day in advance by adding 10 % methanol, diluting with distilled and deionized water and cooling to 4 °C over night. During the run, ice bags were applied to prevent overheating. Transfer efficiency was determined by evaluation of remaining protein standard color on the gel, which was not

observable. To further evaluate the transfer I could have Coomassie stained the gel, but the gel was discarded directly after use. Anther method of evaluating transfer efficiency is to stain the membrane with Ponceau Red, which allows for non-specific visualization of all protein bands present on the membrane.

Membranes were cut into separate pieces and blocked in TBS-T and 5 % non-fat milk to prevent downstream non-specific binding of antibodies, but this could also have been done using BSA. Milk is generally better for preventing background signals, but BSA allows for visualization of proteins with lower expression. Primary antibody was diluted in block buffer and incubation was performed overnight (about 10 hours) at 4 ºC for myosin and PLAU, but 1 hour in room temperature for actin and tubulin. The reason for this was that the anti-actin and anti-tubulin antibodies were expected to have greater specificity for their respective epitopes than the anti-myosin and anti-PLAU antibodies. All incubations were performed with gentle agitation. Anti-myosin and anti-PLAU was diluted 1:1000, whereas anti-actin and anti-tubulin was diluted 1:10 000 and 1:20 000, respectively. This provided good bands when 10-20 µg protein per lane was loaded. Secondary antibody was diluted 1:10 000 in TBS-T for low unspecific detection (background), but this could possibly have been even more diluted. I did not reuse primary antibodies, but could have done this by adding 0.5 % NaN_3 (sodium azide) directly to the primary antibody solution to prevent bacterial growth, which then would be usable 3-5 times if stored at 4 ºC. Regarding washing of membranes, I chose a regimen with 2 quick washes, then 15 min and 3 times 5 min washes with gentle agitation. 3 times 10 minutes would probably do just as well. After the final wash, membranes were exposed to substrate for the chemiluminesence reaction. As we used the HRP conjugated secondary antibodies, ECL Plus solution was applied, and light was detected on film. In the process of adapting this protocol to my needs, I started off using too much primary and secondary antibody for actin and tubulin, resulting in rapid depletion of the substrate. This appeared as a transparent band within a black band on the film. Myosin and PLAU, on the other hand, did not turn up until at a rather high antibody concentration (i.e. low dilution). Still, most of the problems I encountered working with western blotting was related to the isolation protocol, which required pedantic work skills. There was great uncertainty to how big amount of protein needed to be loaded onto the gel and how to best dissolve the proteins before BCA measurements were performed. To increase the protein concentration available for gel separation, I chose to dissolve precipitated proteins in about 55 µl SDS-Tris buffer, which is quite low. This resulted in difficulties in dissolving the proteins, which we resolved by pre-

BCA heating, as mentioned. However, we did not know how processing (heating, cooling, and repeated thaw-freezing cycles of samples) affected protein stability. To avoid excessive repeated freeze-thaw cycles, I decided to perform precipitation, dissolution, SDS-PAGE separation, membrane transfer, blocking and incubation with primary antibody on same day, and incubation with secondary antibody and processing on the following day. Membranes not incubating overnight were stored in a small amount TBS-T at 4 ºC.

In retrospect I realize that specificity of the antibodies used for protein detection should have been confirmed properly for my protocol, similar to the stepwise procedure used in published reports (115). This would possibly be required for publication-quality work. Firstly and relatively effortless, I could have loaded manufacturer-provided recombinant protein directly onto the SDS-PAGE gel, followed by immunoblotting and detection. Secondly but slightly more demanding, I could have transiently transfected cells (such as C2C12 cells) with FLAG-tagged human genes of interest, followed by proliferation, differentiation, RNeasy-based protein isolation, SDS-PAGE and immunoblotting. Finally and even more challenging, I could have knocked down these transfected (in the case of C2C12 cells) or endogenous (in the case of human primary skeletal muscle cells) gene products by siRNA.

The membranes were cut into separate pieces according to the protein ladder prior to blocking. Following membrane developing (processing) lane bands visualized were compared to the protein ladder, and approximate molecular weight was estimated. This method is not perfect but possibly sufficient for my use. I could also have non-specifically stained the gel or membrane, and physically measured the migration distance of each standard band in relation to the migration front. These numbers could I then in a graphing program have plotted against the log (molecular weight, MW) for generating a standard curve. Using the same procedure for unknown bands, these could have been extrapolated using the standard curve. This method would probably be more accurate for publication-quality work. However, according to antibody datasheets from manufacturers, actin, myosin, α-tubulin and PLAU migrate at 42, 200, 50 and 37 kDa, respectively. α-tubulin and PLAU therefore migrate similar to bands present in the standard and hence, are easy to identify. Actin and myosin, on the other hand, are identified more or less on visual comparisons with nearby standards (37 and 50 for actin, and 150 and 250 for myosin).

It is important to emphasize the great advantage of isolating proteins using a combined RNA and protein isolating method as I have done, as the experimental design of EPS experiments is

greatly limiting, and the reason is briefly this. Time curve experiments are the most relevant experiments, as they provide information about dynamics rather than before-after effects. Similar to pharmacologists who want to explore the cellular effects of a chemical compound, we want to explore the cellular effects of EPS in the short and long term, and hence need to excecute experiments with time manipulations. Only 6 wells can be stimulated simultaneously, which means that 6 different samples (5 if the zero timepoint control is excluded) are available for analysis in time curve experiments. The application of EPS in studying for example dynamics in energy metabolism, cell signalling, gene regulation and protein production and secretion is a potentially valuable tool for investigating myocellular molecular effects of physical exercise. Importantly, this tool is even more valuable as RNA and protein data from exact same samples can be evaluated. Although this may be true, I am fully aware of the shortcomings of the protein isolation method. Individual proteins usually require individually tailored western blotting protocols for optimal results, i.e. for nuclear proteins, mitochondrial proteins, cytoplasmic proteins and membrane bound proteins. Fractioning of cellular concent and increasing concentration of certain proteins is just one of several optimalizations for better and more easily reproducable results. Hence, as discussed for RNA isolation, we do not know if our method selectively isolates proteins with certain properties and subcellular localizations.

6.1.7 ELISA

ELISA is a protocol-based technique and relatively easy to perform compared to western blotting. The PEDF-ELISA was performed according to manufacturers instructions, as described in methods, and there were no practical difficulties related to the procedure, much thanks to helpful coworkers.

Fig. 5.8 shows the concentration of PEDF in conditioned medium during myogenic differentiation, measured with ELISA. The concentration is a result of secretion from cells and removal related to medium renewal. As for LDH, serum may already contain PEDF, and also, membrane damage may cause release of PEDF. However, the greatest influence on medium PEDF concentration does secretion and removal have. When initiating differentiation, I replaced 100 % of the proliferation medium with fresh differentiation medium, and then I changed 50 % of the medium the remaining days of differentiation. This was done primarily to prevent variations in nutrient availability, but probably affects PEDF

concentration as well. If no medium was changed for 2-3 days, which is standard for many differentiation experiments, PEDF would probably accumulate during this time and introduce measurement errors. I believe that changing 50-100 % of the medium daily provides the most easily interpretable results. This argument is strengthened by the fact that RT-qPCR and ELISA data show similar results for PEDF.

It should be emphasized that ELISA measurements does not provide the same degree of specificity that western blotting, as different kits contain different epitope-recognizing antibodies. For example, the proteins measured can be only certain post-translationally modified forms, or all forms. Hence, unless thoroughly characterized, this might introduce uncertainty to the interpretation of the results.

6.1.8 Cell integrity: LDH measurements

Unlike the complexity of *in vivo* toxicity, cell culture *in vitro* toxicity is mainly a cellular event (116), and can be quantified by measuring LDH in conditioned medium (117). LDH is an cytoplasmic enzyme found in all cells in humans, important for the interconversion of pyruvate and lactate (118). The enzyme is especially important for anaerobic muscle work. Cellular hypoxia results in blockage of the last step in the electron transport chain and subsequent $NADH+H^+$ and $FADH_2$ accumulation. This results in tricarboxylic acid (TCA cycle) inhibition and glycolytic NAD^+ regeneration, including the redirection of pyruvate to lactate, which is released from the cell. This lactate molecule can be taken up by cells with sufficient oxygen supply, converted to pyruvate, and redirected where needed. In cardiomyocytes, this pyruvat may be completely oxidised via the TCA cycle and electron transport chain for ATP regeneration, whereas hepatocytes may activate gluconeogenesis for glucose production and release (118).

Cell damage results in release of intracellular content into the blood stream, including release of enzymes. LDH is normally found in serum, and the concentration of LDH in serum increases when there is cell and organ damage present, such as following a myocardial infarction or during hepatic steatosis (119). Similarly, cultured cells release LDH upon membrane damage, increasing the LDH concentration in the medium. This was measured with an LDH kit, as described in Methods.

The LDH in samples must still possess enzymatic properties (120). LDH-containing conditioned medium is incubated with the reaction mixture from the kit. In the first step NAD^+ is reduced to $NADH+H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. In the second step the catalyst (diaphorase) transfers $H+H^+$ from $NADH+H^+$ to the yellow-colored tetrazolium salt INT, which is reduced to red-colored formazan (fig. 6.2). An increase in the amount of dead or plasma membrane-damaged cells results in an increase of the LDH enzyme activity in the culture supernatant. This increase in the amount of enzyme activity in the supernatant directly correlates to the amount of formazan formed during a limited time period. Therefore, the amount of color formed in assay is proportional to the number of lysed cells. The formazan dye formed is water-soluble and shows a broad absorption maximum at about 500 nm, whereas the tetrazolium salt INT shows no significant absorption at these wavelengths (120).

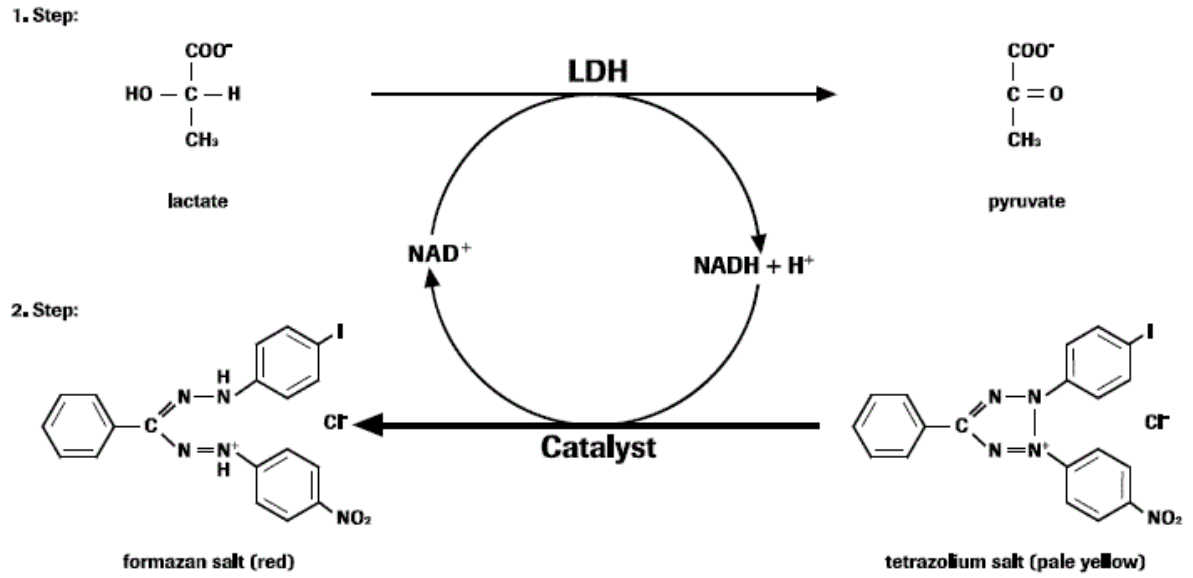


Figure 6.2. LDH kit: mechanism of action. Illustration taken with permission from (120).

There were significant differences between incubation groups at all measurement days. After adjustment, the proliferation media still showed larger LDH release on day 1 and 4. The tendency of SFM to release more LDH on day 7 was visually confirmed during daily inspections of the cell cultures, as large amounts of myotubes were detached from the surface. However, none of the groups present LDH release above 10 %, which can be viewed as a tolerable upper limit for cell culture integrity. To make sure the optical density of samples fell

within the range of capacity of the spectrophotometer, a dilution of the samples of interest was often required. In the case of my samples, I performed a 10X dilution of all samples used for 100 % reference values, and also samples of proliferation medium, but not samples of SFM, 1.0 % HS or 2.0 % HS. This could result in the changes seen between incubation groups on day 1 (the high LDH levels in the proliferation medium), but it is unlikely. I would expect this to be consistent for the remaining days as well, but it is not.

Researchers use a variety of methods for evaluating cell culture integrity. Other arabitrarily used names include cell survival, cell killing, cytotoxicity, toxicity and viability (117). Methods to measure cell culture integrity include permeability assays, functional assays, morphological assays and reproductive assays (117). The LDH measurement used in this thesis is a permeability assay, measuring the cellular membrane integrity. This enzyme assay is relatively easy to perform and, because LDH is highly expressed, more usable than enzymes with lower expression level (120). Other assays include uptake or exclusion of dyes, and labelling of cells with radioactive isotopes or fluorescent dyes (117, 120). Disadvantages to the use of these assays include use of radioactivity and the need for labelling of cells. Also, staining and counting of cells are time consuming and inefficient, and not usable with large sample numbers. Kato I. et al. have used cellular creatine kinase (CK) activity as a measure of cell injury in an *in vitro* system for measuring muscle cell toxicity of antibiotics (121), showing that CK release is also good for cell culture integrity measurements. Nikolic N. et al. evaluated cell culture integrity during EPS by a combination of protein content, LDH measurements, live imaging for nuclei counting, and staining with tryptan blue (62). In summary, a combination of different methods is probably best for evaluating integrity of cell cultures.

6.2 Differentiation of human skeletal muscle cells

One of the main objectives of this thesis is to characterize and evaluate the degree of differentiation in the primary cell cultures used in our laboratory. In terms of understanding the mechanisms underlying differentiation, new insight has emerged the last decade. Differentiation is no longer regarded as irreversible, as we now know that dedifferentiation may be induced both by genetic and chemical manipulation (88, 89). Research in amphibian muscle regeneration has shown that muscle cells in culture can be dedifferentiated (122, 123), and that this dedifferentiation is mediated, at least in part, through phosphorylation of

retinoblastoma protein (pRb) (104). Differentiation is a complex process connected with other core processes in the cell, such as proliferation and growth, apoptosis and senescence, tightly controlled via the cell cycle machinery (56). In my work, I have used the ratio between nuclei inside myotubes and total number of nuclei to evaluate differentiation. In addition to methods discussed earlier, other morphological indices of differentiation could be number of nuclei within each myotube, number of myotubes with visual striation or number of myotubes with contractile force generation (upon stimuli) (124). Technically, my method of choice is probably easiest to perform. However, distinguishing single myotubes can be borderline impossible, especially when cells are overlapping in large spider webs (see fig. 5.2). Quantifying the contractile ability of myotubes requires special equipment, such as an electic pulse stimulator and detector equipment (125). Hence, the morphological evaluation, despite its subjective bias discussed earlier, is possibly sufficient for visual evaluation of differentiation. It goes with the story that researchers often evaluate the myotube nuclei-accumulation with phase contrast light microscopy of live cells. We, on the other hand, chose to stain cultures with Romanowsky stain to highlight membranes and nuclei to overcome the problem of potentially not discriminating nuclei in myoblasts from nuclei in myotubes. Moreover, I found that proliferating myoblasts until 80-90 % confluency, followed by 7 days of differentiation resulted in formation of multinucleated syncytial myotubes. The highest fusion index was consistently observed with 1.0 % HS. Hence, I concluded that a differentiation medium consisting of DMEM and F12 (1:1 ratio) with 1.0 % HS provided better or equally good differentiation of cultured human myoblasts than FBS (0.5-1.0 %) or UG (0.2-0.4 %). I have not managed to find any reports that has compared addition of different types of serum on *in vitro* human muscle differentiation. Moreover, although low concentration of dexamethasone has been shown to promote differentiation (74), I did not find better differentiation with addition of high concentration of dexamethasone. This is in line with the well-known muscle-atrophy effects of glucocorticoids. Unexpectedly, adding insulin to HS or SFM did not increase the fusion index, and I have not found any similar reports on cultured human skeletal muscle cells. Interestingly, at most 67 % of all nuclei in the cultures were located within myotubes.

In order to evaluate the dynamics of differentiation, I investigated the mRNA expression of PAX7 and myogenin. They were chosen because of their early and late expression pattern (43). As expected, I found that *PAX7* decreased during differentiation for all cells grown in serum-reduced differentiation media. However, the proliferation medium had higher *PAX7*

mRNA expression levels throughout differentiation, also as expected. The initiate decrease in *PAX7* mRNA expression (and possibly protein levels) activate pathways that converge in activation of myogenin and the initiation of the myogenic apparatus (43). Further, there is a major increase in *MYOG* in all serum-reduced media, evidence for a strong activation of the myogenic apparatus in the cell cultures.

Furthermore, I investigated expression of the archetype muscle-specific proteins myosin and actin (126). *MYH1* shows a remarkable expression level between incubation treatments, as also shown in fig. S2 in supplemental materials. It seems as if 2.0 % HS provides a higher mRNA expression of *MYH1*, especially at day 7 and 8. This might indicate that serum concentration of the differentiation medium contributes to the fiber type of the culture, but it could also be due to methodological errors. Note that day 8 of differentiation in SFM was not applicable because of lack of RNA due to cell detachment and cell death, this time point was removed for all other incubation groups as well, and hence not shown here (fig. S2). To further investigate the possible phenomenon of muscle fiber type switch, I could have measured *MYH2* and *MYH7* on the same samples, to see if the opposite occurred (i.e. that 2.0 % HS had markedly decreased, and that SFM and 1.0 % HS increased levels of *MYH7*). This was not done because of shortage of time. It should be mentioned, however, that follow-up investigations of pipetting skills revealed no obvious mistakes or outliers for 2.0 % HS (fig. S6). Moreover, actin is, in cooperation with the myosins and numerous other proteins, important for the contractile ability of skeletal muscle cells. The increase in mRNA expression of *ACTA1* seen during differentiation is promoting a build-up of contractile proteins. Both the fast twitch type myosin and the muscle-specific isoform of actin is confirmed on protein level, strengthening the mRNA data greatly. This is good evidence for claiming that the cell cultures are well differentiated to the muscle tissue phenotype. The loading control α-tubulin also shows a remarkable pattern, with only a weak band on day 0 of differentiation, followed by identically powerful bands day 1-7 of differentiation. The result var repeated several times, and it is unexpected, as tubulin is shown to be equally expressed on all days of differentiation in similar experiments (77). This discrepancy may be a result of different isoforms of tubulin.

Finally, I wanted to investigate transcription of genes important in muscle-specific metabolic activity, such as the coactivator PGC1α and glucose transporter GLUT4, and I found increases during differentiation. Even the proliferation medium, with close-to-zero visible

myotube formation, had a 100-times increase in *PPARGC1A* mRNA expression. However, this was delayed 1-2 days compared to SFM, 1.0 % HS and 2.0 % HS. All differentiation media increased to a peak expression level, and then decreased. *SLC2A4* (GLUT4) increased drastically when differentiated in SFM, with a peak at day 5. The other differentiation media, including the proliferation medium, showed a slow but steady increase in *SLC2A4* mRNA expression. The reason for this result is probably that cells grown in serum free conditions have to rely on ATP regeneration from glucose and amino acid oxidation alone, as there is no supply of fatty acids from serum (i.e. carrier proteins, such as albumin). There is also no insulin present in SFM, so the induction of *SLC2A4* transcription must be insulin independent, possibly initiated by AMPK activation, Ca^{2+} influx, nuclear receptor activation or repression, or other mechanisms. Metabolism probably shifts towards glucose and amino acid metabolism during this fatty acid "famine", partly by induction of genes for glucose and amino acid catabolism, and partly by post-translationally modifying proteins of the intermediary metabolism, such as Pyruvate Dehydrogenase kinase (PDK4), which is an important regulator of pyruvate entry into the TCA cycle (127-129).

Taken together, these data indicate that all serum-reduced media strongly activate myogenesis, including mRNA transcription and translation of the muscle specific contractile apparatus, and mRNA transcription of myogenic markers and muscle specific metabolic genes. Differentiation of human muscle cells may therefore be done by proliferating until about 80-90 % confluency, with subsequent reduction of the serum concentration. The use of 1.0 and 2.0 % HS will both provide satisfactory differentiation within 7 days. SFM seems to promote similar changes (both morphologically and on mRNA expression level), but appear to cause a faster differentiation. However, LDH analyses on day 7 revealed, as seen morphologically, a toxic effect on the cultures. Hence, control of the cell culture integrity and differentiation is probably best achieved by 1.0-2.0 % HS.

As I here present one method of differentiating muscle cells, it is appropriate to mention *in vitro* differentiation in a general setting. Freshney proposes a six step procedure to general cell culture differentiation: 1) select correct cells by specialized isolation and selective media; 2) choose appropriate matrix (coating), and proliferate cultures to high density; 3) change to media designed for differentiation, i.e. serum or Ca^{2+} concentration; 4) add agents known to induce differentiation, i.e. retinoids, DMSO or cytokines; 5) add interacting cell types, i.e. add fibroblasts isolated from same tissue, and; 6) elevate cells in a filter well to provide nutrients

from all sides (58). The sequence which these are presented may not be similar for all cells, and not all steps may even be required. Furthermore, the scheduling of events can be important. For example, some hormones may be effective in short exposures, also dependent on the cell density and cell interactions (58).

Finally, to illustrate the complexity of the differentiation process, I have summarized a vast amount of mechanisms into an integrated image (fig. 6.3). Most published reports (that I have been able to locate) focus mainly on TFs, and MRFs in particular, as important in different parts of the myogenic differentiation. However, less interest has been on elucidating the signalling pathways resulting in transcriptional activation of the myogenic machinery. The signalling pathways involved in early and late myogenesis probably have important immediate (i.e. post-translational modifications), intermediate (i.e. cellular behavior) and long-term effects (i.e. gene regulation). Knight and Kothary have reviewed what they call *the myogenic kinome* – protein kinases critical to mammalian skeletal myogenesis (76), and signalling pathways that should be mentioned in relation to *early* myogenesis include the PKA (WNT-Frizzled) signalling pathways, MAP-kinase pathways, Akt (insulin/IGF) pathways and cyclin/CDK (cell cycle regulation) pathways. These are particularly important at embryonic migration and for priming of cells for a myogenic fate, and also for driving the cell cycle. In *later* myogenesis many of the same signalling pathways have important roles, such as the MAP-kinase pathway, which gets input both from cell-cell interactions and other extracellular signals. The Akt pathways also has many important roles in differentiation. Knight and Kothary conclude with the need for further investigations, as there are many other potentially important signaling pathways (76). Furthermore, the resulting change in gene expression will transcribe important regulators for cell cycle exit. MyoD is important for transcription of Cyclin D3, pRb and p21. Collectively, these will push the cell in favor of differentiation. Additionally, efficient shift from proliferation to differentiation is promoted via miRNA feed-forward mechanisms. However, I have not been able to locate specific roles for myokines, but they may possess roles at several sites, as indicated.

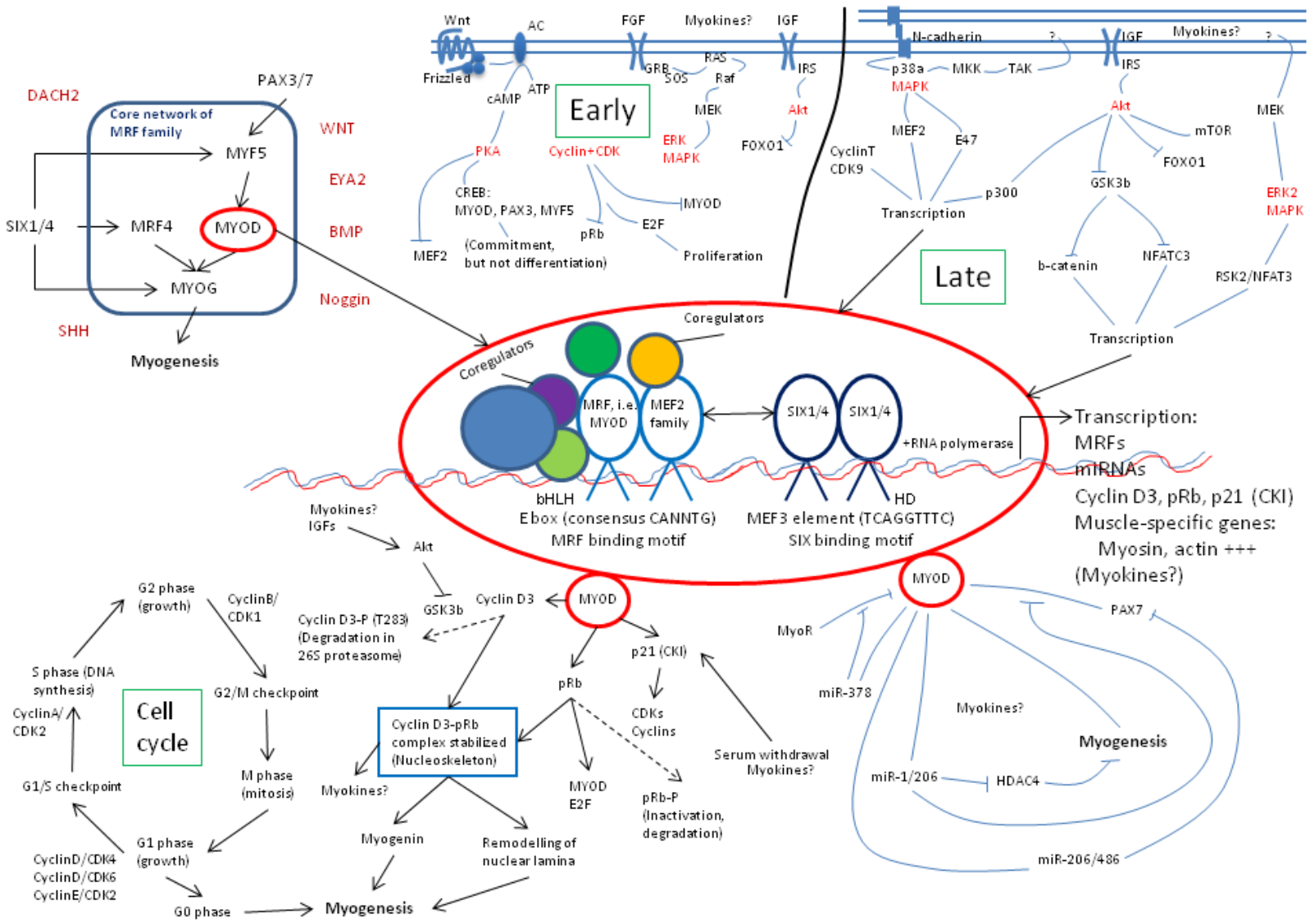

DACH2
PAX3/7
Core network of MRF family
MYF5
WNT
EYA2
BMP
SIX1/4
MRF4
MYOD
MYOG
Noggin
SHH
Myogenesis
Wnt
AC
FGF
Myokines?
IGF
Frizzled
cAMP
ATP
GRB
SOS
RAS
Raf
MEK
ERK MAPK
IRS
Akt
FOXO1
Early
PKA
Cyclin+CDK
CREB: MYOD, PAX3, MYF5
(Commitment, but not differentiation)
MEF2
pRb
E2F
MYOD
Proliferation
N-cadherin
?
p38a MAPK
MKK
TAK
MEF2
E47
CyclinT CDK9
p300
Transcription
Late
mTOR
FOXO1
GSK3b
b-catenin
NFATC3
MEK
ERK2 MAPK
RSK2/NFAT3
Transcription
Coregulators
Coregulators
MRF, i.e. MYOD
MEF2 family
SIX1/4
SIX1/4
+RNA polymerase
bHLH
Ebox (consensus CANNTG)
MRF binding motif
HD
MEF3 element (TCAGGTTTC)
SIX binding motif
Transcription:
MRFs
miRNAs
Cyclin D3, pRb, p21 (CKI)
Muscle-specific genes:
Myosin, actin +++
(Myokines?)
Myokines?
IGFs
Akt
GSK3b
Cyclin D3
MYOD
Cyclin D3-P (T283)
(Degradation in 26S proteasome)
p21 (CKI)
pRb
CDKs
Cyclins
Cyclin D3-pRb complex stabilized (Nucleoskeleton)
Myokines?
MYOD
E2F
pRb-P
(Inactivation, degradation)
Myogenin
Remodelling of nuclear lamina
Myogenesis
Serum withdrawal
Myokines?
Cell cycle
G2 phase (growth)
CyclinB/ CDK1
G2/M checkpoint
M phase (mitosis)
G1 phase (growth)
G0 phase
CyclinD/CDK4
CyclinD/CDK6
CyclinE/CDK2
G1/S checkpoint
S phase (DNA synthesis)
CyclinA/ CDK2
MYOD
MyoR
PAX7
miR-378
Myokines?
Myogenesis
miR-1/206
HDAC4
miR-206/486

Figure 6.3. Some mechanisms important in myogenic differentiation (Adapted from several references). *Upper left quadrant*: MYF5, MRF4, MYOD and MYOG are the core network of TFs (MRFs), influenced by PAX3/7 and SIX1/4 in adult skeletal muscle (43). MYOG is the initiator of myogenesis and is therefore under regulatory control of upstream MRFs, which exhibit a great deal of redundancy. MYOD is well characterized (MYOD is further described in the centre and lower panels), and $MYOD^+$ cells are considered activated or commited to a myogenic fate. Much research is focused on MYOD, but other MRFs probably have similar functions. Other upstream signals (WNT, BMP, etc) are also important in embryonic development (43). *Upper centre quadrant*: Signalling pathways important in early myogenesis, resulting in migration, proliferation, activation and determination (76). Wnt-Frizzled signalling activates PKA which phosphorylates and activates CREB, resulting in MyoD, Pax3 and Myf5 transcription. At the same time MEF2 is inhibited, leaving the MRFs with no dimerization partner (see below). Cyclins activate CDKs which phosphorylate and inhibit pRb, resulting in MyoD inhibition and degradation, and E2F release and subsequent transcriptional activation (proliferation). Growth factors such as FGF and IGF activate the MAP-kinase and IRS-PI3K-Akt signalling pathways, both promoting proliferation (76). *Upper right quadrant*: Signalling pathways important in late myogenesis, resulting in cell cycle exit and myogenic differentiation (76). Cell-cell contacts and extracellular factors activate the MAP-kinase pathways, which in a network communication-manner, including CDK and Akt signalling pathways, activate MRF-mediated gene transcription. IGF-activated Akt also phosphorylate and activate mTOR and inhibit FOXO, collectively resulting in increased protein synthesis, differentiation and hypertrophy. The GSK3β-mediated inhibition of β-catenin and NFATC3 is inhibited upon Akt-mediated phosphorylation, which, together with RSK2/NFAT3 activation by the MAP-kinase pathway result in transcription of genes involved in differentiation, fusion and cell cycle exit (76). *Centre and right centre panel*: The basic helix-loop-helix MRF-TFs, such as MyoD, heterodimize with members of the MEF2 family at the E box motifs on DNA (43, 44). Protein complexes form, including coactivators (i.e. CDK9), histone modifiers (i.e. p300) and RNA polymerase (76). Via their homeodomain (HD), SIX1/4 TFs bind nearby motifs and contribute to MRF-mediated transcriptional activation of MRFs, miRNA, cyclins D3, pRb, p21 (and other CDK inhibitors, CKIs) and other muscle-specific genes (43-45, 130, 131). *Lower left quadrant*: MyoD-mediated transcription of cyclin D3, pRb and p21 is an important event in myogenic differentiation (131). Akt-mediated inactivation of GSK3β, combined with CKI-mediated inactivation of CDKs, rescue cyclin D3 and pRb from phosphorylation, inactivation and degradation, resulting in cyclin D3-pRb complex-formation and subsequent stabilization in relation to the nucleoskeleton (130-132). As also shown in upper centre quadrant, actions of MyoD and E2F are now reversed. Stabilization of the cyclin D3-pRb complex results in a remodelling of the nuclear lamina and myogenin induction (130). The upstream events result in cell cycle exit (to G0 phase) and subsequent myogenesis. *Lower right quadrant*: miRNA promote efficient shift from proliferation to differentiation (45). MyoD-mediated transcription of miRNAs result in inhibition of HDAC4, PAX7 and MyoR, promoting silencing and degradation of these transcripts (45, 47, 48). As indicated, myokines possibly have important roles at several steps during activation, proliferation and differentiation of myoblasts.

6.3 Expression of myokines during myogenesis

Signaling peptides secreted from muscle cells are known as myokines (36). Interest in myokine research has grown following and parallel to the mapping of secretomes of conventionally considered non-secretory organs (organokines), such as the liver and adipose tissue (133, 134). Researchers hypothesize that myokines may mediate the health promoting effects of exercise, at least in part by counterbalancing the detrimental effects of many adipokines (36). Some proteins, such as IL-6, may be secreted from adipose as well as muscle

tissue, suggested to be known as adipo-myokines (135). Also the arche-adipokine adiponectin is shown to be secreted from skeletal muscle, at least in various mouse and rat models (136). I did not, however, detect adiponectin mRNA in my human primary cell cultures (data not shown).

Researchers have used primary cultures to identify novel myokines during 7 days myogenic differentiation (34, 41). Haugen et al. concludes that their cell culture model may be used to identify novel myokines on the basis of production of IL-6, although the mRNA expression level is unchanged during differentiation (34). Norheim et al. consider their cell culture to be a model of resting, non-contracting muscle cells, but do not identify IL-6 with their proteomics approach (41). However, none of these investigators discuss the usability of the differentiating muscle cell culture system in an embryonical-regeneration-physiological setting. Myokines may clearly be important during myogenesis. In view of the above discussion, differentiating primary cell cultures and cell lines are not directly skeletal muscle. It can be argued that they may be thought of as an intermediate between embryonic muscle tissue development and regenerating adult skeletal muscle. Importantly, the process of forming myotubes during *in vitro* differentiation and fusion of myoblasts closely resemble embryonic development of muscle tissue. Muscle fibers are namely formed before birth, and not in adult skeletal muscle. Satellite cells, on the other hand, fuse with existing fibers in adult skeletal muscle. Furthermore, Norheim and coworkers identified 17 new proteins potentially secreted via the classical secretory pathway in media from cultured human skeletal muscle cells (33). Most of the identified proteins are important in muscle tissue remodelling, and possess extracellular matrix degradation or synthesis function (33). SPARC is not only shown to have anti-tumor activity, but is a matrix scavenger chaperone (38-40). Also, collagen alpha-1 chain, lumican, fibronectin 1, extracellular matrix protein 1 and several members of the peptidase A1 family (cathepsin B, D, H and L1) were identified, all important for matrix remodelling. Hence, I speculate that the secretome identified during differentiation of myoblasts to myotubes (with standard conditions: normal glucose and fatty acid supply, etc) are predominantly auto- and paracrine myokines and matrix proteins, important for development of muscle tissue, and not endocrine myokines. Hence, in order to detect and investigate function of endocrine myokines during activity-related muscle contraction, an exercise model of differentiated myotube cultures is more appropriate. Researchers should use EPS alone, or in combination with addition of *exercise substances*. These can be physiological substances such as cathecholamines, glucocorticoids and pancreatic hormones,

or pharmacological modulators such as ionomycin (increase [Ca^{2+}] intracellularly), AICAR (AMPK activator), forskolin (increase [cAMP] intracellularly), PPARδ and α activators, PGC1α and PGC1α4 activators (61, 62). A carefully designed cocktail of such substances can possibly also be beneficial.

In view of the previous discussion, I wanted to examine the mRNA expression on extablished and potential myokines during differentiation. Of course, I had to measure IL-6. IL-6 is not only a pro-inflammatory cytokine in acute and chronic disease, but a well-established myokine in everyday physiology (137). Summarized, IL-6 is important in metabolic regulation of lipid and glucose homeostasis, muscle hypertrophy, and also the compensatory increase in insulin secretion during insulin resistance (36). I show that *IL6* increases in a time-dependent manner from day 4 to 7 of differentiation. As the glucose, fatty acid and amino acid concentrations are relatively physiological in 1.0 % HS, there is no obvious reason for the cells to crave extra supply of either macronutrient. Also, there is no obvious reason for the cells to be insulin resistant. Hence, I speculate that the increase in *IL6* mRNA expression seen here represent its role in hypertrophy (autocrine and paracrine signalling), rather than its metabolic and hormonal (endocrine) roles. Next, I wanted to examine IL-8. As discussed, there is yet no definite answer to what role IL-8 plays in muscle physiology (135), but it is tempting to speculate. Human IL-8 has, as its murine homologue CXCL1, been linked to the local angiogenic effects of exercise (137). IL-8 can bind to chemokine (C-X-C motif) receptor (CXCR) 1 and -2 on endothelial cells, and in case of the latter receptor, induce angiogenesis. Still, conclusions on this issue is yet to be made. I found no particular pattern of mRNA expression of *IL8* during myogenic differentiation, neither a decrease nor increase. Still, mRNA is detectable, and it is very likely that this is being translated to secretory IL-8 protein, which is an important observation.

As the research community has welcomed the discovery of FNDC5 with discussions and criticizm (138, 139), I wanted to investigate the pattern of FNDC5 mRNA expression during differentiation. FNDC5 was originally discovered as a PGC1α-dependent transmembrane protein in skeletal muscle cells (138). This protein was shown to be posttranslationally cleaved upon physical exercise, releasing Irisin, which enters the bloodstream. The researchers presented evidence for this protein to activate the thermogenic programme in white adipose tissue (WAT) through peroxisome proliferator-activated receptor (PPAR)-α activation (138). Currently, there is an ongoing debate regarding the serum concentration of

Irisin in relation to acute and chronic exercise (139). I found that the mRNA expression of *FNDC5* rapidly increased when initiating differentiation, which thereafter declines and slowly increases and stabilises at approximately 5.5-fold of day 0 of differentiation. As this protein is probably not directed for secretion immediately, the increase in mRNA expression may represent a myogenic stock up of protein for membrane storage. FNDC5 might also have unknown important roles in myogenesis, such as myoblast activation or fusion. As FNDC5 activates peroxisome proliferator-activated receptor (PPAR)-α in adipocytes, the same may possibly occur in myocytes. This may implicate FNDC5 and its posttranslationally modified variants (cleavage, glycosylation, glycation, etc) as regulators of fatty acid metabolism in myocytes, and should be elucidated in future cell culture experiments. Furthermore, the last year, several published reports have presented evidence of circulating Irisin levels to be important in obesity, energy expenditure and diabetes type 2, as well as being secreted from adipocytes (140-143). Also, interestingly, myostatin knockout mice, which exhibit both increased muscle mass and leaner body composition, have been showed to activate the AMPK-PGC1α-Fndc5 pathway in muscle, suggesting a myostatin-Irisin crosstalk (144).

ANGPTL proteins are regulators of fatty acid metabolism, important in regulation of lipoprotein lipase activity, and hence important for circulating levels of triacylglycerol (145-147). 7 different ANGPTLs are described, and more potential family members are being discovered, such as Lipasin (148). To date, ANGPTL2, -3 and -4 are best known. Tissue panels show that ANGPTL7 are expressed in skeletal muscle (149), and may therefore be important in local triglyceride clearance. I found that *ANGPTL7* steadily increases 134-fold in mRNA expression during differentiation. This observation may represent an increasing demand for fatty acids as fuel source as differentiation progresses, but may also be a result of many other processes, such as membrane synthesis. Again, the increase might be a build up of the ANGPTL7 protein stores, if there are any, or ANGPTL7 might have a role in myogenesis that is yet to unreveal. The drastic increase in mRNA expression is nevertheless interesting, and verifies ANGPTL7 as a potential important protein in muscle physiology.

Serpins are a superfamily of conserved proteins that, in most cases, inhibit serine proteases of the chymotrypsin family, and are involved in a broad range of bodily functions, including hormone metabolism, immune regulation, blood pressure regulation, apoptosis and angiogenesis (150). PEDF (SERPINF1) is a potent anti-angiogenic and growth promoting factor (151). Because of its role in anti-angiogenesis, among other things, it has been linked to

antitumor activity (152). Also, PEDF has been shown to be secreted from adipocytes, liver and other cells, and is associated with obesity and insulin resistance (153, 154). Norheim et al. has previously shown that PEDF is a novel myokine (41). I found that *PEDF* mRNA expression increases 5-fold by day 3 of differentiation, decreases, and stabilises at a 3-fold increase compared to day 0 of differentiation. This result was also confirmed on protein level by measuring PEDF concentrations in conditioned medium using ELISA for PEDF. Mean PEDF concentrations increased to 293 ng/mL on day 5 of differentiation, then declined, indicating a lag time compared to the mRNA expression curve. This result is in accordance with a recent paper (77). Here, *in vitro* PEDF secretion is inhibited by brefeldin incubation, which suggests classical secretion, as opposed to non-classical secretory pathways (i.e. shedding from cell membrane, such as FNDC5-Irisin) (77). My PEDF expression results are definitely compatible with the idea of muscle as a organ that regulate angiogenesis both by activation and inhibition, given that angiogenesis is PEDF major role in human skeletal muscle. This is comparable with the above-mentioned myokine myostatin, which inhibits muscle hypertrophy, hindering undesirable levels of muscle mass. PEDF might also, indicated by the serpins diverse roles in humans, have yet unknown roles in muscle physiology.

PLAU (commonly known as uPA) is a serine protease important in plasminogen activation, and hence for fibrinolysis (155). The receptor for PLAU (uPAR) exists in several forms, including various cleaved soluble forms, collectively abbreviated suPARs (155). Serum levels of suPARs reflect inflammation and elevated levels are found in several diseases, such as cancer and HIV (155). PLAU has been implicated in muscle regeneration after damage and in muscle hypertrophy related to strength training (156, 157). This implication of PLAU is independent of its membrane-bound receptor. I found that *PLAU* mRNA expression decreased drastically on initiation of differentiation, and that this continued to stabilise at a 10-fold decrease compared to day 0 of differentiation, which was still a high expression level. This result was also confirmed on protein level by western blots of conditioned medium. PLAU levels in conditioned medium were high on day 0 of differentiation, but undetectable day 1-7 of differentiation. This suggests that PLAU is primarily present in the medium when there is no differentiation (myoblast fusion), indicating that it may have myoblast specificity. This should, in future studies, be confirmed using immunohistochemistry. Studies have previously shown that *PLAU* mRNA expression is FGF-2 and serum-dependent, also indicating that the serum withdrawal used in cell culture studies, may be the cause of these results (158). Still, inhibition of PLAU abrogates myogenesis *in vitro* (159). In addition to these *in vitro* results,

our group have mRNA *in vivo* results from before and after an 11 weeks strength training intervention (fig. 6.4). *PLAU* mRNA expression increases from 0.16 till 0.45. This joins the already presented data on PLAU, changing the picture somewhat. It seems as if mRNA expression of PLAU during *in vitro* differentiation of myocytes decreases, whereas strength training increases its expression. Interestingly, *MYH1* mRNA expression levels stabilised at a higher Ct value than *PLAU*, indicating lower expression. It is very probable that PLAU plays important roles in myoblast activation or recruitment. Studies with PLAU-KO and Plasmin(ogen)-KO mice have shown similar phenotypes, exhibiting fibrin accumulation after muscle damage, reduced infiltration of myoblasts (satellite cells), and reduced tissue regeneration (156). Similar studies have shown reduced muscle hypertrophy (157). This implicates PLAU as an important player in muscle physiology, and future studies should be done to evaluate the exact localization of PLAU intracellularly (immunhistochemistry), its potential matrix remodelling roles, and its roles when binding to its receptor(s). A good model would be a muscle-specific PLAU-KO mouse.

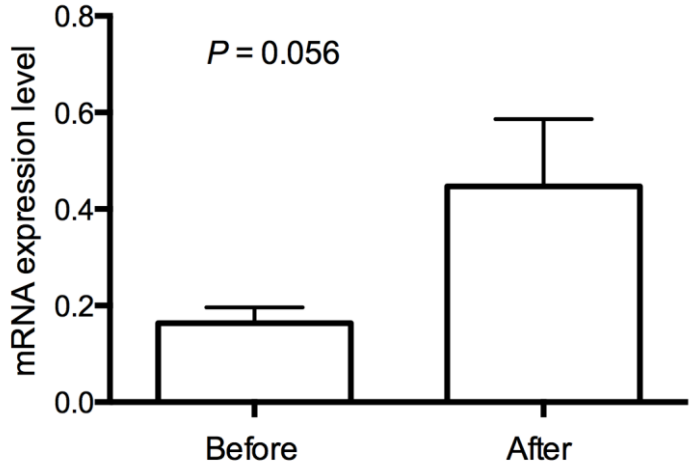


Figure 6.4. *PLAU* mRNA expression levels before and after a 11 weeks strength training intervention. Biopsies were obtained from the upper part of musculus trapezius from untrained male subjects (n=13) before and after an 11 weeks strength training intervention, as described in (41). mRNA expression is related to the endogenous control RPLP0. Error bars represent SEM. Data presented with permission from the investigators.

6.4 Future perspectives I: culturing human skeletal muscle cells

Human muscle cell cultures used in research should be investigated for experimental applicability. As mentioned previously, well-characterized continuous muscle cell lines are easy to culture, whereas human primary and cell line cultures can be more difficult. Hence, I here present steps that can increase the control and reliability of human skeletal muscle cell cultures. This can increase both the reproducibility and trustworthiness of the results produced in the subsequent experiments.

1. Following isolation of satellite cells from biopsies, isolate a *pure* satellite cell fraction with fluorescence-activated cell sorting (FACS) using both positive and negative selection markers. In humans, positive selection for cell surface molecules M-cadherin^{+} and CD56^{+} detects satellite cells (93, 160). Negative selection for cell surface molecules CD34^{-}, CD144^{-} and CD45^{-} removes residual contamination of haematopoietic, endothelial and white blood cells, respectively (41). In mice, many more markers exists, extensively reviewed in (93, 160). Alternatively, purity of cultures used in experiments can be tested using flow cytometry of random samples.

2. Establish proliferation and differentiation media. High growth factor content typically promote proliferation, and low content promote differentiation. Potential factors include the HGFs, FGFs, insulin, IGFs, members of the IL-6 family, NO, PDGF, EDGF, EGF, testosterone and dexamethasone (42, 51, 74).

3. Characterize the satellite cell activation kinetics (161). Yablonka-Reuveni et al double-stained cultures using antibodies for MyoD^{+}/Pax7^{+} cells, and related this to total amount Pax7^{+} cells (161). After FACS, all cells seeded should be Pax7 positive, and most cells should be in a state of myogenic activation (MyoD^{+}) or determination within a few subsequent days. Determining kinetics using RT-qPCR and western blotting of MRFs and progenitor markers are applicable alternatives.

4. Characterize the proliferation kinetics of the culture (161). Besides counting cells, many methods are available, including the [^{3}H]thymidine or BrdU incorporation assays which measures rate of DNA synthesis (42, 161). The proliferation and differentiation media can constitute positive and negative experimental controls, respectively.

Alternatively, genomic DNA and total protein content are examples of indirect cell number measurements (162). These are frequently used for normalization of biological samples. Importantly, growth curves can be determined, which might provide information of how long cultures should be proliferated before initiating differentiation.

5. Characterize the differentiation kinetics of the culture (161). Yablonka-Reuveni et al double-stained cultures using antibodies for $CyclinD3^{+}/MyoD^{+}$ cells, and related this to total amount $MyoD^{+}$ cells (161). Cyclin-dependent kinases (CDKs), CDK inhibitors (CKI), CyclinD3 and pRb protein control terminal myogenic differentiation by regulating cell-cycle withdrawal (into G0 phase) and expression of genes encoding for structural proteins, and may present as potentially good markers of differentiation status of cultures (55, 161). Determining kinetics using RT-qPCR and western blotting of these gene products and proteins are also applicable alternatives.

6. Differentiated cultures should exhibit phenotypic traits of muscle cells *in vivo*. Tube- and fiber-like structures should appear as myoblasts fuse and differentiation progresses. Expression of the transcriptional coactivator PGC1α and the muscle-specific insulin-dependent glucose transporter GLUT4 should increase both on mRNA and protein level. Expression of myosins, actin, dystrophin, troponins and other muscle-specific proteins should increase both on mRNA and protein level. Upon low-frequency EPS stimulation, contraction may be visible in a microscope. Furthermore, the the muscle cell cultures can be evaluated with respect to muscle size. Ruas et al. used the ratio between total protein and genomic DNA to evaluate muscle hypertrophy between cell cultures with increased expression of splice variants of PGC1α (106). Fiber type dominance of the cultures can also be determined on basis of the myosin and ATP-dependent sodium-potassium channel variants (49, 163, 164).

In summary, future work with human skeletal muscle cell cultures should, at least to some extent, be investigated for experimental applicability. This might increase both the reproducibility and trustworthiness of subsequent experiments. The presented protocol is very comprehensive, and I am aware that high-quality research can be performed with far less characterization.

6.5 Future perspectives II: how to study myokines

The myokine research was long dominated by a few scientists, like Bente Klarlund Pedersen in Denmark, who has written most of the reviews in the field (36, 165-168). Exercise was associated with health benefits, and the idea of an *exercise factor* had been in focus (169). Early research showed that electrical stimulation of muscles of paralyzed individuals induced many similar physiological changes as individuals with intact nervous signaling, suggesting that humoral factors secreted from contracting muscle may mediate some exercise-related changes (36, 170, 171). Interestingly, Steensberg et al. measured arterial-femoral venous IL-6 concentrations and blood flow, and showed a muscle-specific increase in IL-6 release with time (172). The arterial concentration was 17 times higher than the amount that accumulated in plasma (172). Since this study, other methods have mainly been used in scientific studies.

6.5.1 Controlled trials, animals studies, cell cultures and systems biology

Contolled exercise intervention studies may be performed on humans and animals, and blood and tissue samples can be collected, in addition to imaging of skeletal muscle, liver and pancreas, adipose tissues and other organs. Beyond the above-mentioned arterial-femoral venous differences, *in vivo* measurements in humans may involve microdialysis for measurement of interstitial concentrations of myokines, which may reveal local (autocrine and paracrine) and systemic (endocrine) distribution differences (36). Infusion of recombinant human myokines in combination with organ function testing may provide important *in vivo* results, especially if performed during various types and intensities of exercise. An example is to measure endogenous glucose production from liver during infusion of recombinant human IL-6, or to measure hepatic and adipose tissue clearance of free fatty acids during infusion of recombinant myonectin (37, 173). Blood samples should be collected at time points during and after exercise to investigate the dynamics of endocrine myokines. Also, in large epidemiological datasets where objective measurements of physical activity level and fasting blood samples may be available, myokines can be investigated as markers of physical activity (chronic exercise). This approach requires validation with experimental studies. Furthermore, tissue samples can be collected as biopsies. This may provide large amounts of biological data, such as mRNA and protein expression, which can be related to blood samples. Also, visualization of proteins in tissue sections using *in situ* hybridization or

immunohistochemistry may give valuable information about presence and localization of myokines. Tissue samples can also be important for determining distribution of specific myokine receptors.

Animal exercise interventions can also be performed, providing valuable molecular understanding and evidence. Myokine knockout mice, general or tissue-specific, may be good models when investigated at rest and during physical activity. These should be supplied with myokine knockdown studies, preferentially organ-specific, using siRNA or antibodies. Moreover, injections and osmotic pump delivery of myokines may allow *in vivo* investigation of biological effects. Such animal studies should be performed before human studies. Furthermore, mice with overexpressed genes, such as muscle-specific Akt overexpression may provide informative results (174).

Cell culture studies can be performed using primary cells isolated from human biopsies. As they are easier to culture, animal cell culture studies are also important, although human cells provide a more direct understanding of human physiology. Cell culture is used for multiple molecular biology research areas, such as mRNA and protein expression, and protein secretion investigations. Also, cultures can be stained using immunohistochemistry to visualize the presence and localization of proteins in relation to exposures in a time-dependent manner. One important use of cell culture is the characterization of molecular mechanisms. By using an arsenal of techniques, including gene knock-down with siRNA/shRNA, protein knock-down using antibodies, gene up-regulation by introducing expression vectors with constitutively active promoters, and addition of recombinant protein, molecular effects can be revealed and characterized. Signaling pathways can be characterized using molecular inhibitors and modulators such as brefeldin, cyclosporine A, cycloheximide and forskolin. Cell cultures also allow single-cell measurements (*n=1 investigations*) by using single-cell qRT-PCR (108). Cell culture studies also may be used to investigate molecular differences between cells isolated from phenotypically different individuals, for example myocytes isolated from healthy vs diabetic individuals. This can include differences in proliferation and differentiation, myokine secretion during differentiation, response to EPS and response to use of exercise mimetics, among others.

Physical activity and exercise can offer one of the most extreme situations the organism ever has to cope with (21). The reductionistic research approach has provided great progress in understanding physical activity and other physiological and patophysiological phenomenon,

but fail to completely appreciate organism complexity (175). Understanding the systems biology (the integrated interpretation of complex physiology of cells, organs and organ systems) is necessary for a complete view. The reductionistic research, such as my own, can only contribute with an insignificant proportion of this view (exemplified in fig. 6.5). The systems biology approach can be used to study complex molecular effects of exercise, and specifically: to *identify and characterize myokines*. MacLellan and coworkers reviewed the system-based approaches to cardiovascular disease (33, 175). They present five main steps, which can be transferred to the myokine field: 1) define the system to investigate, i.e. exercising human muscle tissue; 2) decide which information that should be obtained, i.e. gene expression data; 3) determine interactions between components, i.e. co-variation between genes; 4) investigate dynamics in the system, i.e. time-dependent gene-interactions; 5) validate the findings with experimental perturbations, i.e. *in vitro* or animal research (33, 175). Norheim et al. used a proteomics approach to identify 236 secreted proteins in conditioned medium from cultured human myocytes (41). Furthermore, they found that 15 of these myokines altered mRNA expression in muscle biopsies after 11 weeks of strength training (41). Other researchers have found large secretomes in human muscle cells, rat L6 and murine C2C12 muscle cells using similar methodology (36).

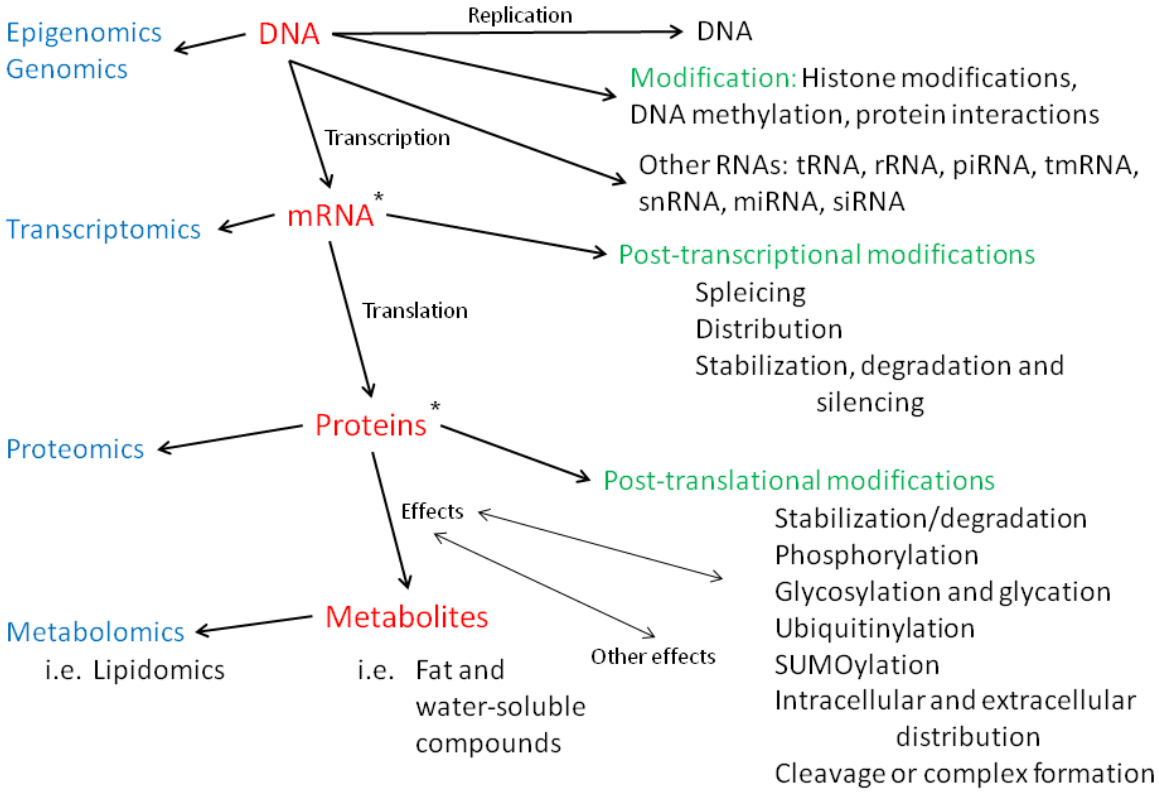


Figure 6.5. Systems biology and the central dogma of biology. Systems biology is the integrated interpretation of complex physiology of cells, organs and organ systems. Scientific discoveries must be interpreted in this context. DNA is replicated to DNA and modified by protein interactions (histones and others), histone modifications, and DNA methylation. Various RNA transcripts are produced, and mRNA can be post-transcriptionally modified by spleicing, stabilization and degradation, silencing and transportation. Translation of mRNA produces polypeptides with primary structure, which subsequently assume secondary, tertiary and quatenary structure and are post-translationally modified (PTMs). The biological effect (function) of the protein can be changed by PTMs. Whereas mRNA, rRNA and tRNA are important for protein synthesis, other RNAs and proteins have a wide range of biological effects, including regulation cycles (feedback/forward). The flux of metabolites through cells, organs and organ systems are controlled by such regulation cycles in order to maintain homeostasis. Short term regulation include reversible covalent modifications of proteins (such as phosphorylation and UDP-GlcNAcylation/glycosylation), intermediate regulation include changes in cellular behavior (such as traffic and mRNA stability), and long-term regulation include genome modification and gene transcription (induced both directly, and by upstream signalling, such as phosphorylation). The epigenomics, genomics, transcriptomics, proteomics and metabolomics are comprehensive analyses (characterization and quantification) of the epigenome and genome, transcriptome, proteome and metabolome in cells or tissues. Novel state of the art-techniques are available for data collection, and advanced bioinformatics can be used to gain insight into a complex understanding of the systems biology. Note that the stars designate the areas of the molecular biology which I have investigated in my thesis, and that there are many more areas that could be explored. Adopted from (33).

6.5.2 EPS and co-culture

The focus of the myokine research field has changed from endocrine myokine function to *all* potential functions of myokines, including paracrine, autocrine and others (such as extracellular matrix components, i.e. collagen). Regardless of how biologically interesting this may be, in my view, it is important to narrow the search, and identify those myokines with important endocrine (systemic) function. This can be done by performing *in vitro* system-based investigations of the myokinome under strickly defined exercise conditions. EPS is such an example, alone or in combination with co-culture. As stated, EPS experiments are of main interest to our research group, and published reports have already shown several uses for EPS. In March 2013 PhD student Marit Hjort summarized the evidence for use of EPS in myotube cell culture to increase sarcomere assembly (176), myokine production that resembles physical activity (177-180), a shift towards oxidative metabolism (Hjort M, unpublished results) and increase insulin sensitivity (Eckardt K, unpublished results). New applications for EPS will probably be discovered as research progresses. For example, EPS has also been used to unravel the molecular basis for IL-6 release during contraction-related glycogen depletion in muscle cells (178). This was an important finding, as IL-6 otherwise have been associated with obesity and insulin resistance. Similarly, other low-grade chronic inflammation-related circulating myokines, such as PEDF and PLAU, needs to be characterized on a molecular level to understand the physiological roles as myokines. A potentially good *in vitro* model of exercise is that of combined EPS and co-culture. Much research is focused on the detrimental effects of adipokines on insulin sensitivity, especially in adipocytes, hepatocytes and myocytes. For example, adding conditioned medium from one cell type, such as adipocyte conditioned medium, into cultures of hepatocytes or myocytes, with subsequent investigation of molecular effects of EPS, might be a good model. Researchers in Jürgen Eckels group in Düsseldorf are currently executing such pioneer experiments, with results confirming its applicability. Shortly, using western blotting to map signal transduction (such as anti-pIRS, anti-pAkt, anti-GSK3), they show that combining conditioned medium from adipocytes with myocyte cultures decrease activity of downstream insulin signaling. Application of EPS to the cultures restored insulin signaling to normal levels (Eckardt K, unpublished results). Other interesting combinations would be to investigate the hepatokinome in relation to muscle insulin sensitivity. For example, does the hepatokinome influence the muscle insulin resistance when adipokine dysregulation (as in obesity) is present? Does the exercise myokinome affect insulin sensitivity in hepatocytes?

How much overlap do the various secretomes exhibit? A model of EPS in combination with co-culture could help answering some of these questions.

7 Conclusion

In this thesis I have examined the process of differentiating cultured human myoblasts to multinucleated myotubes, with purpose of developing an *in vitro* human skeletal muscle model of exercise. My main results are:

1. Romanowsky staining and subsequent fusion index determination is an easy and cheap method of evaluating differentiation. Initiating differentiation before complete confluence is required to prevent overgrowth, and to be able to distinguish between nuclei in myotubes and myoblasts.

2. There is no statistically significant difference in degree of differentiation between various concentrations of serum and between different types of serum. Both 0.5 and 1.0 % HS result in well-differentiated muscle cell cultures, exhibiting at best 67 % of all nuclei inside multinucleated myotubes.

3. Dexamethasone inhibit differentiation in a dose-dependent manner.

4. Increasing concentration of insulin in SFM promotes a tendency towards lower differentiation, but in HS insulin does not influence differentiation.

5. Differentiating in both 1.0 and 2.0 % HS gives reduction in mRNA expression of PAX7, and increase in expression of myogenin, myosin, alpha-actin, PGC1α and GLUT4 throughout differentiation. However, there are no statistical significant difference between the serum concentrations. Protein expression of myosin and actin increase during differentiation, reaching highest expression on day 7.

6. mRNA expression of FNDC5, ANGPTL7 and PEDF all increase during differentiation, whereas PLAU decrease. Expression of IL-8 shows a minor increase, and adiponectin is undetectable.

7. PLAU and PEDF proteins are secreted to the medium in a pattern corresponding to the mRNA expression. PLAU decreases, whereas PEDF increases throughout differentiation.

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Supplemental materials

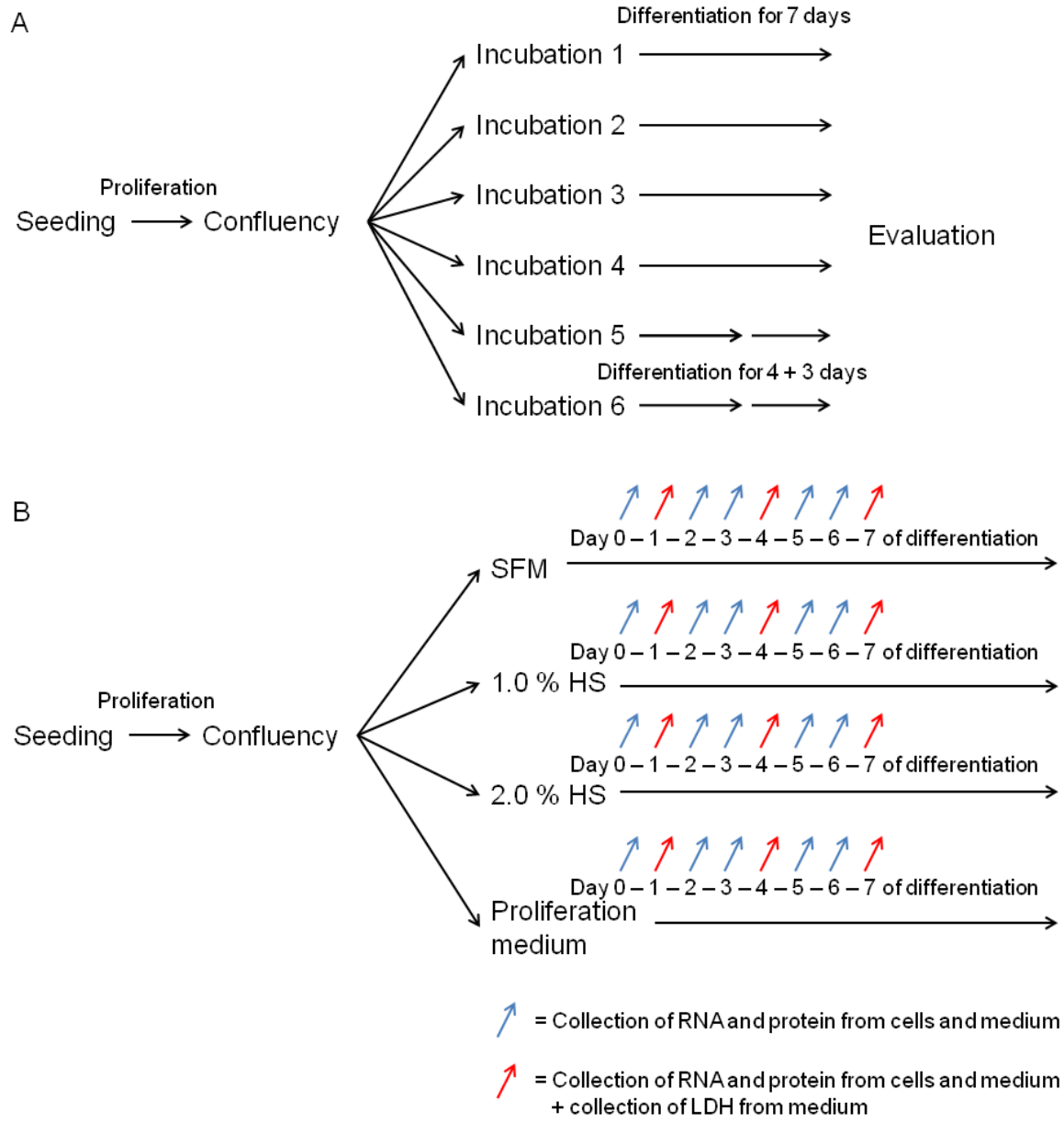

Figure S1. Design of experiments. *A*, general outline of the 4 experiments performed to visually evaluate differentiation, results presented in figure 5.3. Cells were seeded on collagen-coated 6 well plates, proliferated until confluency, and differentiated for 7 days in serum reduced medium. Exact composition of the differentiation media is specified in figure 5.3. The cell cultures were fixed and stained with Romanowsky stain, and evaluated by manual nuclei counting. *B*, outline of the differentiation kinetics experiments, results presented in figures 5.3-5.9. Cells were seeded on collagen-coated 12 well plates, proliferated until confluency, differentiated in 4 different media: DMEM/F12 (1:1 ratio) without any added serum (SFM), DMEM/F12 (1:1 ratio) with 1.0 % HS (1.0 % HS), DMEM/F12 (1:1 ratio) with 2.0 % HS (2.0 % HS), or DMEM/F12 (1:1 ratio) with 20.0 % FBS, 1 µg/mL insulin, 10 ng/mL hEGF, 2 ng/mL hbFGF, and 0.4 µg/mL dexamethasone (proliferation medium). Biological material was collected daily, as indicated by blue and red arrows, and stored as described in Methods.

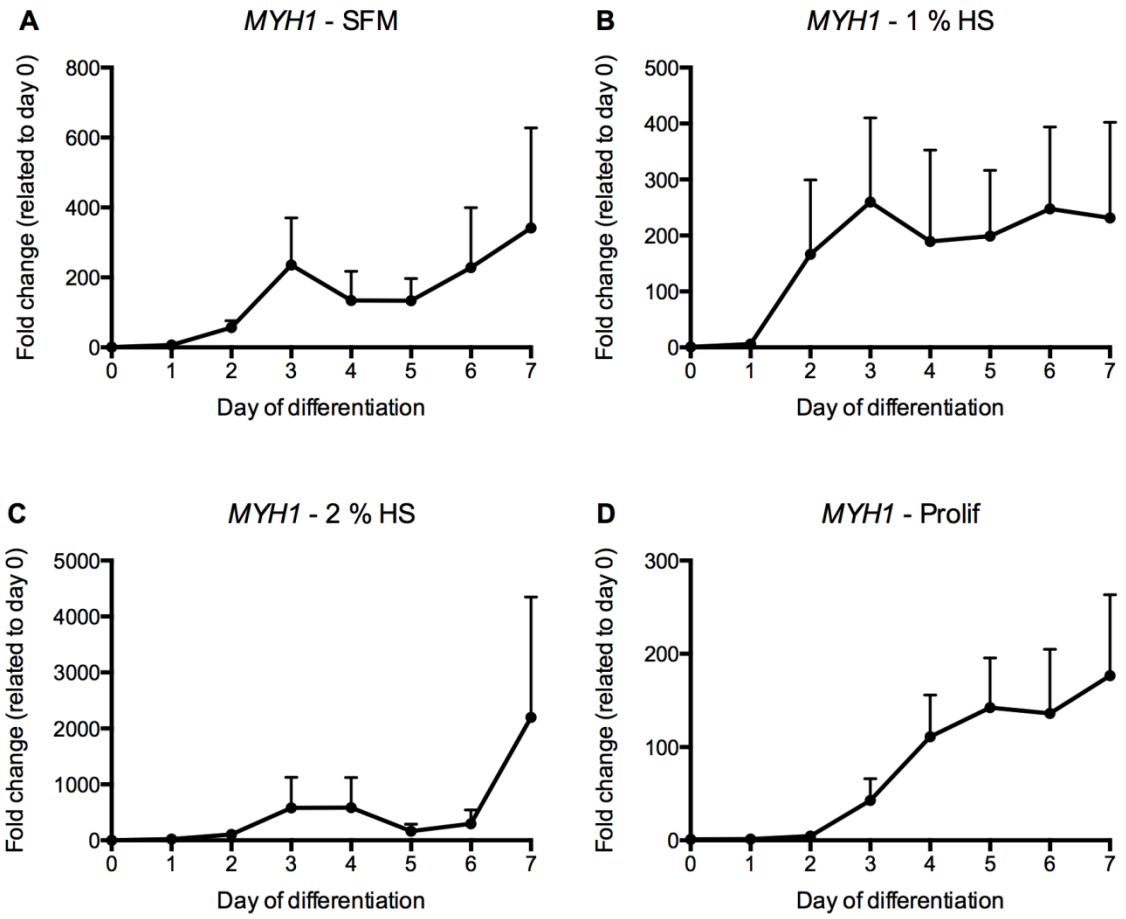

Figure S2. Variation in *MYH1* mRNA expression between groups. Confluent cultures were differentiated for 7 days in either serum free medium or medium containing 1.0 % HS, 2.0 % HS or 20.0 % FBS, 1 µg/mL insulin, 10 ng/mL hEGF, 2 ng/mL hbFGF, and 0.4 µg/mL dexamethasone. The experiments were performed three times, once with LD30 and twice with EO2. All experiments were performed using passage 5. Error bars represent SD. Note: large differences between incubation groups on the y axis – 2.0 % HS seem to provide increased *MYH1* mRNA expression.

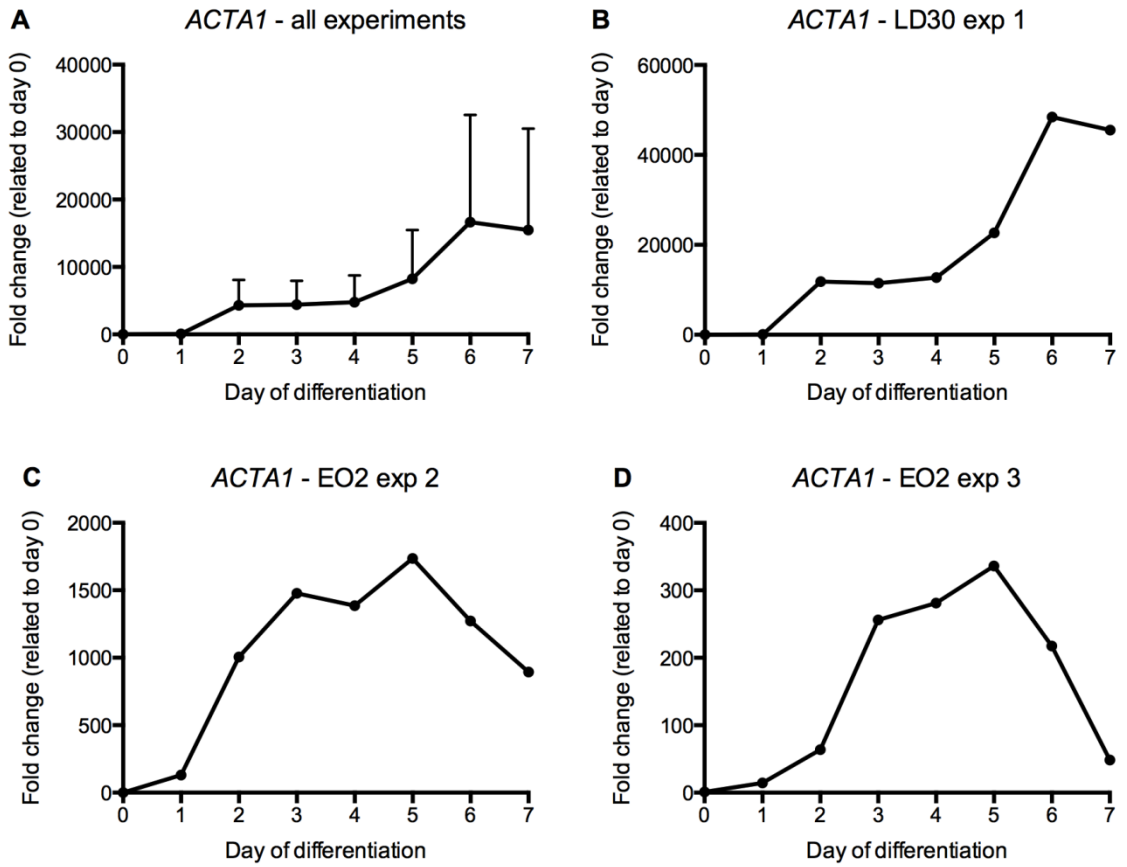

Figure S3. Variation in *ACTA1* mRNA expression response of two donors (LD30 and EO2). Cultured myoblasts were differentiated for 7 days in DMEM/F12 (1:1 ratio) + 1.0 % HS. The experiments were performed three times, once with LD30 (*B*) and twice with EO2 (*C* and *D*). All experiments were performed using passage 5. Error bars represent SD. Note: large individual differences in levels of expression results in large standard deviations.

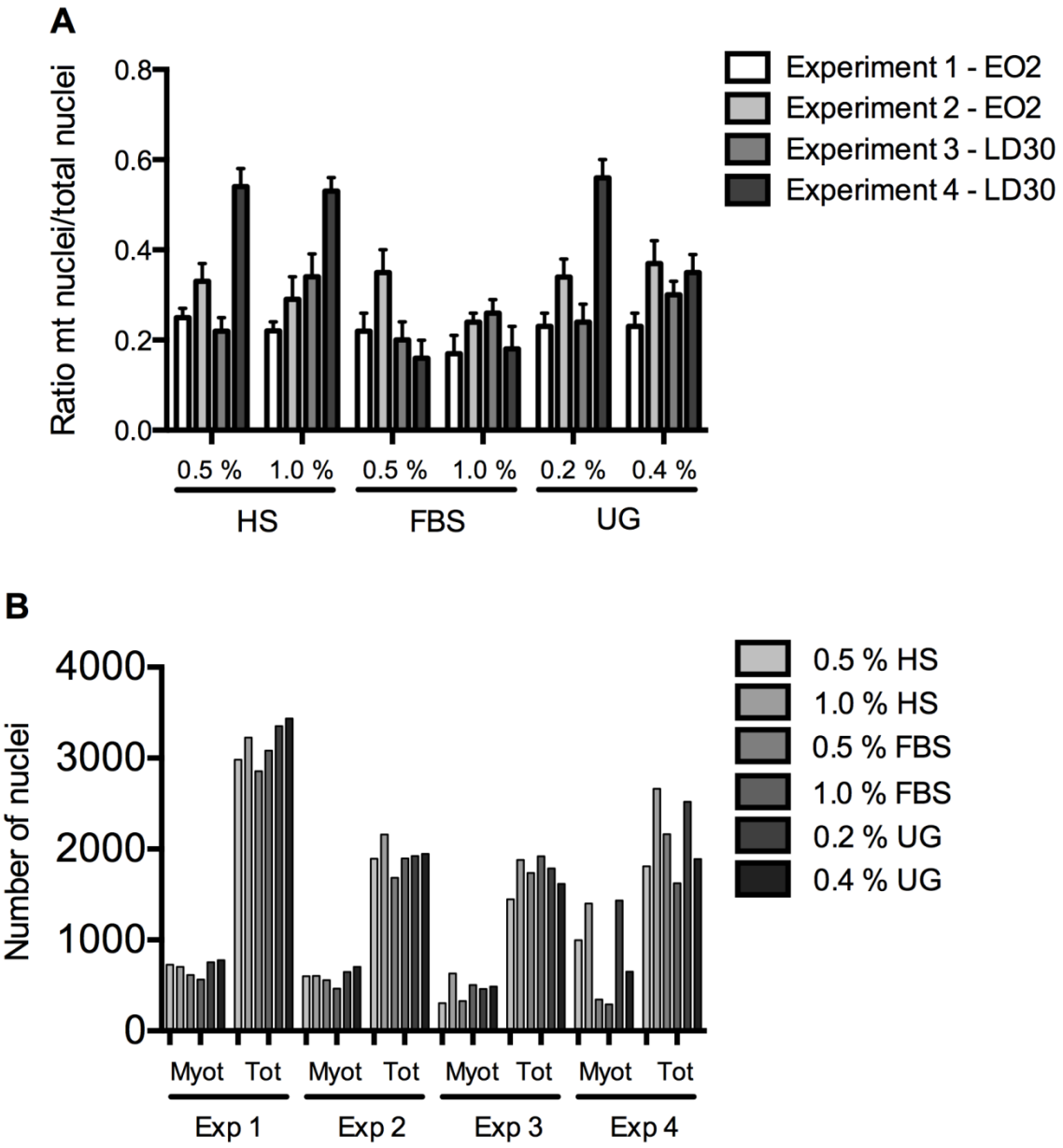

Figure S4. Variation in counting within four similar experiments. *A* and *B*, myoblasts were proliferated until confluence, then cells were differentiated for 7 days in 0.5 % HS, 1.0 % HS, 0.5 % FBS, 1.0 % FBS, 0.2 % UG or 0.4 % UG. Cells were fixed, stained and counted as described in Methods. Results are shown as four individual experiments. All experiments were performed using two donors (EO2 and LD30). *Myot* = Myotube; *Tot* = Total. Note the decisive differences in experiment 4, compared to experiment 1-3.

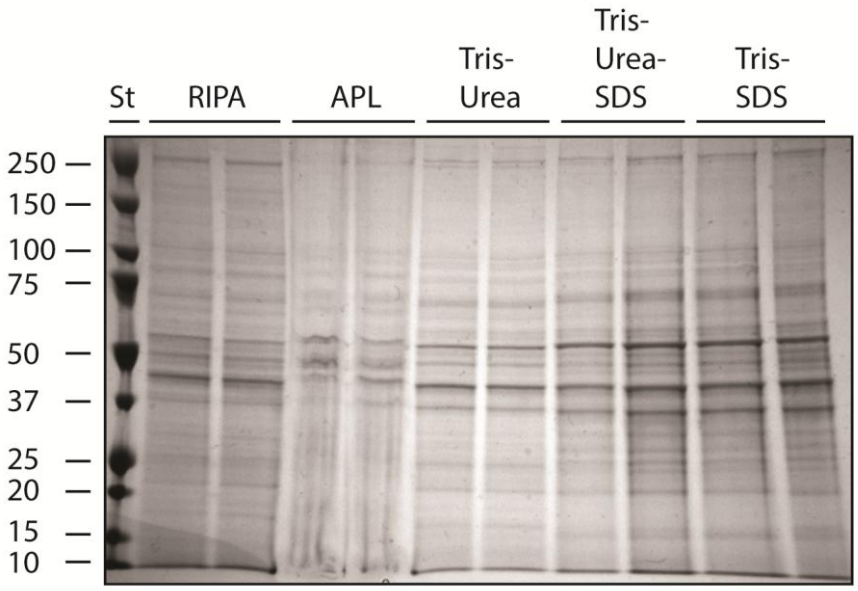

Figure S5. Validation of a Tris-SDS buffer and the RNeasy Mini Kit RNA and protein isolation procedure as compatible for SDS-PAGE. Cells were seeded, proliferated until confluence, and differentiated for 7 days in 1.0 % HS. Intracellular proteins were isolated and prepared by collecting flow through from spin columns, followed by washing and precipitating of the protein fractions, and dissolving in different buffers. The RIPA buffer consisted of 150 mM NaCl, 1.0 % Triton X-100, 0.5 % Na-deoxycholate, 0.1 % SDS and 50 mM Tris (pH 8.0). The Tris-Urea buffer consisted of 100 mM Tris and 8 M Urea (pH 8.0). The Tris-Urea-SDS buffer consisted of 100 mM Tris, 8 M Urea and 2.0 % SDS (pH 8.0). The Tris-SDS buffer consisted of 100 mM Tris and 4.0 % SDS (pH 8.0). APL is the AllPrep RNA/Protein kit (Qiagen, cat. no. 80404), which is a double-column based isolation procedure, and proteins were dissolved according to manufacturers instructions. Protein concentration was measured using BCA assay. Equal amounts of protein was loaded onto SDS-PAGE for separation, and visualized with Coomassie gel staining. A standard protein ladder was added for molecular weight recognition. Note: I participated in the work of this validation, but did not have the main responsibility.

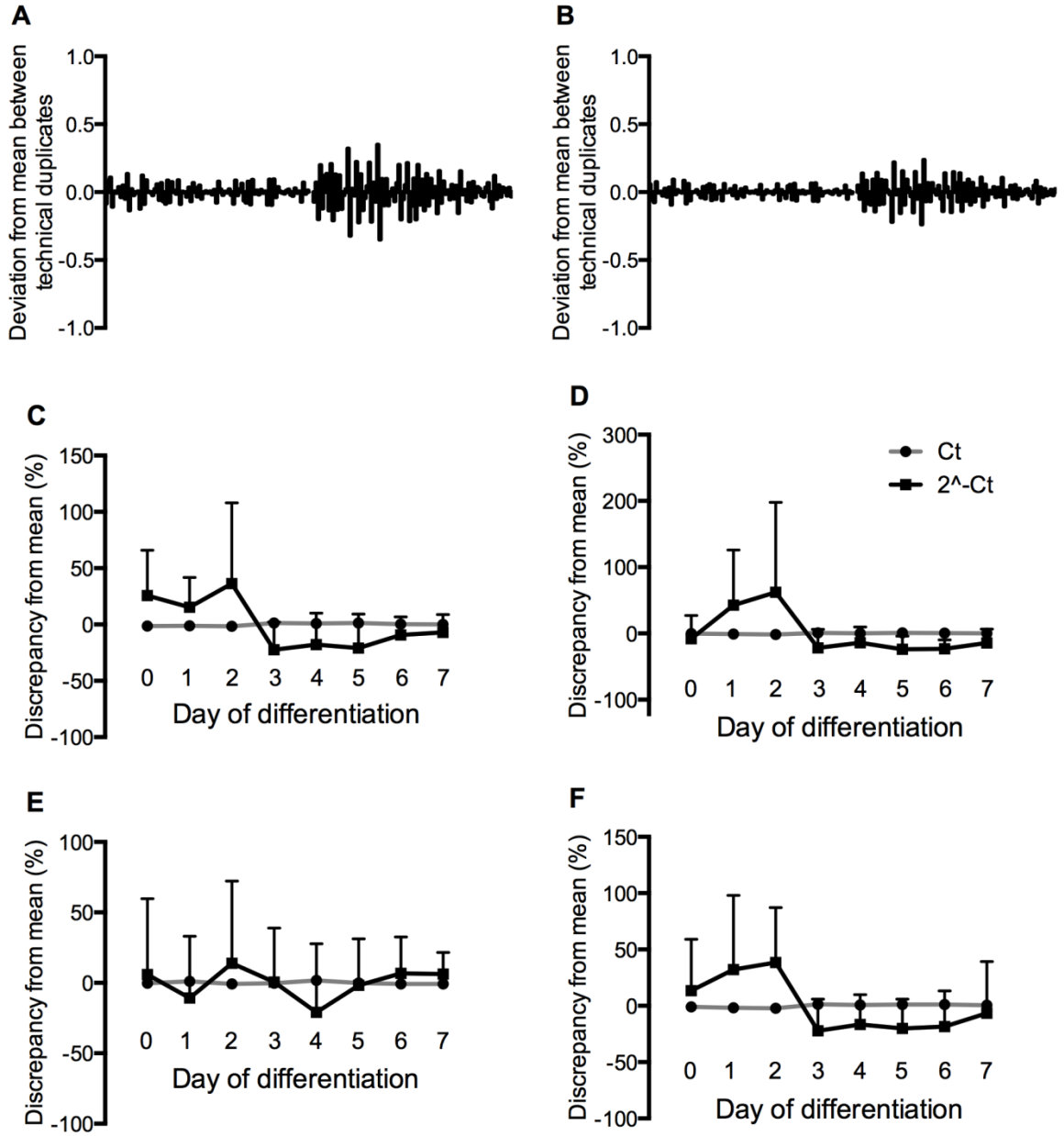

Figure S6. Variation in pipetting and RPLP0. *A* and *B*: Variation in pipetting between technical duplicates. *A*, Ct values; *B*, 2^{-Ct} values. This is a randomly selected dataset from the processing of RT-qPCR data, comprising 8 individual samples and 8 different primers on two 96 well plates (a total of 192 values). Note that the deviation from mean between technical duplicates is not above 0.5, indicating that the pipetting variation is low. This dataset has a mean (min-max) coefficient of variation (CV) of 0.36 (0.02-1.36) % for Ct values and 2.33 (0.34-33.56) % for 2^{-Ct} values. *C-F*: Variation in the endogenous control RPLP0 during differentiation. Confluent cultures were differentiated for 7 days in either serum free medium or medium containing 1.0 % HS, 2.0 % HS or 20.0 % FBS, 1 µg/mL insulin, 10 ng/mL hEGF, 2 ng/mL hbFGF, and 0.4 µg/mL dexamethasone. The experiments were performed three times, once with LD30 and twice with EO2. All experiments were performed using passage 5. Error bars represent SD. Statistical testing have been performed using Kruskal-Wallis test between each time point during differentiation.