Regulation of Mechano Growth Factor in skeletal muscle and heart

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(met een samenvatting in het Nederlands)

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Alles komt goed…

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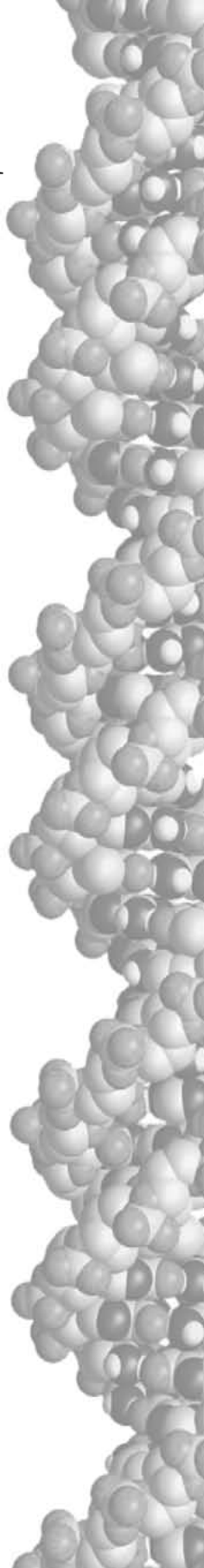
Chapter 1

General Introduction



The discovery of the Mechano Growth Factor

The Mechano growth factor (MGF) was discovered by the research group of Goldspink in 1996. The scientists were especially interested in the factors involved in autocrine regulation of muscle mass, because the hypertrophy after exercise is restricted to the muscles that have been involved in the muscular work, while the resting muscles do not change (41). The rabbit anterior tibialis (TA) muscle was used as an *in vivo* model, because muscle mass increased by 35% within a week when this muscle was electrically stimulated in a stretched position (19). Goldspink *et al.* extracted mRNA from exercised and resting TA muscles and subsequent sequence analysis showed that the stretched rabbit TA muscle strongly expressed two types of the insulin-like growth factor 1 (IGF-1) mRNA. The first one identified was IGF-1Ea as a muscle specific IGF-1 isoform of the systemic liver-type. The second one differed from IGF-1Ea by the presence of a 52 bp insert in the E domain (figure 1). Because this splice variant was only present in the stretched muscle it was named the Mechano Growth Factor (MGF). Later MGF was classified as IGF-1Eb in rodents and IGF-1Ec in humans (23). In contrast to normal muscle, the mRNA of MGF is not detectable in dystrophic muscles when subjected to stretch and/ or electrical stimulation (21).

Generation of MGF by alternative splicing of the IGF-1 gene

The IGF-1 gene is highly conserved among mice, rats and humans (35). The IGF-1 gene is the product of a single-copy gene located in humans at the long-arm on chromosome 12. The gene contains six exons and five introns (figure 1), spanning more than 80 kb (8). IGF-1 transcripts can be derived from two different promoter sequences adjacent to exon 1 or 2. Transcripts encoded by exon 1 are the major isoforms in skeletal muscle and most other peripheral tissues, whereas the exon 2-initiated transcripts are predominantly expressed in the liver and represent the majority of circulating IGF-1. The sequence of exon 1 is highly conserved among mammalian species and contains multiple initiation sites and regulatory. Among these exon 1 promoter regulatory elements are sequences responsive to muscle regulatory factors (MRFs), calcineurin/ NFAT-responsive elements, cAMP and prostaglandins that function in skeletal myocytes. Exon 1 or 2 also encode the first part of the signal peptide. Exon 3 and 4 are invariant and encode the remaining portion of the signal peptide and the mature IGF-1 peptide. The rest of the E-peptide is encoded by exons 5 and (or) 6. Most IGF-1 transcripts skip exon 5 and splice exon 4 directly to exon 6. The MGF isoform results from a novel splice acceptor site in the intron between exons 5 and 6, altering the structure of the C-terminus. The inclusion of exon 5, which is 49 nucleotides in length in human

(and 52 bp in rodents), causes a frame shift in the open reading frame of the subsequent exon and gives rise to a premature stop codon within exon 6 (20, 36).

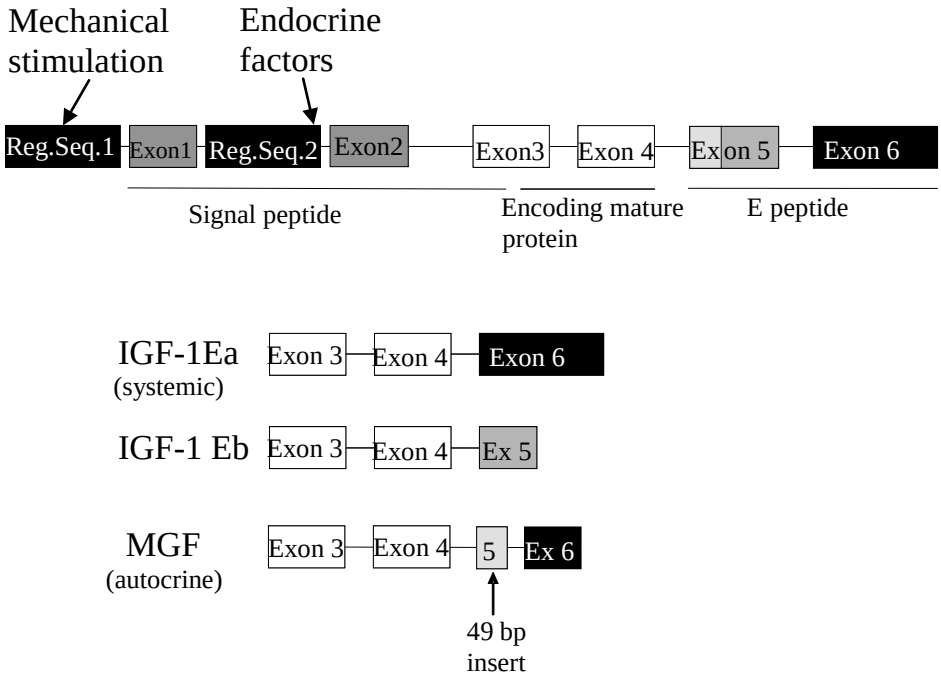


Figure 1. *Schematic representation of the structure of the human IGF-1 gene and its splice variants* (Adjusted and redrawn from: (6, 17, 20)).

Regulation of MGF expression in skeletal muscle

To better understand the regulation of MGF *in vivo*, multiple animal models have been used. For example, when TA muscles of rats were subjected to mechanical loading, MGF as well as IGF-1Ea mRNA were both induced (25, 26). MGF was induced at day 1 and decreased in time to control levels. In contrast, IGF-1Ea increased progressively in time to a maximum level at day 7. Other experiments were performed on human subjects who underwent different resistance training regimes and MGF and IGF-1Ea were determined on mRNA level. The results of these studies were comparable to that of the animal studies: the increase in MGF preceded that of IGF-1Ea (23).

To investigate how mechanical signals influence IGF-1 gene splicing in regenerating/ developing muscle, Cheema *et al.* have grown C2C12 cells (immortalized muscle cells) in a three-dimensional culture and subjected the myotubes to different regimens of mechanical strain (13). IGF-1Ea was found to be constitutively ex-

pressed in myoblasts and myotubes and its expression was up-regulated 8 hrs after a single ramp stretch but was reduced by repeated cyclical stretch. In contrast, MGF showed no expression in static cultures, but was up-regulated by a single ramp stretch too, but also by cycling loading. This result was in alignment with *in vivo* results, where levels of MGF were too low to be detected in unstretched muscle and MGF was only detected, once a muscle had been stretched (19). These studies indicate that MGF can be induced by mechanical loading *in vitro*.

Severe muscle stretch could easily lead to tissue damage and therefore, MGF might be induced by local or systemic factors involved in the response to damage, apart from direct induction by mechanical loading. As an *in vivo* approach to investigate if MGF is also expressed after muscle damage, TA muscles of rats were injected with the local anaesthetic bupivacaine and MGF and IGF-1Ea mRNA levels were determined (25, 26). At the site of injection, muscle tissue was fully destroyed. MGF and IGF-1Ea mRNA were found to be both up-regulated after severe muscle damage. These results suggest that MGF is not only expressed after mechanical loading of skeletal muscle but also after severe muscle damage.

The group of Popov hypothesized that MGF is induced during muscle contractions because of the production of heat and lactic acid (28). When C2C12 cells were cultured at temperatures of 40 ºC and/or low pH (6.3-6.7), MGF expression on mRNA and protein level was increased 14-fold in myoblasts and 10-fold in myotubes. The C2C12 cells were also subjected to other stress stimuli like hypoxia, hyperosmosis, hyperoxia or lipopolysaccharide exposure. None of these other stress stimuli caused any activation of MGF synthesis neither in myoblasts nor in myotubes (28). In conclusion, MGF can be induced by severe muscle damage *in vivo* and by hyperthermia and acidification *in vitro.*

Regulation of MGF during regeneration of the skeletal muscle

Adult mammalian skeletal muscle is a stable tissue with little turnover of nuclei (11). Minor lesions occur every day leading to a slow turnover of its multi-nucleated muscle fibers. Nonetheless, mammalian muscle has the ability to complete a rapid and extensive regeneration in response to severe damage. Damage can be inflicted by direct trauma like extensive physical activity or by indirect trauma like innate genetic defects. Muscle regeneration is characterized by two phases: a degenerative phase and a regenerative phase.

Necrosis of the muscle fiber is the first step in muscle degeneration. This event is generally triggered by disruption of the myofiber sarcolemma resulting in increased myofiber permeability. Disruption of the myofiber is reflected by increased serum levels of muscle proteins such as creatine kinase. This protein is in intact fibers restricted

to the myofiber cytosol (3). It is also hypothesized that an increased calcium influx after sarcolemmal or sarcoplasmic reticulum damage results in a loss of calcium homeostasis and increased calcium-dependent proteolysis that drives tissue degeneration (7). Calpains are calcium-activated proteases that can cleave myofibrillar and cytoskeletal proteins and are therefore thought to play a role in the process of degeneration (7). Thus disrupted myofibers undergo autolysis depending on the extent of the injury.

The early phase of muscle injury is usually accompanied by the activation of inflammatory cells (39). Factors released by the injured muscle fiber attract neutrophils to the site of inflammation and in a later stage also macrophages. Macrophages phagocytose the necrotic parts of the muscle fiber and they may also affect other aspects of muscle degeneration by activating myogenic cells (31). Thus muscle fiber necrosis and an increased number of non-muscle mono-nucleated cells within the damaged region are the main histopathological characteristics of the early steps in the process of muscle injury.

Muscle degeneration/necrosis is followed by the activation of a muscle repair process. Cellular proliferation is an important event necessary for muscle regeneration. Many nuclear radio-labeling experiments have demonstrated the contribution of dividing myogenic cells to regenerating myofibers and it is well accepted that following the myogenic proliferation phase, new muscle fibers are formed due to differentiation of myogenic cells and fusion to existing damaged fibers (4). These myogenic cells are called satellite cells. Once fusion of satellite cells with the existing myofiber is completed, the newly formed myofibers increase in size and myonuclei move to the periphery of the muscle fiber (figure 2). Under normal conditions, the regenerated muscle is morphologically and functionally indistinguishable from the undamaged muscle.

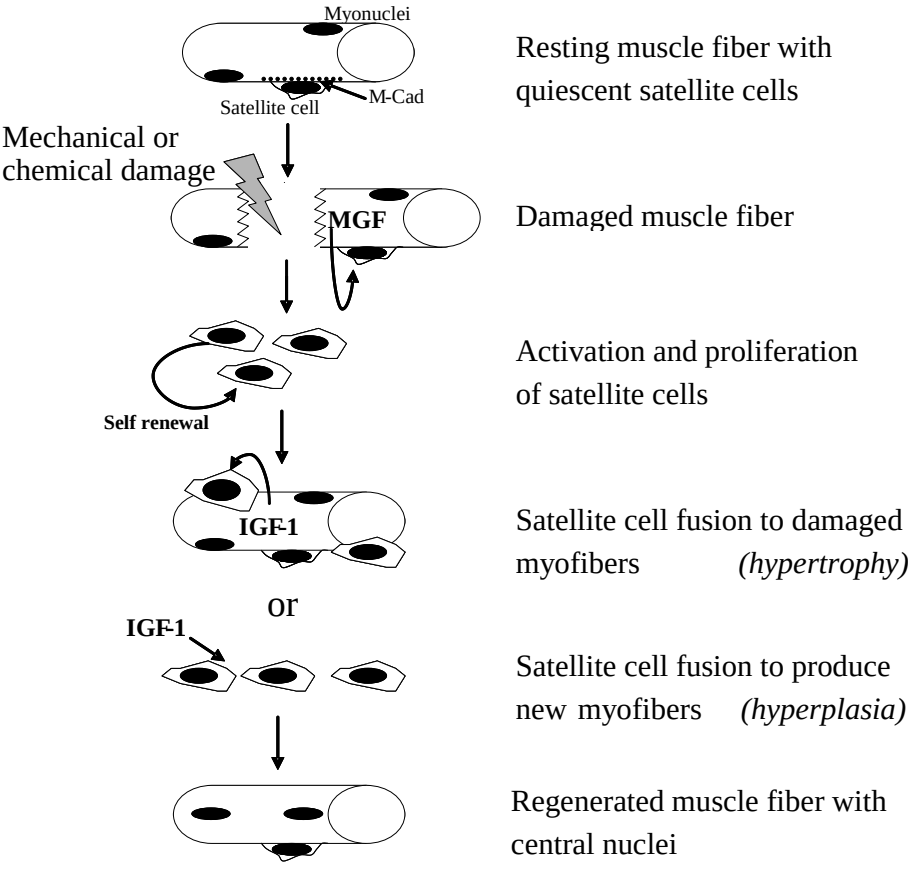


Figure 2. *Hypothetical model of muscle regeneration and the role of MGF and IGF-1Ea in the activation and differentiation of satellite cells.* Skeletal muscle damage is caused mechanically by contractions or chemically by injection of myotoxins, but also during muscle diseases like Duchenne muscular dystrophy. In response to injury, the muscle fibres produce MGF and satellite cells become activated and start to proliferate. Some of the satellite cells will re-establish the quiescent satellite cell pool through a process of self-renewal. Other satellite cells will migrate to the damaged region and, depending on the severity of the injury, fuse to the existing myofiber or align and fuse to produce a new myofiber. IGF-1Ea plays a role during the differentiation of the satellite cells into mature muscle fibres. In the regenerated myofiber, the newly fused satellite cell nuclei will initially be centralized but will later migrate to assume a more peripheral location M-Cad: M-Cadherin. (Redrawn and adjusted from: (24)).

As mentioned before MGF and IGF-1Ea mRNA are both induced by muscle damage. Remarkably, the mRNA and protein levels of the proliferation marker MyoD and the satellite cell marker M-Cadherin (M-Cad) were increased simultaneously with MGF, suggesting that MGF is associated with muscle satellite stem cell activation. The results also suggested that IGF-1Ea, which was expressed later in time, is associated with muscle differentiation (42). However, the use of an *in vivo* system does not exclude any systemic factors. This study does not consider the fact that another factor could activate the satellite cells and that the induction of MGF represents a stress response to muscle damage by severe contractions or by trauma.

Although the muscle IGF-1 isoforms derive from the same gene, their expression pattern differs and they were characterized separately. The function of MGF, accept being mechano-sensitive, remained unclear, until 2002. Goldspink and Yang studied the function of MGF in an *in vitro* cell model (42). Goldspink and Yang found that C2C12 cells transfected with MGF cDNA were inhibited to differentiate and remained in their proliferated state while in cells transfected with IGF-1Ea cDNA differentiation was stimulated relatively to control cells. Addition of synthetic/exogenous MGF was shown to inhibit differentiation of C2C12 cells once more. Interestingly, the proliferation induced by addition of MGF could not be inhibited by blocking the IGF-1 receptor, suggesting that MGF works by another mechanism than IGF-1Ea. These findings support the idea that MGF and IGF-1Ea should be treated as two independent growth factors with separate functions.

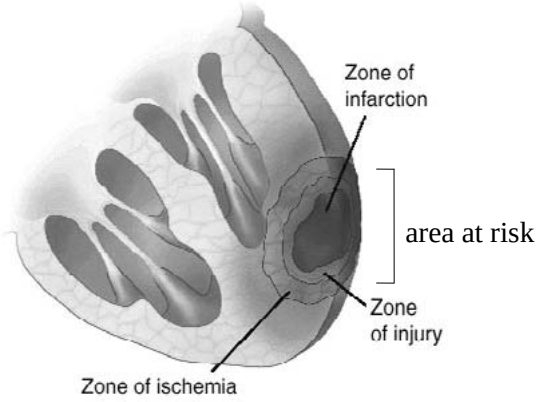
Goldspink and colleagues found that during the aging process, MGF expression decreases in time (16). Elderly men were unable to up-regulate MGF in response to exercise. Therefore, with advancing years, the elderly become more deficient in repairing muscle damage leading to a progressive loss of tissue function, also known as sarcopenia. This was confirmed by another study of Cutlip *et al.* (5). In that study the decrease in MGF expression with aging was associated with an increased susceptibility of satellite cells to apoptosis (27).

Regulation of MGF during ischemia in brain and heart

MGF can be described as a local tissue repair factor as well as a growth factor. In this context MGF expression was analyzed after an ischemic stroke, which is the third leading cause of death in developed countries with no treatment available. It was shown that MGF is a potent neuro-protective agent as functional copies of MGF cDNA protected facial neurons after nerve damage. Pre-treatment with an IGF-1Ea construct was less effective than with MGF (2). Zablocka *et al.* were the first to show ischemia-induced changes in endogenous MGF protein expression in the hippocampus together with strong neuro-protective effects of the autonomous MGF C-terminal peptide *in*

vivo and *in vitro*. Immuno-localization of MGF showed that MGF is found in the ischemic resistant areas and not in the vulnerable areas. Again MGF acted independently of the IGF-1 receptor (15) but further the mechanism still remained unclear.

The group of McMahon aimed to determine if MGF would reduce the area "at risk" of the myocardium (figure 3) and would improve cardiac function in the post-infarcted heart (10). Following loss of cardiac muscle tissue due to myocardial infarction, viable cardiomyocytes in the peri-infarct zone are unable to re-enter the cell cycle and thus cannot replicate to regenerate the lost tissue. Instead, lost tissue is replaced by scar tissue in the infarct zone (1) and by hypertrophy of the viable cardiomyocytes bordering the damaged area (32, 40). In large infarcts this leads to dysfunction of the chamber wall and eventually to heart failure. Myocardial infarct was induced in sheep. The animals were pre-treated with mature IGF-1, the MGF E peptide or full length MGF. McMahon *et al.* demonstrated that MGF had a cardio-protective effect that improves heart function after a relatively large myocardial infarction. MGF reduced post-infarct apoptosis in the peri-infarct region, leading to a reduction in infarct expansion. Moreover, immuno-staining for activated caspase 3 (apoptosis marker) was absent at the peri-infarct border in MGF-E-peptide treated hearts, but present in controls. Unfortunately, this study does not include any immuno-histochemistry of MGF. Therefore it is unknown which cells (cardiomyocytes, endothelial cells, smooth muscle cells or fibroblasts) express MGF in the area "at-risk".



Feature	Time
Onset of ATP depletion, anaerobic glycolysis, accumulation of noxious breakdown products	Seconds
Loss of contractility (→ acute heart failure) myofibrillair relaxation, glycogen depletion, cell and mitochondrial swelling (all reversible)	< 2 min
ATP reduced To 50% of normal To 10% of normal	 10 min 40 min
Irreversible cell injury, primarily structural defects in the sarcolemmal membrane	20-40 min
Microvascular injury	> 1 hr
Onset necrosis	2 hrs
Severe necrosis	1-3 days
Beginning disintegration of dead myofibres, with dying neutrophils, early phagocytosis of dead cells by macrophages at infarct border	3-7 days
Well-developed phagocytosis of dead cells; early formation of fibro vascular granulation tissue at margins	7-10 days
Well-established granulation tissue with new blood vessels and collagen deposition	10-14 days
Increased collagen deposition, with decreased cellularity	2-8 wks
Dense collagenous scar	> 2 mo

Figure 3. *Approximate time of onset of key events in ischemic cardiac myocytes.* Myocardial ischemia also contributes to arrhythmias, usually due to ventricular fibrillation caused by myocardial irritability. This might lead to sudden death. Figure adjusted and redrawn from textbook: (29).

In conclusion

Although the muscle IGF-1 isoforms derive from the same gene, their expression pattern differs and they should be treated as two independent growth factors with separate functions. Table 1 shows the similarities and differences between MGF and IGF-1.

Table I. *Summary of similarities and differences between MGF and IGF-1.*

Feature	Mechano growth factor	Insulin-like growth factor
Gene	Encoded by exon 3, 4 part of 5 (Insertion of 49 bp in human and 52 bp in rodent) and exon 6, which is stopped early (36)	Encoded by exon 3, 4, and 6 (36)
Protein	Different E-peptide at C-terminus of protein (20, 36)	Same mature peptide as MGF (20, 36)
Expression	Expressed only after mechanical loading in tissue and declines during ageing (16, 18, 19, 22, 25)	Expressed constitutively, declines during ageing (16, 18)
Location	Expressed in skeletal muscle (17, 19), heart (10, 40) and brain (2, 15)	Expressed in many different tissues, like skeletal muscle (17, 19), heart (33, 38) and brain (12), but also in liver (34).
Function	- Action autocrine, paracrine - Activation and proliferation of stem cells (42) - Protection of neurons (2, 15) and cardiomyocytes during ischemia (30, 37)	- Action autocrine, paracrine but also endocrine - Differentiation of stem cells (42) - Fusion of muscle stem cells to each other or existing fibers (42) - Protection of neurons (9) and cardiomyocytes (33, 38), but less potent than MGF

Autocrine action is between similar cells, paracrine action refers to an effect on nearby located target cells and endocrine function refers to IGF-1 production in the liver and released into the systemic circulation, as part of the regulation of the GH/ IGF-1 axis.

Aim, outline and experimental approach

Aim of the thesis

The overall objective of this thesis is to determine the regulation of MGF in skeletal and cardiac muscle. In this thesis the following specific objectives will be addressed:

1. To determine the regulation of MGF by mechanical loading in skeletal muscle.
2. To assess the regulation of MGF during hyperthyroidism in skeletal muscle and in heart.
3. To evaluate the regulation of MGF upon ischemic injury in the heart.

Outline of the thesis

Chapter two describes that only 15 minutes of *in vitro* lengthening contractions induced a three-fold increase in MGF mRNA, while isometric contractions did not. These results show that MGF expression is dependent on the lengthening component of the contraction and support the view that MGF is a mechano-sensitive growth factor. The proliferation markers MyoD, Proliferating Cell Nuclear Antigen (PCNA) and c-kit were increased, simultaneously with MGF after lengthening contractions but not after isometric contractions. These findings suggest activation of satellite cells directly or indirectly by MGF.

Chapter three describes an *in vivo* mouse model for hyperthyroidism. Hyperthyroidism causes among others an increase in metabolic rate and cardiac hypertrophy. After daily T3 injections for 12 days, MGF expression was significantly up-regulated in the heart and not after a single injection of T3. To determine whether the increase of MGF was mechano-sensitive, an *in vitro* model was used. Treating neonatal rat cardiomyocytes with T3 increased the beating frequency and in parallel induced MGF expression. If the T3 treated cells were placed under contractile arrest, MGF expression was down-regulated. These results indicate that MGF is involved in the mechanotransduction of the heart.

Chapter four describes that MGF is expressed during development in an *in vitro* model for early embryonic development. P19 mouse embryonic stem cells have the ability to differentiate into a variety of cell types, including cardiac lineages. Because MGF was expressed during the differentiation of P19 cells into cardiac cells, these results suggest that MGF plays a role during heart development. During pathologic cardiac hypertrophy for example caused by myocardial infarction, the heart undergoes a remodelling process and therefore turns on a fetal gene program. Chapter four also shows that endogenous MGF is significantly expressed in the heart after ischemia-

induced injury. In an *in vitro* assay where a (non-beating) atrial mouse cell line, HL-1, was treated with desferrioxamine (DFO) to mimic hypoxia, MGF was induced. These studies indicate that MGF can act as a stress response factor.

Finally in chapter five, general conclusions are drawn from the different experiments described in chapter two, three and four, and potential implications for application of MGF are discussed, as well as suggestions for further research.

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Chapter 2

Lengthening contractions induce expression of mechano growth Factor and active satellite cells in mouse EDL muscle

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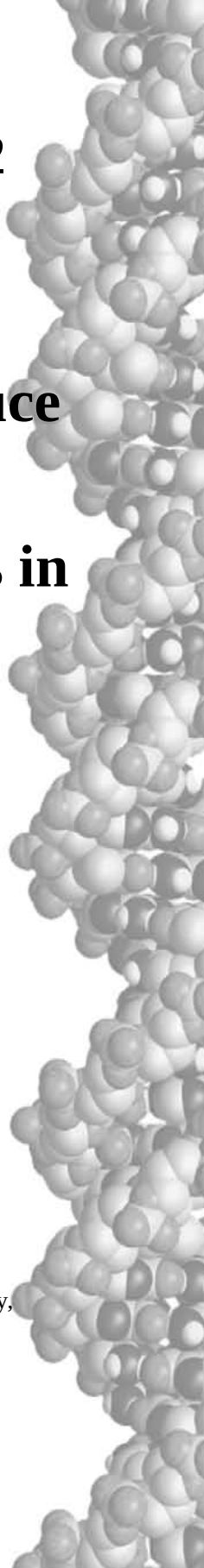

Abstract

The insulin-like growth factor (IGF-1) gene is involved in exercised-induced hypertrophy. The IGF-1 gene can undergo alternative splicing to generate different products, like the mechano-growth factor (MGF) expressed in mechanically overloaded muscle. The aim of this study was to determine the correlation between different modes of contractions and MGF expression in time. Therefore mouse EDL muscles were isolated and lengthening and isometric muscle contractions were applied. mRNA expression levels were determined by real time PCR for MGF and IGF-1Ea, and for Proliferating Cell Nuclear Antigen (PCNA) and MyoD as specific markers for satellite cells activation. In addition Myogenin expression was determined as marker for early differentiation. Lengthening contractions for 15' induced MGF expression after 4 hrs and IGF-1Ea expression after 6 hrs. Isometric contractions did not induce MGF and only a small amount of IGF-1Ea. PCNA and MyoD were expressed at the same time after lengthening contractions, indicating that satellite cells started to proliferate. Myogenin is not expressed within 10 hrs after contractions. These results provide evidence that MGF expression is induced in muscles which are subjected to lengthening contractions and suggest that MGF is involved in the activation of satellite cells.

Introduction

Hypertrophy of skeletal muscle is induced by increased load resulting from resistance exercise (7, 16, 36). Skeletal muscle loading during activities includes periods of muscle shortening and lengthening as well as periods where muscles are activated without external length changes, called isometric contractions (13). Each of these three types of load can stimulate muscle adaptations including muscle hypertrophy, depending on sufficient intensity, frequency and duration of the resistance exercise stimulus (5, 30, 35, 38), although one mode of exercise appears to be more effective than another in inducing hypertrophy (2, 32, 38). Local cellular and molecular responses might specifically promote this growth process (39). Muscle hypertrophy is characterized by an increase in the volume of the cytosol, while the cytosol: myonuclei ratio remains constant (25, 31). These new myonuclei in the hypertrophied muscle originate from fusion of satellite cells with muscle fibers (4, 12), since muscle fibers are terminally differentiated and therefore post-mitotic (8). In normal adult muscle, the satellite cells are quiescent and located between the basal lamina and the plasma membrane (1). The gene expression profile of quiescent satellite cells and their progeny is largely unknown. Activated satellite cells up-regulate MyoD or myf5 or both before entering the S-phase (23). PCNA is synthesized during DNA synthesis and its expression is proven in proliferating satellite cells in skeletal muscle (22). During skeletal muscle growth the MyoD protein is expressed before PCNA (20). Subsequently, satellite cells express Myogenin and MRF4 characteristic for differentiation (20).

More evidence emerges that muscle contractions are associated with satellite cell proliferation (19, 30, 33, 34). How this mechanotransduction takes place is unclear. Previous studies have defined the effects of growth factors (3, 8, 39) alone or in combination with other factors (13) and have provided insight into the activation, proliferation, differentiation and self-renewal of satellite cells (reviewed in (18)). The present study focuses on the insulin-like growth factor (IGF-1), because IGF-1 has been implicated to be involved in satellite cell activation and fusion of satellite cells with damaged or growing muscle fibers (6, 16). It has been shown that the IGF-1 gene is spliced in response to mechanical signals producing isoforms which have different modes of action (17, 27). One variant is the systemic-like IGF-1Ea and a second isoform the mechano growth factor (MGF) (14). Yang and Goldspink have shown in myoblasts in culture that IGF-1Ea promotes an increase in cellular mass and induces the myoblasts to fuse and form myotubes. In contrast, MGF increases cellular proliferation and inhibits terminal differentiation (41). Cell cultures are limited for modeling activation of satellite cells because their isolation disrupts intercellular relationship between the fiber and the quiescent satellite cell. In previous *in vivo* studies electrical neuromuscular stimulation applied to stretched rat muscles up-regulated MGF and IGF-1Ea (28). Or *in vivo*

human studies, where subjects performed different exercise-protocols, MGF and IGF-1Ea were up-regulated as well (26). Unfortunately, one cannot rule out any systemic or environmental factors and most of these studies did not include time points of measuring MGF or IGF-1ea mRNA earlier than 24 hrs.

Although it is unknown when exactly and how the splice variant MGF is induced in response to contractile loading, these *in vivo* results combined with the *in vitro* results are indications that exercise-induced MGF may be responsible for satellite cell activation. As MGF is thought to play a role in satellite cell proliferation and IGF-1Ea to regulate the differentiation of myoblasts into myotubes (41), we imply a sequential necessity for expression of these growth factors, as well as a temporally parallel pattern for cell cycle regulatory factors, such as MyoD, PCNA and Myogenin upon muscle contraction.

Therefore in the present study electrically stimulated lengthening and isometric contractions were performed on isolated EDL muscle and the time course of mRNA expression for MGF and IGF-1Ea, as well as for specific markers of activated satellite cells like MyoD and PCNA was determined, beginning from one hour after the exercise-protocol.

Material and methods

Experimental animals

Protocols for animal use were reviewed and approved by the local Animal Ethical Committee of Utrecht University. Eight week old male BalbC mice were obtained from Charles River and were fed standard laboratory chow and water *ad libitum*.

Isolated muscle contractions

Mice were euthanized by cervical dislocation and the Extensor Digitorum Longus (EDL) muscles were dissected and placed in ice cold Ringer's buffer (in mM: 116 NaCl, 4.6 KCl, 1.16 KH_2PO_4, 2.5 $CaCl_2$, 1.16 $MgSO_4$ and 25.3 $NaHCO_3$, pH 7.4) rapidly. Tendons from both ends of the muscle were tied with a ligature. The muscle was mounted in a 1200A/ 300B intact muscle test system (Aurora Scientific Inc., Canada). The muscle was held vertically in a water-jacketed bath. Both force and length were controlled and measured with this system. The length of the muscle was adjusted to allow maximum tetanic isometric force to be generated (optimal length (l_0)). During the entire experiment isolated muscles were incubated in Ringer's buffer at 25ºC and were gassed continuously with 95% O_2 and 5% CO_2. To investigate the gene expression in time, muscles were frozen in liquid nitrogen either immediately after the contraction protocols or after 1, 2, 3, 4, 5, 6, 8 or 10 hrs of subsequent rest and stored at -80ºC until further use.

Stimulation protocols

The stimulation protocol was started after 1 h of equilibration at l_0 at 25ºC. Tetanic contractions were produced by trains (300 ms) of supramaximal current pulses (0.5 ms duration and with an amplitude of 700 mA), which were applied every third second for 15 minutes via platinum electrodes placed on each side of the muscle (duty cycle of 5%). Both at the start and at the end of the contraction protocol, maximum isometric force was measured. For lengthening contractions the velocity applied was 0.5 l_0 /s. In the experiments applying isometric contractions, the muscles were positioned in l_0 and the length of the muscle did not change. All muscles were compared with their untreated contra-lateral control muscle, positioned simultaneously at l_0 at 25ºC in an identical set-up.

RT-PCR analysis

Total RNA was prepared from muscle using Trizol (Gibco Life Technologies, Paisley, UK), previously described by Chomczynski *et al (10)*. The muscle membranes were disrupted in a MagNA Lyser (Roche, the Netherlands) for 30 sec at 6000 rpm. RNA was quantified by spectrophotometry (260 nm) and checked on a 1% agarose gel. RNA was stored at -80 ºC until further use.

cDNA was synthesized using the SuperScript II kit (Invitrogen, the Netherlands) according to the manufacturers' instructions. Samples were diluted to 10 ng/µl work stocks and stored at 4°C. Real time PCR was performed on a MyiQ cycler (Biorad, Luxembourg) using SYBRGreen Supermix (Biorad, Luxembourg); cDNA input was 50 ng and the final concentration of the primers (forward and reverse) used was 180 nM. The sequences of primers used for real time PCR are described in table I. Product specificity during PCR was verified by melting curve analysis of the products. For each amount of RNA tested, duplicate Ct values were obtained and averaged. Quantification was performed using a mathematical model of relative expression ratio in real-time PCR, the $2^{-\Delta\Delta Ct}$ –method (29) and was calculated with help of the Genex-software (Biorad, Luxembourg). As we found no significantly alteration by the contraction regimens in GAPDH expression, GAPDH was used as a reference gene (21).

Table I. PCR primers for real time PCR analyses

Primer	Sequence	Size (bp)
IGF-1Ea forward	GCTTGCTCACCTTTACCAGC	301
IGF-1Ea reverse	AAATGTACTTCCTTCTGAGTCT	
MGF forward	GCTTGCTCACCTTTACCAGC	353
MGF reverse	AAATGTACTTCCTTTCCTTCTC	
MyoD forward	AGGACACGACTGCTTTCTTC	113
MyoD reverse	CCCAACAGTACAATGACAAAGG	
Myogenin forward	GCAATGCACTGGAGTTCG	145
Myogenin reverse	ATGGTTTCGTCTGGAAGG	
GAPDH forward	GAAGGTCGGTGTGAACGG	101
GAPDH reverse	TGAAGGGGTCGTTGATGG	
PCNA forward	GTTGGTAGTGTCGCTGTAGG	122
PCNA reverse	ATGGTGGCGGAGTTGTGG	

Immunostaining

Primary antibodies used in the present study were as follows; a rabbit anti-Ki67 clone SP6 (a marker molecule for active proliferating cells, determined by numerous previous studies (11, 18); 1:50; Labvision RM-9106-S0, Fremont, CA, USA) and Alexa 633 Phalloidin (a marker for muscle actin fibers; 1:25, Molecular Probes, Carlsbad, CA, USA). Secondary antibodies were raised against rabbit, used at 1:100.

Ten-micrometer cryotome cross-sections of the contralateral control and the ipsilateral exercised EDL muscles at t=0 and t=4 hrs were cut at the midbelly level using a cryostat at -20ºC and thawed on 3-amino propylethoxysilane-coated slides. The sections were fixed with ice-cold acetone for 10', washed two times for 5' with PBS containing 0.2% Triton (pH 7.4). To block any non-specific reaction, the sections were incubated with PBS containing 10% bovine serum albumin (BSA, Sigma, St. Louis, USA) for 30' at room temperature. The primary antibodies were appropriately diluted with PBS containing 1% BSA and 0.2% Triton and incubated overnight at 4ºC. The following day, the sections were washed, blocked again and incubated 1 hr with secondary antibodies diluted in PBS with 0.2% Triton and 1% BSA at room temperature, washed with PBS, and incubated with Alexa 633 conjugated Phalloidin in PBS, washed again and counterstained with 4,6-diamidino-2-phenylindole (DAPI; 1 ng/ml; Vector Labs, Burlingame, USA) and mounted in FluorSave (Calbiochem, San Diego, USA). Cross sections were imaged using a Zeiss 510 META Confocal Scanning Light Microscope (Zeiss, Jena, Germany) using sequential excitation of 488, 633 and 408 nm.

An average of >1,000 propidium iodide nuclei were counted from four to five different fields for each time period assessed. The percentage of PCNA-positive or TUNEL-positive nuclei was calculated as the total number of PCNA- or TUNEL-positive nuclei divided by the number of propidium iodide-positive nuclei (n = 4, >1,000 cells counted). All negative control sections (PBS substituted for primary antibody) had an absence of signal.

ß-Glucuronidase assay

Muscle ß-glucuronidase activity, a biochemical indicator of muscle damage, was measured (24). EDL muscles were homogenized and a protein concentration of 50 ng/ml was incubated with 1 mM phenolphthalein- ß-glucuronidase in a total volume of 250 µl incubation medium (70 mM acetic acid at pH 5.0) for 60 minutes at 37ºC. Incubations without protein were used as a negative control. A liver homogenate was used as a positive control. Phenolphthalein served as a standard. The reaction was stopped by adding 250 µl ice-cold 5% TCA. Samples were neutralized by adding 500 µl glycine at pH 12.9. Absorbance was measured at 540 nm. Specific ß-glucuronidase activity was defined as µmol/s/kg protein (24).

Statistical analysis

Expression values relative to GAPDH for the exercised and control muscle were calculated (for each gene). The values of the control muscle were subtracted from those of the exercised muscle, resulting in a relative expression level for each exercised muscle. Each time point represents the results of EDL muscles from 4 mice and in the figures the mean values +/- SEM are expressed. For statistical analyses multivariate analyses (control versus exercised group) were performed with Bonferonni post hoc for each gene. For all statistical tests statistical significance was set at $P < 0.05$. The experimental design provided sufficient statistical power (above 75%) with n=4.

Results

In vitro contractions

Mouse EDL muscles were subjected to a 15' contraction protocol consisting of lengthening or isometric contractions. After the 15' contraction period the tetanic force produced was reduced to about 30% of the maximal force in both protocols (figure 1). However, muscles subjected to isometric contractions recovered in 1 to 2 hrs after the last contraction and muscles subjected to the lengthening protocol did not recover within the 10 hrs analyzed (figure 1). However the muscle could still generate a tetanic contraction (data not shown).

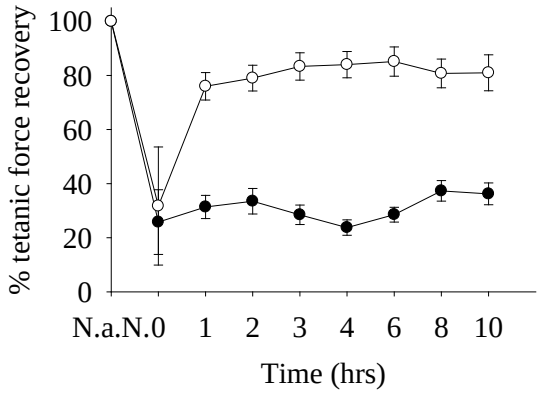

Figure 1. *Tetanic force recovery after lengthening (●) and isometric contractions (○).*
Each time point represents data of recorded tetanic force. Maximum tetanic force before start of the contraction protocols was set at 100%. In the figures the mean values of 4 mice per time point +/- SEM are expressed.

Effect of contractions on expression and splicing of IGF-I

MGF mRNA expression following lengthening contractions, was significantly increased 4-fold after 3 to 4 hrs and declined after 6 hrs (figure 2A). As shown in figure 2B, MGF mRNA was not significantly increased after isometric contractions.

Figure 2B shows the mean expression difference between the contracted muscle and its contralateral control of IGF-1Ea mRNA levels following lengthening contractions. The mRNA levels of splice variant IGF-1Ea started to increase 30-fold after 6 hrs and peaked significantly after 8 hrs (20-fold, $P<0.05$) followed by a continuous level of expression relative to t=0. Isometric contractions did not significantly induce IFG-1Ea mRNA expression in this time range as depicted in figure 2D, however at 3 hrs there is an upward trend of IGF-1Ea expression.

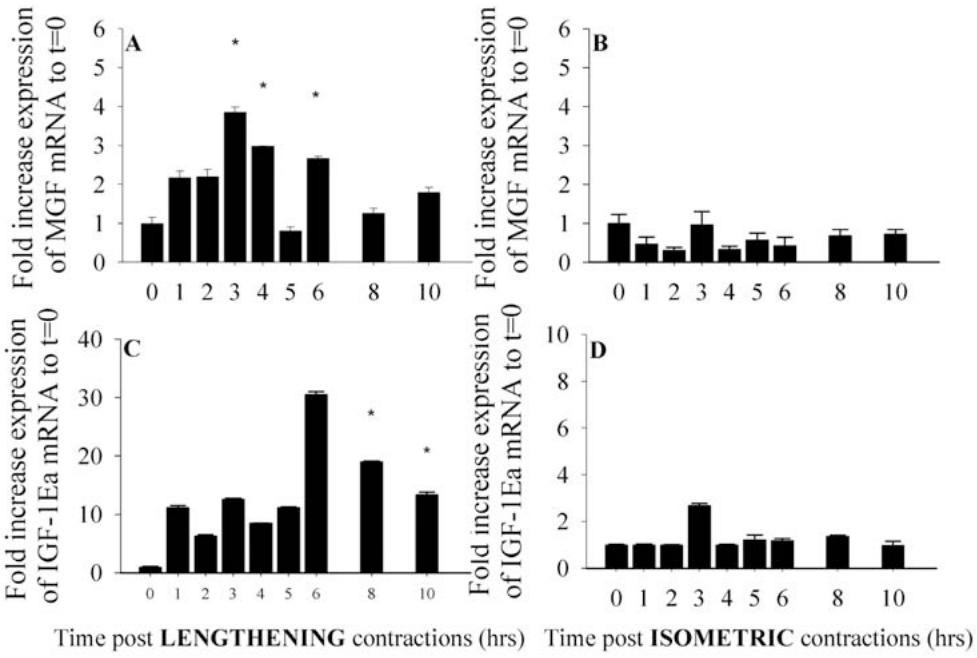

Figure 2. *Temporal pattern of mRNA expression and splicing of IGF-1 upon lengthening and isometric contractions.*
For each time point, expression values of the contralateral control EDL were subtracted from those of the ipsilateral exercised muscle, resulting in a relative expression level (n=4 mice, each time point). A, MGF expression after lengthening contractions, B, MGF expression after isometric contractions, C, IGF-1Ea expression after lengthening contractions and D, IGF-1Ea expression after isometric contractions. Data are expressed as mean values +/- SEM are expressed.
* Significantly different from t=0 (P<0.05).

Markers for satellite cell activation, proliferation and differentiation

As depicted in figure 3A MyoD expression increased in two peaks relative to t=0 after 15' of lengthening contractions. First, MyoD is significantly induced after 2 hrs by 3-fold, and was down-regulated 5 hrs after lengthening contractions ($p<0.05$). The second up-regulation by 4-fold started after 8 hrs relative to t=0. No significant increase of MyoD expression was detected following isometric contractions (figure 3B). Myogenin mRNA levels were not elevated after lengthening or isometric contractions (figure 3C and D). As shown in figure 4A, PCNA mRNA levels were significantly increased 3-fold 3 hrs after 15' lengthening contractions and peaked after 4 hrs relative to t=0 ($p<0.05$). After isometric contractions no PCNA mRNA increase was detectable.

To confirm proliferation of satellite cells, as indicated by PCNA mRNA expression (a marker for S-phase); immunohistochemistry was performed using a Ki67 antibody (a nuclear marker for G0-exit, i.e. activated proliferating cells) in combination with Phalloidin (a marker for actin fibers). Figure 5 shows that in control muscles there were some (1.40 ± 0.34 % of total nuclei per cross section) satellite cells proliferating as marked by the double staining of nuclei with DAPI and Ki67, however most cells, satellite and myogenic cells, were quiescent. The number of activated satellite cells did not increase 4 hrs after 15' isometric contractions (1.47 ± 0.30 % of total nuclei per cross section). In contrast, 4 hrs after 15' lengthening contractions, number of proliferating satellite cells increased 2.6-fold, marked by DAPI and Ki67 co-positive nuclei surrounding individual muscle fibers. There was no difference in Ki67/DAPI positive nuclei between controls and muscles 1 h after 15' of lengthening or isometric contractions (data not shown). No difference between control and exercised skeletal muscle architecture was visible at 4 hrs post contraction. Muscles were not edematous, with Phalloidin staining homogeneous throughout the muscle fibers, and centrally located myonuclei present, suggesting no injury was induced by the contraction protocols.

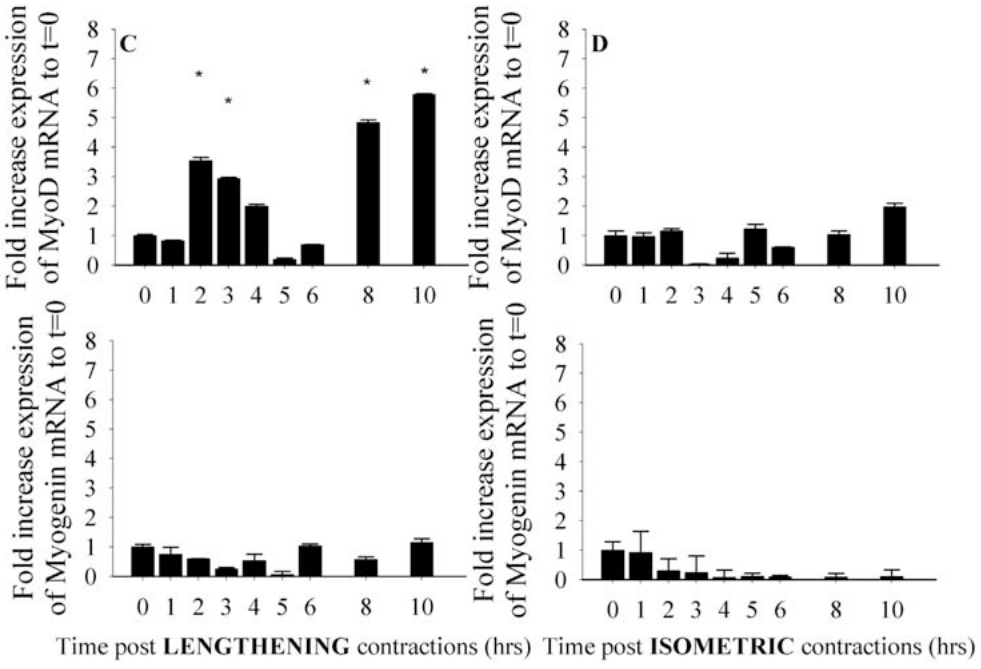

Figure 3. *Temporal effect of contractions on mRNA expression of markers of satellite cell proliferation and differentiation.*
For each time point, expression values of the control EDL were subtracted from those of the exercised muscle, resulting in a relative expression level. Each time point represents results of EDL from 4 mice. A, MyoD expression after lengthening contraction, B, MyoD expression after isometric contractions, C, Myogenin expression after lengthening contractions and D, Myogenin expression after isometric contractions. Data are expressed as mean values +/- SEM are expressed. * Significantly different from t=0 (P<0.05).

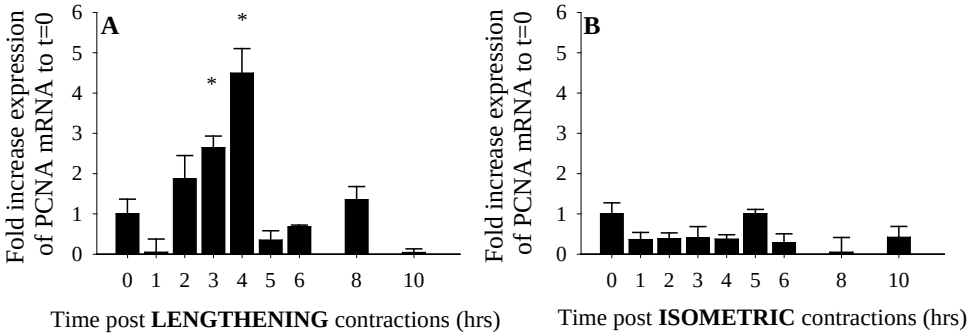

Figure 4. *Effect of contractions on mRNA expression of PCNA a marker of satellite cell activation.*
For each time point, expression values of the control EDL were subtracted from those of the exercised muscle, resulting in a relative expression level. Each time point represents results of EDL from 4 mice. A, PCNA expression after lengthening contraction, B, PCNA expression after isometric contractions. Data are expressed as mean values +/- SEM are expressed. * Significantly different from t=0 ($P<0.05$).

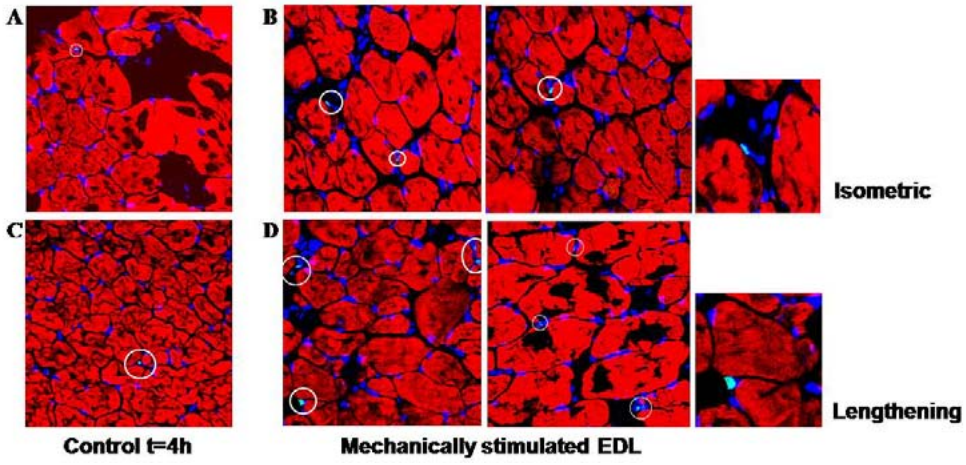

Figure 5. *Representative immunofluorescent micrographs of cross cryosections of mouse EDL muscle showing nuclear localization of Ki67 after 4 hrs of a 15' lengthening or isometric contraction protocol.*
All nuclei, including centrally located myonuclei and satellite cell nuclei, are stained by DAPI. Satellite cells which were activated to proliferate are marked by DAPI and Ki67 double positive staining. A and C, contralateral control EDL muscle 4hrs after contraction protocol started with the ipsilateral EDL muscle (40x); B, DAPI and Ki67 double immuno-staining (encircled) after 15' isometric contractions (40x); D, DAPI and Ki67 double immuno-staining (encircled) after 15'lengthening contractions (40x). Extra inset: enhanced magnification of double fluorescent signal.

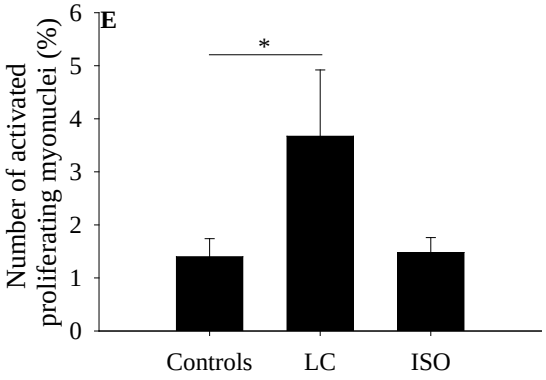

Figure 5E. *Representative immunofluorescent micrographs of cross cryosections of mouse EDL muscle showing nuclear localization of Ki67 after 4 hrs of a 15' lengthening or isometric contraction protocol.*
E, Increased cellular proliferation in exercised EDL muscle, as assessed by PCNA expression, was coincident with a significant increase in the total number of Ki67 positive nuclei compared with control non-exercised muscle. * Significantly different from contralateral controls (controls) (P<0.05).

ß-Glucuronidase assay

ß-Glucuronidase activity reflects the histopathological state of muscle well and is thus a reliable indicator of muscle damage (24). The tibialis anterior muscle (TA) was used as a positive control by mechanically stimulating the muscle by pulling it hard 3 times. The TA from the other leg was frozen in immediately. There was a 2.5 fold increase in ß-Glucuronidase activity of the mechanical stimulated TA muscle. No ß-Glucuronidase activity was observed after 15'of lengthening or isometric contractions at t=0 and t=4 hrs (table II).

Table II. *ß-Glucuronidase activity (µmol/s.kg protein) in muscle of mouse following (forced) lengthening contraction.*

Condition	Muscles after forced lengthening contraction TA (3)	Time post lengthening contractions		Time post isometric contractions	
		0 h (2)	4 h (2)	0 h (2)	4 h (2)
Exercised	3.3 ± 0.8	1.0 ± 1.3	0.1 ± 0.6	0.5 ± 1.0	1.6 ± 1.0
Control	1.5 ± 0.5	1.4 ± 1.3	0.8 ± 0.6	1.6 ± 0.6	0.5 ± 0.4

A sample from the liver was tested as a positive control; 13.0 ± 2.0. Data are means ± SD, *n* is given in *parentheses*.

Discussion

The objective of the present study was to determine in isolated EDL muscle the relation between contraction type and MGF expression with satellite cell activation. To our knowledge, the present study is the first study showing that MGF expression is not only selective to the lengthening component of the contraction but also a single eccen-

tric contraction regimen of only 15' is enough to induce MGF in isolated whole-mounted skeletal EDL muscle. Lengthening contraction-induced MGF together with MyoD appears functional in cell cycle activation of quiescent satellite cells, as supported by G0-exit and PCNA expression prior to IGF expression.

We investigated systematically the temporal expression pattern of the two splice variants IGF-1Ea and MGF as stimulated *ex vivo* by either lengthening or isometric contractions of the isolated EDL muscle. Lengthening contractions specifically led to MGF expression at 3 hrs, followed by IGF-1Ea expression after 8 hrs, whereas both splice variants, MGF and IGF-1Ea, were not induced upon isometric contractions. These results of *ex vivo* contractions are in agreement with previous cell culture studies, reporting MGF expression prior to IGF-1Ea. (9, 15, 41). This is in contrast to the results of the study of Haddad *et al*., where sequential bouts of resistance exercise induced both IGF-1 and MGF (16). However as their study was performed *in vivo*, systemic effects cannot be eliminated.

To investigate if contraction-induced MGF and IGF-1Ea expression is functional in activating satellite cell proliferation and/or differentiation, the expression patterns of MyoD, PCNA and Myogenin were investigated. MyoD is up-regulated concomitantly with the proliferation of satellite cells (40), PCNA is synthesized during DNA replication (22) and Myogenin is induced during early differentiation (3). In the present study, the expression of MyoD was induced within 2 hrs after 15' of lengthening contractions. A second peak of MyoD expression occurred 8 hrs after 15' of lengthening contractions. After isometric contractions no MyoD was expressed.

Wozniak *el al*. (37) also found two peaks of MyoD activation upon stretching of single EDL muscle fibers in culture (37). The two peaks were explained by the hypotheses that a) a single population of satellite cells requires more than one MyoD stimulus and are transiently activated to enter proliferation or b) that two or more populations of satellite cells enter proliferation after stretch due to differences or asynchrony in cell-dependent activation characteristics. This is in concert with the study of Kitzmann *et al*. (28), using synchronization of myoblasts in culture. MyoD levels peaked as cells passed through mitosis and early G1 of a new cell cycle. The second peak of MyoD expression was observed by Kitzmann *et al* in the late G2/M phase, where cells exit the cell cycle and induction of differentiation can take place (23). Therefore our first MyoD mRNA peak at 2 hrs may represent satellite cells activated from their quiescent state G0 to enter the cell cycle G1. This activation is indeed confirmed on the protein level by immuno histochemistry, showing an increase in Ki67/DAPI-positive nuclei of cells around the muscle fibers. This suggests that MyoD at 2 hrs initiated the satellite cells to proliferate, which is supported by the sequential expression of PCNA, peaking at 4 hrs, indicating that satellite cells entered the S-phase

and started to duplicate. Interestingly, the first peaks of MyoD and PCNA occurred simultaneously with MGF expression, suggesting that MGF is functional in activating quiescent satellite cells. This is sharpened by the effect that not only MGF but also PCNA peaked after 4 hrs of lengthening contractions and not after isometric contractions. The second peak of MyoD at 8 hrs after lengthening contractions coincided with IGF-1Ea expression. One might hypothesize based on both Wozniak *et al. (37)* and Kitzmann *et al.(23),* that the satellite cells can then be activated by a second, other stimulus for a subsequent action, i.e. IGF-1Ea may induce differentiation of myoblasts. Indeed PCNA is not expressed simultaneously with MyoD and IGF-1Ea, indicating that the satellite cells are no longer in the S-phase. However, differentiation of the myoblasts has not started as Myogenin expression has not been detected either after lengthening or isometric contractions within the 10 hrs observed.

Furthermore, we can exclude that MGF expression was induced by damage instead of by mechanical load. The absence of ß-glucuronidase activity after 15' of lengthening or isometric contractions confirmed that there was no injury of the muscles. Moreover, McKay *et al.* who subjected humans to a muscle damage protocol found, that the *in vivo* MGF expression was not up-regulated within 24 hrs. Both 15' of lengthening and isometric contractions caused a similar decrease in maximal isometric tetanic force. The recovery period of isometric contractions was short (1-2 hrs). Muscles subjected to lengthening contractions did not recover at all. Nevertheless both after isometric and after lengthening contraction protocols the muscle could still produce a tetanic contraction, proving that the muscle is physiologically stable.

In conclusion, this study shows that a single short bout of 15' of lengthening contractions can initiate various molecular responses in whole-mounted skeletal EDL muscle, independent of any circulating systemic factor. As a result satellite cells were activated to proliferate by locally and contraction-induced MGF expression. Subsequent IGF-1Ea expression might regulate differentiation initiation of satellite cells into myotubes. Furthermore, MGF mRNA expression was not induced by damage implying that MGF unlikely was a regenerative response but contributed to the adaptive growth response of muscle to resistance exercise.

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Chapter 3

Thyroid hormone induced cardiac Mechano Growth Factor expression depends on beating activity

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Abstract

The mechano growth factor (MGF), a splice variant of the insulin-like growth factor (IGF-1) gene, was first discovered in mechanically overloaded skeletal muscle and was shown to play an important role in proliferation of muscle stem cells. Since then, the presence and effects of MGF have been demonstrated in other tissues: MGF has been shown to act neuro-protective during brain ischemia and pre-treatment with MGF before myocardial infarction improves cardiac function. Because MGF plays a permissive role in exercise-induced skeletal muscle hypertrophy, we hypothesize that MGF is commonly involved in cardiac hypertrophy.

To investigate the regulation of MGF expression in heart, mice were treated with thyroid hormone (T3) for 12 days to induce physiological cardiac hypertrophy. MGF mRNA expression was specifically increased in mid regions of the septum and left ventricular wall. Interestingly, MGF expression strongly correlated with the increased or decreased beating frequency of hyperthyroid and hypothyroid hearts. To further investigate the mechanical dependent induction of MGF, neonatal rat cardiomyocytes were isolated and exposed to T3. Upon T3 treatment, cardiomyocytes increased both contractile activity measured as beats per minute, and MGF as well as IGF-1Ea mRNA expression. Importantly, when cardiomyocytes were contractile arrested by KCl, simultaneous exposure to T3 prevented the up-regulation of MGF, whereas IGF-1Ea was still induced. These studies demonstrated that MGF but not IGF-1Ea expression is dependent on beating activity. These findings suggest that MGF is specifically stimulated by mechanical loading of the heart to mediate the hypertrophic response to thyroid hormone.

Introduction

The Insulin-Like Growth Factor (IGF) is named after the primary structural homology of pro-insulin (36). Although 90% of circulating IGF-1 is synthesized in and secreted by the liver (4), many types of cells, for example in the hippocampus and skeletal muscle fibres, are capable of local IGF-1 production (2, 15). Mammalian IGF-1 acts predominantly as a growth, survival and differentiation factor (12, 20, 40).

The multiple functions of IGF-1 are reflected in the complex structure and regulation of the *Igf-1* gene (1). IGF-1 can be systemically up-regulated for example by elevated growth hormone levels (3, 16). However, locally the *Igf-1* gene can also be alternatively spliced and can yield multiple mature transcripts (37). In total 3 different IGF-1 protein isoforms are generated: IGF-1Ea, IGF-1Eb and IGF-1Ec. The N terminus of the proteins differs, especially for IGF-1Ec. This isoform is also called the mechano growth factor (MGF) and was first discovered in mechanically overloaded skeletal muscle and was shown to function as a kick-starter of satellite cell proliferation during muscle hypertrophy (6, 18, 42). The precise mechanism of splicing of the *Igf-1* gene is not clear, although in skeletal muscle after mechanical stress or muscle damage the *Igf-1* gene is first spliced towards MGF and later in time towards IGF-1Ea (9, 17). This temporal difference suggests that the splice variants MGF and IGF-1Ea could act as individual growth factors. Since then, the presence and/or effects of MGF have been demonstrated in other tissues: recent studies show that endogenous MGF has a strong neuro-protective effect during brain ischemia (13), exogenous MGF has pro-migratory activity on human myogenic precursor cells during transplantation as a therapeutic approach in muscular dystrophy (33) and that synthetic MGF could improve heart function after myocardial infarction (8).

Since MGF is functional during skeletal muscle growth upon mechanical stress, we hypothesized that MGF is induced during cardiac hypertrophy. Therefore we determined MGF mRNA expression in an *in vivo* mouse model of cardiac enlargement by thyroid hormone (T3). It is well known that T3 has a profound effect on the cardiovascular system as reviewed by Klein and Ojamaa (25). Alterations in T3 levels are associated with effects on heart frequency (24) and changes in myocardial Ca^{2+}-homeostasis, by positive regulation of sarcoplasmic reticulum Ca^{2+}-ATPase (Serca2) (7). The long term effect of T3 on the heart includes the induction of hypertrophy (10), which has traditionally been attributed to the increased hemodynamic load and compensatory increase of heart frequency (24). Interestingly, T3-mediated cardiac enlargement is associated with proliferation of myocytes (30), providing a suitable and intriguing model for investigating MGF expression in cardiac striated muscle. In contrast to the heart, skeletal striated muscle does not undergo hypertrophy under influence

of T3 (14), although T3 shows comparable effects on the contractile and Ca^{2+}-transporting proteins in both muscles.

The aim of the present study was to investigate whether MGF mRNA expression was up-regulated specifically in the hearts of hyperthyroid animals and down-regulated in hypothyroid animals, without effects on skeletal muscle. We determined heart rate and relative heart weight to examine the correlation of MGF mRNA expression and mechanical activity and/ or hypertrophy. To discriminate between direct T3 actions and indirect effects of mechanical load on MGF transcription, we used an *in vitro* system where we exposed neonatal rat cardiomyocytes to T3 alone, or in combination with KCl to induce contractile arrest (29), and analyzed cell lysates for MGF mRNA in parallel to IGF-1Ea mRNA expression.

Material and methods

Experimental animals

Animals (C57Bl/6OlaHsd mice) were 8 weeks old by the start of the T3 treatment. Animals were obtained from Harlan and were fed standard laboratory chow and water *ad libitum* and were kept in a light and temperature regulated environment, with a 12 h light/dark cycle. All animals were treated according to the national guidelines and with permission of the Animal Experimental Committee (DEC) of Utrecht University, Utrecht, The Netherlands. After an acclimatization period of one week the mice were separated in randomly selected groups.

Long term T3 treatment

The mice were daily injected i.p. with the same dosage of T3 (10 µg/ 100 g body weight) or vehicle for 6 or 12 days. T3 was dissolved in a 0.9% NaCl solution containing 0.05 mM NaOH and 1% BSA. All chemicals were from Sigma, St. Louis, USA. To determine the heart frequency ECGs were recorded under inhalation anesthesia (2,5% isoflurane in 300cc/min O_2) and controlled body temperature; between euthyroid and hyperthyroid conditions rectal temperature was matched at 37º Celsius using heating pads. To eliminate circadian influences, animal handling, including ECGs were restricted between 1:00 and 3:00 PM. The electrodes were configured to contact three paws, providing an ECG signal equivalent to Einthoven lead I (Cardiosmart ECG recorder, Hellige GmbH, Freiburg GE). Data from continuous recordings of 20–30 signals with clear R peaks were used for heart rate analysis. The isolated beating heart was put in a Petri dish with PBS/Heparin and pumped itself clean from blood. The heart was frozen in total or divided into smaller sections, which were: atria, right and left ventricle and septum. Both the left ventricle and the septum were further dissected into base, middle and apex. As control tissue, the Soleus muscle was isolated from the two

hind limbs, as well as the Extensor Digitorum Longus (EDL) muscle after removing first the Tibialis Anterior muscle. All sections were snap frozen in liquid nitrogen and stored at -80ºC until further use. We measured as a direct end parameter of T3 biological activity, the Na, K-ATPase content in skeletal muscle samples as described previously (41). The number of 3[H] ouabain binding sites in soleus muscle was 497 ± 15 in control mice (n=5) and 658 ± 67 pmol/g wet wt in 12-d T3 treated mice, i.e. an increase of 32% (n=5; *P=0.06*), indicating that T3 had exerted its effect in the mice.

Short term T3 treatment

The mice were injected only once with T3 (10 µg/ 100 g body weight) and were sacrificed after 3, 4, 8, 24, 48 or 72 hrs. There were 2 vehicle treated groups at 3 hrs (n=11) and at 48 hrs (n=5). Hearts and EDL muscles were isolated as described above.

Hypothyroidism induced by 5-Propyl-2thiouracil (PTU)

PTU is an anti-thyroid drug which inhibits both the synthesis of thyroid hormones in the thyroid gland, and the conversion of thyroxine (T4) to its active form, triiodothyronine (T3), in peripheral tissues (5). Six week old mice were randomly divided into 2 groups. The euthyroid control group (n=3) was fed *ad libitum* and the PTU treated group (n=6) was fed *ad libitum* with food containing 0.15 % PTU (Teklad Premier Laboratory Diets, Illinois, USA) for 7 weeks (5, 7), which results in a hypothyroid condition as evident from low plasma T3 and T4 levels. The last 12 days of this experimental period all animals received daily injections with the vehicle solution that is used in all experiments in the present study. Subsequently at 13 weeks of age, hearts were isolated as described before.

Culture of Primary rat cardiomyocytes

Primary neonatal rat ventricular cardiomyocytes (NRCM) were isolated from hearts of 1- to 2-day-old Wistar rats (11). Hearts were excised and large vessels and both atria were removed. Ventricular tissue was minced with scissors and remaining blood was washed out. Tissue fragments were transferred to a sterile glass Erlenmeyer and incubated under stirring in 9 ml of trypsinization buffer consisting of 1.16 M NaCl, 200 mM HEPES, 13 mM NaH_2PO_4, 61 mM D-glucose, 53.6 mM KCl, 17 mM $MgSO_4$, 0.05% pancreatine (all from Sigma, St. Louis, USA), 200 units/ ml collagenase IV, pH 7.35) for 20 min at 37°C. Next, the suspension was gently triturated, and the first supernatant was discarded from the sedimented tissue and replaced by fresh enzyme solution. This was repeated for 4 cycles of 20 min, wherein supernatants were collected and supplemented with 10 ml of differential plating medium composed of DMEM with 15% FCS (Life Technologies) and 0.5% Gentamycin (Gibco). To enrich the population

of myocytes, cell pellets were suspended in differential plating medium and plated for 1 hour in uncoated culture flasks. In contrast to cardiomyocytes, non-myocytes rapidly adhere to the plastic and therefore can be removed from the cell preparation. The non-adhering cells were collected by centrifugation, resuspended in fresh plating culture medium supplemented with 17% M199, 0.5% gentamycin and 100 units/ ml penicillin/ streptomycin and plated on 0.1% gelatine (Sigma, St. Lious, USA) coated wells. The neonatal cardiomyocytes were plated at a high density; 500,000 cells per well in a 48-well plate. The next day cells were washed with PBS and maintenance medium (DMEM, 20% M199 (Invitrogen, the Netherlands), 0.5% gentamycin) was added. During experiments cells were kept in maintenance medium and treated with 50 nM T3 in 0.05 mM NaOH with or without 50 mM KCl (32). Cells were harvested after 24, 48 and 72 hours. Cell pellets were stored at -80ºC until further use. Myocyte monolayers typically began synchronously contracting 48 to 72 hrs after plating. The effect of T3 and/ or KCl on myocytes contractile rate was determined using an inverted microscope to visualise the cells. Beats were counted per minute for 3 different fields per well.

RT-PCR analysis

Total RNA was isolated from mouse hearts or myocytes cell lysates using an RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA was quantified by spectrophotometry (260 nm) and checked on a 1% agarose gel. cDNA was synthesized using the iScript^{TM}cDNA Synthesis kit (Biorad, Luxembourg) according to the manufacturer's instructions. Samples were diluted to 10 ng/ ml work stocks in RNAse free water and stored at 4°C. Real time PCR was performed on a MyiQ cycler (Biorad, Luxembourg) using SYBR Green Supermix (Biorad, Luxembourg); cDNA input was 50 ng and the concentration of the primers (forward and reverse) used was 10 µM. The sequences of primers used for real time PCR are described in table I. Product specificity during PCR was verified by melting curve analysis of the products. For each amount of RNA tested, triplicate Ct values were obtained and averaged. Quantification was performed using a mathematical model of relative expression ratio in real-time PCR, the $2^{-\Delta\Delta Ct}$ –method (31, 35) and was calculated with help of the Genex-software (Biorad, Luxembourg). No alterations were found in GAPDH mRNA expression in response to thyroid hormone neither within 6 or 12 days of treatment in the *in vivo* experiments nor within 72 hrs in the *in vitro* experiments; GAPDH was therefore used as a reference gene in cardiac tissue and cardiac cells.

Table I. *PCR primer sequences for real time PCR analyses.*

Primer	Sequence
IGF-1Ea forward	GCTTGCTCACCTTTACCAGC
IGF-1Ea reverse	AAATGTACTTCCTTCTGAGTCT
MGF forward	GCTTGCTCACCTTTACCAGC
MGF reverse	AAATGTACTTCCTTTCCTTCTC
VEGF forward	AGGCTGCTGTAACGATGAAG
VEGF reverse	CTCCTATGTGCTGGCTTTGG
HIF1α forward	GGCTCACCATCAGTTATTTAC
HIF1α reverse	GTCGCCGTCATCTGTTAG
Serca2 forward	TGCTGCTGGGAAAGCTATGG
Serca2 reverse	GTGTTCTCTCCTGTTCTGTTGC
GAPDH forward	GAAGGTCGGTGTGAACGG
GAPDH reverse	TGAAGGGGTCGTTGATGG

Statistical analysis

For the animal experiments, each time point represents the mean values +/- SEM. The values of the T3 treated groups are expressed relatively to the vehicle group. For statistical analyses between groups unpaired Student's *t* tests were performed. Two-way ANOVA with Holm-Sidak post-testing was used for data analysis between thyroid hormone and respective vehicle treatment when all different cardiac regions were compared. Mann-Whitney Rank Sum t-Test was used in comparisons of eu- and hypothyroid mice.

Cell culture experiments were presented as means of three individual experiments +/- sem. Values were expressed relatively to time point t=0. For statistical significance per time point between controls and the experimental group, Student's *t* tests were performed. For statistical analyses in time, multivariate analyses were performed with Bonferonni post hoc tests. For all statistical tests, significance was set at $P< 0.05$.

Results

Long term T3 treatment induces an increase of heart frequency and MGF mRNA expression in vivo

To study the effect of thyroid hormone (T3) on MGF mRNA expression, mice were injected with 10 μg/ 100 g body weight T3 daily for 12 days. Physiological parameters as heart frequency, body temperature, body weight and heart weight were measured and mRNA expression of MGF and IGF-1Ea were determined in cardiac and skeletal muscle. Daily T3 treatment for 12 days significantly increased the basal metabolic state as shown by the significant increase of mean body temperature (1ºC relative to controls, table II). Also heart rate was significantly increased from 581 to 638 bpm relative to controls (table II). Body weights remained stable and were similar between controls and T3 treated mice, indicating this treatment rendered the mice relatively mild hyperthyroid. Heart weight/ body weight ratio increased significantly by 18% upon T3 treatment (table II). As shown in figure 1A these effects were associated with a two-fold significant increase in cardiac MGF mRNA, while skeletal muscle MGF and IGF-1Ea mRNA was unaffected. IGF-1Ea mRNA expression in the heart was slightly induced, but not significantly.

Table II. *Characterization of physiological parameters after long term (12d) T3 injections versus vehicle injected controls*

Groups / Parameters measured	Mean Body Temperature (ºC)	Heart Frequency (BPM)	Absolute heart weight (mg)	Rel. heart weight (HW/100gBW) (%)
Controls (n=10)	37.0 ± 0.24	526 ± 21	136 ± 3	0.51 ± 0.01
Hyperthyroid mice (n=12)	37.9 ± 0.18 *	612 ± 20 *	160 ± 6 *	0.59 ± 0.01 *

Asterisks indicate significance of difference of T3 *vs.* control ($P<0.05$).

To determine in which specific cardiac compartments MGF mRNA expression is induced during hyperthyroidism, hearts of 12 days T3 treated animals were divided into smaller sections. The atria were separated from the ventricles and the right and the left ventricle free wall were dissected from the septum. Additionally, the left ventricle free wall and the septum were divided in a base, middle and apex slice. As shown in figure 1B, the increased MGF mRNA expression in the hyperthyroid heart was pri-

marily due to increased expression in the middle of the left ventricle free wall and in the middle part of the septum. Because the data was depicted relatively to controls, it cannot be deduced from this figure, that basal MGF mRNA was significantly higher expressed in the atria than in the ventricles. Another interesting finding was that in the middle of the septum together with the increase of MGF mRNA, a significant up-regulation of Hypoxia Induced Factor-1α (HIF-1α) and Vascular Endothelial Growth Factor (VEGF) mRNA expression ($P<0.05$) was seen (data not shown). Figure 1C shows a significant difference in IGF-1Ea mRNA expression in the hyperthyroid heart in the middle of the septum but not in the other heart compartments between T3 treated mice and the controls.

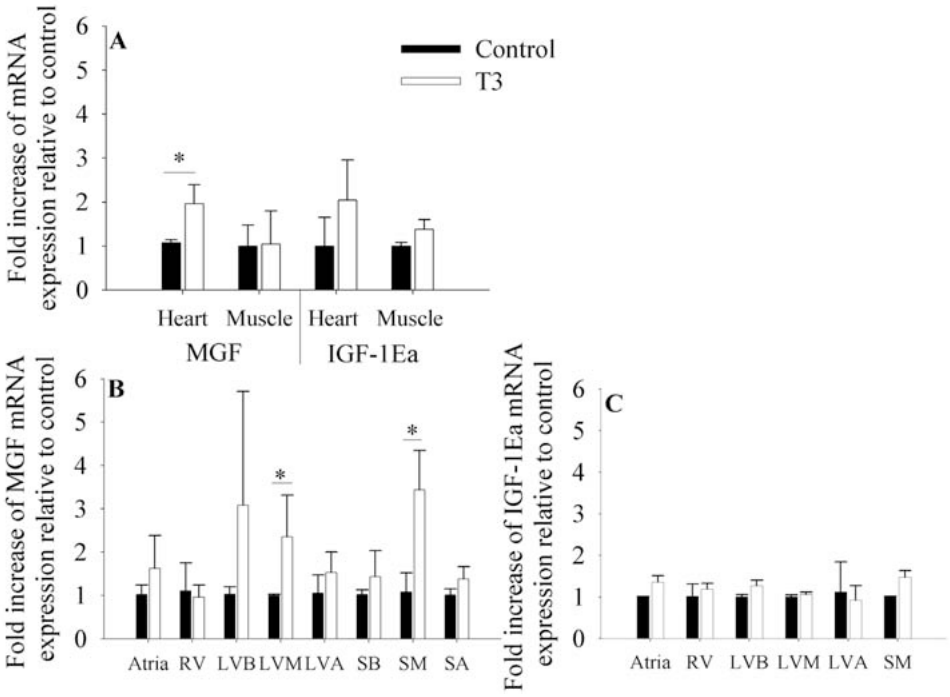

Figure 1. *Cardiac MGF and IGF-1Ea mRNA expression in mice injected daily with T3 for 12 days*

A, RT-PCR analysis of MGF and IGF-1Ea in heart and skeletal muscle of 8 week old mice treated for 12 days daily with T3 (n=5)**.** B and C, MGF and IGF-1Ea mRNA expression in different heart compartments after the same T3 treatment relatively to control (n=4 and 3 resp). Abbreviations: RV = right ventricle, LVB = left ventricle free wall basis, LVM = left ventricle free wall middle, LVA = left ventricle free wall apex, SB = septum basis, SM = septum middle and SA = septum apex. PCR was normalized to GAPDH content in each sample. Data were expressed relatively to controls as means ± SEM. Asterisks indicate significance of difference of T3 *vs.* controls ($P<0.05$).

To investigate if shorter exposure to T3, without reaching a stage of overt hypertrophy, could also induce MGF mRNA expression, mice were treated with the same dose of T3 daily for only 6 days. As shown in figure 2, MGF mRNA expression in the whole heart was increased 4-fold relative to the controls. MGF and IGF-1Ea mRNA expression was again not induced in skeletal muscle. IGF-1Ea mRNA in the heart was slightly induced but statistically not significant after 6 days of T3 treatment.

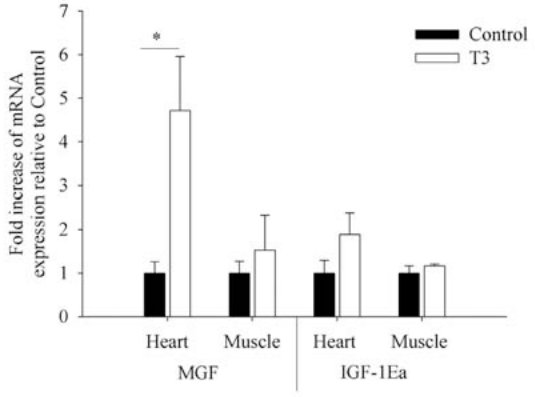

Figure 2. *Cardiac MGF and IGF-1Ea mRNA expression in mice injected daily with T3 for 6 days*
RT-PCR analysis of MGF and IGF-1Ea in heart and skeletal muscle of 8 week old mice treated daily with T3 for 6 days. PCR was normalized to GAPDH content in each sample. Data were expressed relatively to controls and are means of n=3 animals ± SEM. Asterisks indicate significance of difference of T3 *vs.* controls ($P<0.05$).

Next, we evaluated whether only a single T3 injection can acutely induce MGF mRNA expression. Mice were injected once with T3 and sacrificed at different time points as can be seen in figure 3. In these experiments, body temperature and heart rate were not elevated in contrast to 6 to 12 days of daily T3 injections (data not shown). Mice injected with a single dose T3, show a slight but not significant increase in MGF mRNA expression at 4 hrs and 72 hrs, whereas IGF-1Ea mRNA expression did not change (figure 3).

Together, these findings show that MGF is induced during hyperthyroidism, especially in the mid-regions of the septum and left ventricle, indicating that MGF action might be important in specific cardiac regions where mechanical forces are particular intense during increased beating activity of the heart.

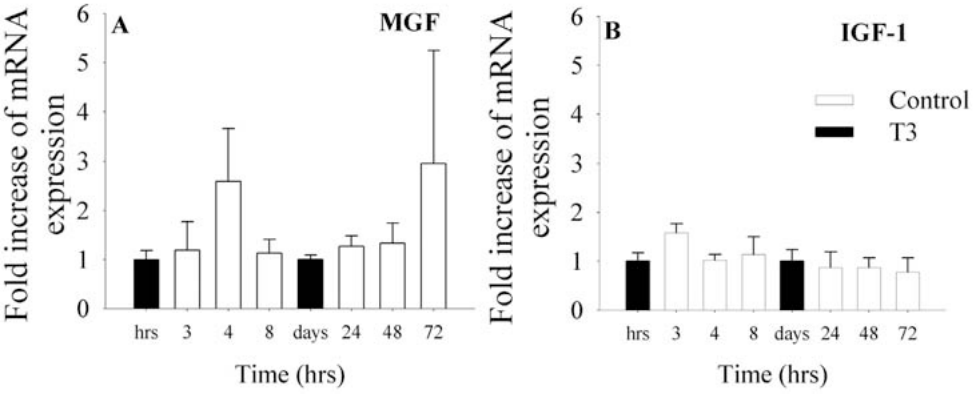

Figure 3. *Cardiac MGF and IGF-1Ea expression in mice single injected with T3*
A and B, RT-PCR analysis of MGF and IGF-1Ea in hearts of 8 week old mice treated one time with the same dose of T3. Animals were sacrificed after 3, 4 and 8 hrs (n=5, 3, 3) with a control group at 3 hrs (n=11). Subsequently, animals were sacrificed at 24, 48 and 72 hrs, with controls at 48 hrs (n=5 per time point). PCR was normalized to GAPDH content in each sample. Data were expressed relative to controls and are means ± SEM. Asterisks indicate significance of difference T3 *vs.* controls ($P<0.05$).

Hypothyroid mice show decreased ventricular expression of MGF and IGF-1Ea mRNA

To determine whether MGF mRNA expression would show the reverse response in the hypothyroid state, mice were put on a PTU diet for 7 weeks. Hypothyroid mice had a significant decrease in heart frequency from 455 ± 30 (n=6) in controls to 313 ± 20 (n=3) bpm in the hypothyroid mice (data not shown). As shown in figure 4 parallel with the decrease in heart frequency in PTU-mice, ventricular specific mRNA expression of MGF and IGF-1Ea decreased significantly by 50%. This decrease was not observed in the atria (data not shown).

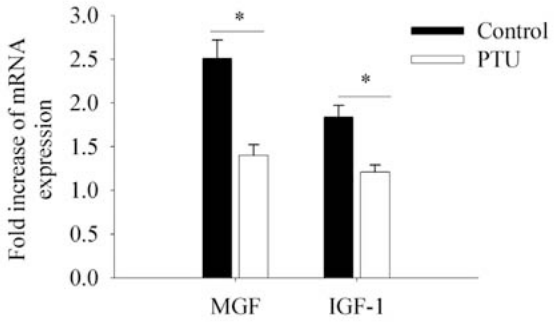

Figure 4. *Cardiac MGF and IGF-1Ea expression after PTU diet* RT-PCR analysis of MGF and IGF-1Ea in hearts of 13 week old mice been on a PTU diet for 7 weeks (n=6) and control mice receiving normal food (n=3). Both groups received vehicle injections. PCR was normalized to GAPDH content in each sample. Data were expressed relative to controls as mean ± SEM. Asterisks indicate significance of difference of PTU *vs.* controls ($P<0.05$).

MGF expression is dependent on mechanical beating activity

To better discriminate if MGF mRNA expression induced by mechanical stress is as part of direct or indirect actions of T3, neonatal ventricular cardiomyocytes were isolated and treated with T3 *in vitro*. Cardiomyocytes started to beat 24h after plating in an asynchronous manner followed by a synchronized and increased beating activity at 48 to 72 hrs (figure 5A). T3 treatment resulted in marked increase in beating frequency. From these same samples MGF and IGF-1Ea mRNA was measured and the results are depicted in figure 5B and 5C. The induction of MGF mRNA occurred already at 24 hrs at the time when cardiomyocytes started to beat. At 72 hrs MGF mRNA expression was significantly increased 40-fold compared to control cardiomyocytes. IGF-1Ea mRNA expression showed the same expression pattern as MGF, however IGF-1Ea mRNA expression was two times lower than that of MGF mRNA (figure 5C).

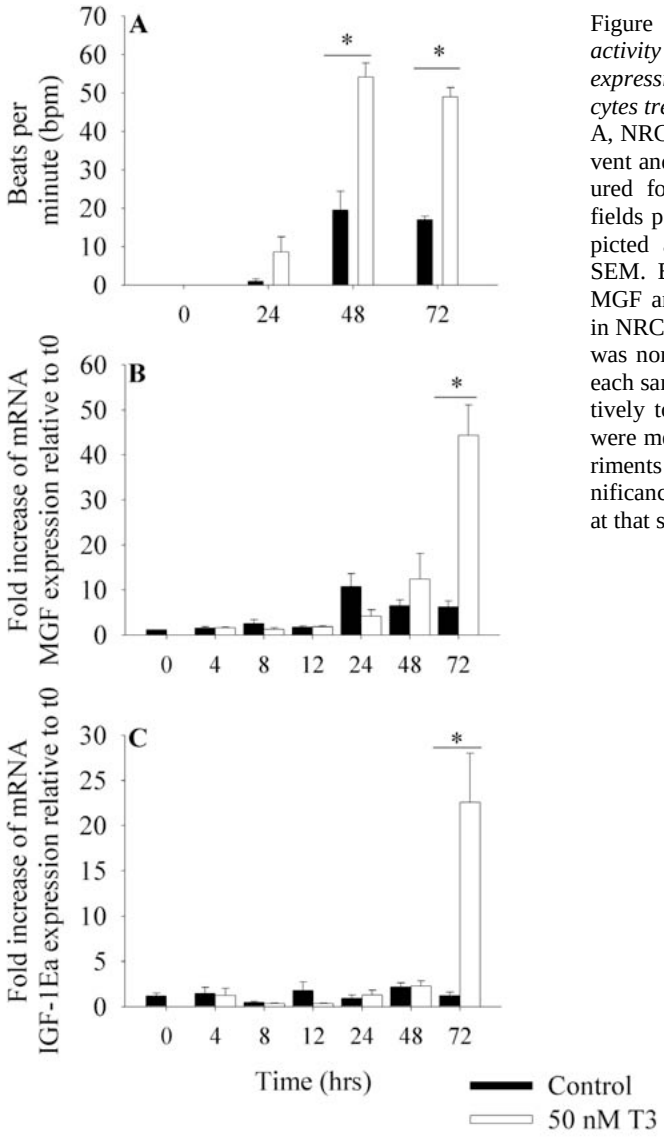

Figure 5. *Time course of contractile activity and MGF and IGF-1Ea mRNA expression in neonatal rat cardiomyocytes treated with T3*
A, NRCM were treated with T3 or solvent and contractile activity was measured for 1 minute in three different fields per well in triplo. Data were depicted as beats per minute (bpm) ± SEM. B and C, RT-PCR analysis of MGF and IGF-1Ea mRNA expression in NRCM treated with T3 in time. PCR was normalized to GAPDH content in each sample. Data were expressed relatively to controls on time point 0 and were means of three independent experiments ± SEM. Asterisks indicate significance of difference T3 *vs.* controls at that specific time point ($P<0.05$).

To evaluate whether MGF mRNA expression is dependent on the mechanical activity cardiomyocytes were contractile arrested by adding KCl to the T3 containing medium. Contractile activity in these cardiomyocytes was significantly decreased 50-fold relative to cells treated with T3 (figure 6A). Parallel to the contractile arrest, MGF mRNA expression was significantly 50-fold reduced compared to cardiomyocytes treated with T3 alone (figure 6B). In contrast, IGF-1Ea was not reduced after supplementation of KCl. As shown in figure 6C, Serca2 was also up-regulated in

cardiomyocytes exposed to T3 for 72 hrs and remained up-regulated in cells exposed to T3 and KCl, indicating that T3 is still active at 72 hrs.

These findings demonstrate that MGF induction in ventricular cardiomyocytes is dependent on the mechanical beating activity.

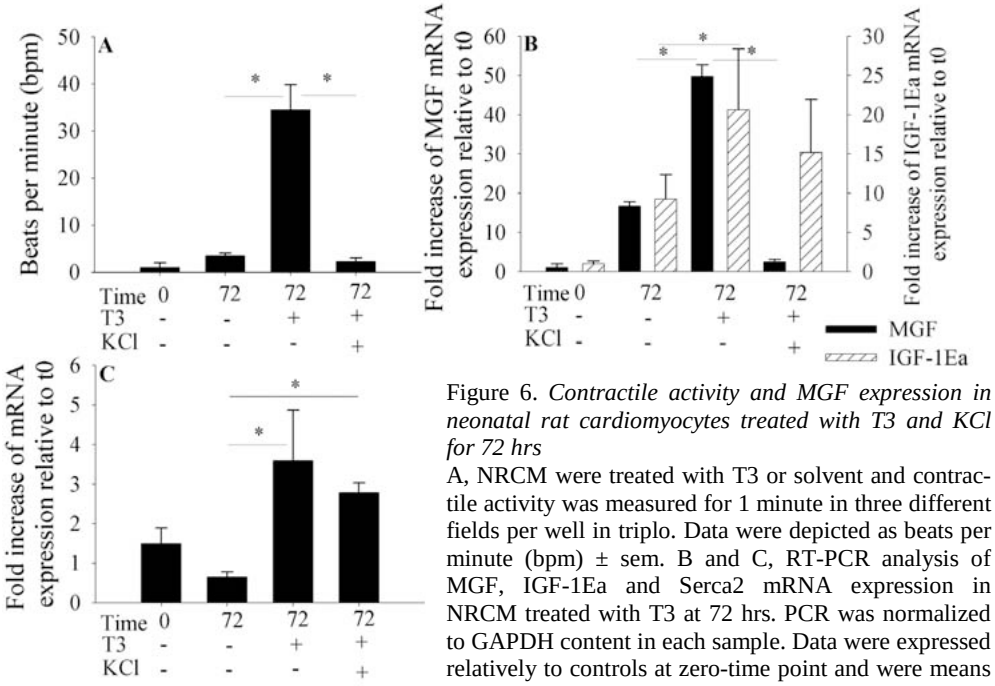

Figure 6. *Contractile activity and MGF expression in neonatal rat cardiomyocytes treated with T3 and KCl for 72 hrs*

A, NRCM were treated with T3 or solvent and contractile activity was measured for 1 minute in three different fields per well in triplo. Data were depicted as beats per minute (bpm) ± sem. B and C, RT-PCR analysis of MGF, IGF-1Ea and Serca2 mRNA expression in NRCM treated with T3 at 72 hrs. PCR was normalized to GAPDH content in each sample. Data were expressed relatively to controls at zero-time point and were means of three independent experiments ± SEM. Asterisks indicate significance of difference T3 *vs.* control ($P<0.05$).

Discussion

This study discovered that MGF mRNA expression was up-regulated in the heart and not in skeletal muscle of mice treated with T3 by daily injections for 12 days, which coincided with an increase of heart frequency. This up-regulation of MGF was particularly observed in the mid part of both the left ventricle free wall and the septum. In contrast to hyperthyroid animals, MGF and IGF-1Ea mRNA expression in the hypothyroid heart were decreased in parallel with a decrease in heart frequency. These findings were also confirmed *in vitro* in cardiomyocytes exposed to T3. Importantly, when cardiomyocytes were treated with T3 and contractile arrest was induced by KCl, MGF mRNA expression was prevented, whereas IGF-1Ea levels were not influenced. Our results show that MGF and not IGF-1Ea regulation in cardiomyocytes is dependent on heart beating activity.

Studies of Goldspink *et al.* in intact skeletal muscle *in vivo* and cultured murine myoblasts (9, 15, 42) show that the IGF gene is first spliced towards MGF and subsequently to IGF-1Ea after mechanical stress or muscle damage. MGF plays a role during skeletal muscle hypertrophy by activating skeletal muscle stem cells and initiating proliferation, followed by differentiation, induced by IGF-1Ea. To test whether MGF also plays a role during cardiac hypertrophy, we determined MGF mRNA expression in hearts of T3 treated mice. After 12 days of daily T3 injections, MGF but not IGF-1Ea mRNA expression was significantly increased in parallel with the increase in heart rate and body temperature. Although, we were not able to confirm MGF mRNA expression at the protein level due to the lack of a commercially available specific antibody, the data of the
research group of Popov *et al.* showed that in myoblasts MGF mRNA measurements do exactly reflect MGF protein levels (27). They showed that MGF synthesis at both mRNA and protein level is activated in murine myoblasts when cultured at a higher temperature (28). From our *in vivo* experiments it cannot be defined what induces increased MGF expression in the mouse hyperthyroid heart; whether MGF is induced by a direct action of T3, or indirectly as a consequence of the change in body temperature and/ or the increase in mechanical loading of the heart. We therefore tested these parameters *in vitro* in isolated NRCM under constant temperature and increased beating activity specifically by adding T3 (39), and found that MGF expression is specifically dependent on beating activity.

To identify the location of the increased cardiac MGF mRNA expression, hearts were further dissected into different compartments. The up-regulation of MGF mRNA in hyperthyroid mice was primarily located in the mid parts of the left ventricle free wall and the septum. This could be explained by the increased mechanical stretch of the ventricle wall due to the increased workload of the heart for sufficient cardiac output to compensate the body's higher metabolic demand (26). It has been shown that mechanical stress applied to the heart is associated with an early expression of HIF-1α and its target VEGF (23). These genes are upstream regulated by Stretch Activated Channels (SAC) and the PI3K/Akt/Frap pathway. The precise mechanism from SACs to PI3K has not been clarified yet [33]. Also in the present study VEGF and HIF-1α (data not shown) were detected in the middle of the septum, i.e. localized in the same areas as MGF mRNA, suggesting MGF might be commonly regulated. Examination of the promoter region of the *Igf-1* gene revealed a HIF response element and not a thyroid response element, suggesting that MGF might be directly induced by HIF-1α during mechanical stretch of the heart. More research is required including promoter binding studies to understand the molecular regulation of MGF expression.

When MGF and IGF-1Ea mRNA expression were measured in hypothyroid mice, we saw that both isoforms decreased in parallel with the heart frequency, suggesting a general down-regulation of the *Igf-1* gene. As changes in T3 status have major effects on the GH/IGF axis, reduced thyroid hormone levels are accompanied with down-regulation of GH gene expression and therefore also a reduced synthesis of IGF-1 (34).

The data on the hyper- and hypothyroid heart support our hypothesis: reduced mechanical load is associated with decreased MGF expression, and increased mechanical load is accompanied by higher expression of MGF. Interestingly, Kawada and Ishii recently reported that MGF expression was reduced in cardiac hypertrophy in a rat model of peripheral vascular disease (22). Unfortunately, their study did not include measurement of heart frequency. Another possible explanation is the basal difference between these two animal models of cardiac enlargement: physiological versus pathological hypertrophy. The maladaptive response of cardiac growth, as described by Kawada *et al*. (22), and being associated with decreased MGF expression, is distinct from our model for

several reasons: T3-mediated cardiac hypertrophy is not a persistent pressure overload induced hypertrophy with loss of nitric oxide (NO) availability. Moreover when T3 is administrated to mice, NO is acutely increased with consequent vasodilatory changes, locally, e.g. in the brain (19), and systemically (25). Neither have we seen marked changes in IGF-1. The only increase, found in the middle section of the septal wall is a moderate change that is reported for adaptive physiological growth exerted by exercise (38). This together implies that MGF may play a beneficial role in adaptive growth of the heart.

Our results show that a single T3 injection did not induce MGF or IGF-1Ea mRNA expression in cardiac nor in skeletal muscle. Moreover, a single T3 injection did not elevate heart frequency either. The latter is in line with the study of Johansson *et al*. who found that only after a second T3 injection heart frequency increased at 48 hrs (21). The issue of short-term effects of T3 on heart was further pursued *in vitro* to allow discrimination between direct or indirect T3 actions e.g. increased body temperature and hemodynamic loading. To test specifically if MGF mRNA expression was induced by elevated beating activity, the NRCM were treated with T3. The cells started to contract synchronously after 48 hours of culture and T3 increased the pacing rate by 30-fold. Subsequently, MGF mRNA expression increased at 72 hrs after starting the treatment with T3. However, from this experiment it was not clear whether MGF expression is induced by T3 directly, or induced as a consequence of the beating frequency. We therefore performed a contractile arrest experiment induced by KCl in presence of T3. MGF mRNA in the cardiomyocytes was down-regulated to control

levels, while Serca2 an important gene that is positively transcribed by T3, was still up-regulated, indicating that T3 action was still present. These results show that the down-regulation of MGF mRNA in NRCM was specifically dependent on mechanical beating action. In all *in vitro* experiments, except for those with contractile arrest, IGF-1Ea mRNA expression followed the same pattern as MGF. It is likely that two different mechanisms of alternative splicing are activated to explain the different expression pattern of MGF and IGF-1Ea, but the precise mechanism remains unclear.

In conclusion, this study shows that endogenous MGF expression is dependent on the beating activity in a mouse model of physiological cardiac hypertrophy induced by thyroid hormone. These findings suggest that MGF is induced in mechanically stressed cardiomyocytes and potentially mediates the adaptive hypertrophic response towards thyroid hormone treatment.

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Chapter 4

Induction of the Mechano Growth Factor during myocardial tissue formation and ischemic injury

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Abstract

Cardiomyocytes actively proliferate during embryogenesis and withdraw from the cell cycle during neonatal stages. The Mechano growth factor (MGF), an isoform of the Insulin-like growth factor, is involved in regulation of cellular proliferation and survival of several cell types. MGF is induced during cardiac enlargement and exogenous MGF improved cardiac function after myocardial infarction. However, regulators of MGF induction are unknown. The aim of this study was to investigate the regulation of MGF expression during cardiac development and injury. Here we show that the expression of MGF increases during early cardiac embryonic development, decreases during postnatal development, and re-emerges shortly after acute myocardial infarction in adult mice. Since myocardial infarction results in an insufficient supply of oxygen and nutrition towards the infarcted area, we evaluated the influence of hypoxia or starvation on MGF expression. We identified that hypoxia induced MGF by exposing cardiomyocyte cell cultures to desferrioxamine, which stabilizes the hypoxia-inducible factor-1α (HIF-1α). In contrast, starvation of cardiomyocytes decreased MGF levels.

These findings demonstrate that MGF is induced during early embryonic development and upon ischemic injury of the heart. Nutritional shortage is not the critical stress factor for MGF induction, but hypoxia/HIF-activated pathways are likely to be important for MGF regulation.

Introduction

Cardiovascular diseases are among the leading causes of death in the Western World. The most important presentation of cardiovascular disease is local ischemia, which leads to cellular apoptosis, necrosis, and tissue hypoxia, and in severe situations, organ dysfunction. Ischemia is a result of the absent or diminished blood flow in coronary vessels, leading to a mismatch between cardiac metabolic supply and demand.

The adult heart has a cellular response to adapt to injury after an infarct: loss of contractile mass due to apoptosis of cardiomyocytes and hypertrophy of the remaining non-injured adult cardiomyocytes to increase the muscle fiber. The existence and quantitative amount of endogenous cardiac regeneration is currently under intense dispute, no clear picture has yet emerged. Recently, cardiac progenitor cells and the signalling pathways controlling their proliferation and differentiation in the adult organ have come into focus. Cardiac progenitor cell transplantation has been shown to decrease infarct size and improve cardiac performance in a rat model of myocardial infarction (4, 32). These findings suggest that cardiac progenitor cells may play an important role in cardiac regeneration.

In mammalian heart development, the insulin-like growth factor-1 (IGF-1) plays a distinct role. It promotes cell mitogenesis and proliferation in early developmental stages and causes after binding to the IGF-1 receptor on embryonic stem cells, expression of a number of early cardiac-specific transcription factors such as the zinc finger GATA proteins and the co-activator of GATA-4, Nkx-2.5 (24, 37). These transcription factors are essential for embryonic cardiac development, but also affect adult cardiac remodelling. In the adult IGF-1 is involved in myocardial hypertrophy. Interestingly, cardiac hypertrophy is characterized by a change in the gene expression pattern that recapitulates the neonatal profile, thereby re-activating genes in the adult heart in response to pathological stimuli that are also involved in cardiac development (25). An emerging concept is that cardiac progenitor cells contribute to the replacement of adult mammalian cardiomyocytes after injury (11). These observations lead to the hypothesis, that endogenous cardiac progenitor cells might be controlled by similar pathways that initially govern cardiac development.

Recently, we have demonstrated that an alternative splice form of IGF-1, the Mechano Growth Factor (MGF), was induced during cardiac enlargement upon thyroid hormone treatment (36). MGF was specifically up-regulated in the septum and left ventricle, and the levels of MGF expression depended on the beating activity of the heart. MGF is expressed 4 weeks after a myocardial infarction in rats (33), moreover a recent study in sheep by Carpenter *et al.* (6) showed that pre-treatment with exogenous MGF improved cardiac function after infarction. Although these studies indicate that MGF is

involved in cardiac remodelling, it is unclear how MGF itself is regulated during cardiac remodelling or even during cardiac development. Because many processes e.g. ischemia, cell death, inflammation, angiogenesis, remodelling take place simultaneously after a myocardial infarction and partly overlap each other, it is unknown what exactly triggers the expression of MGF. During an ischemic insult, insufficient blood flow leads to inadequate oxygenation and metabolic supply of the surrounding tissue. This in turn leads to tissue hypoxia (reduced oxygen). We tested whether the lack of oxygen supply or the shortage of nutrients causes MGF induction or if MGF is part of an early stress response.

The precise mechanisms regulating MGF expression in the (re)generation of heart tissue is incompletely understood. Therefore MGF was assessed in the context of cardiac lineage determination in an *in vitro* P19 embryonic stem cell model as well as during hypoxia of cardiomyocytes as well as in an *in vivo* mouse model of myocardial infarction. Here, we report on two splice variants of IGF-1 (38), MGF and IGF-1Ea regulation during early cardiac development and in response to myocardial infarction *in vivo*. Furthermore, we identified a hypoxia-dependent mechanism of MGF regulation in cardiomyocytes.

Materials and methods

Experimental animals

All experiments with BALB/c mice (34.2 +/- 0.5 g, 10 to 12 weeks old, The Jackson Laboratory, Bar Harbor, Me) and wild-type (WT) mice (26.2 ± 0.5 g, 10 to 12 weeks old, BALB/c, Harlan, Indianapolis, U.S.A.) were performed in accordance to the national guidelines and with permission of the Animal Experimental Committee (DEC) of Utrecht University, Utrecht, The Netherlands.

Surgical Protocol: MI

Mice were anesthetized with isoflurane, and intubated using a 24-gauge intravenous catheter with a blunt end. Mice were artificially ventilated at a rate of 105 strokes/min using a rodent ventilator with a mixture of O_2 and N_2O (1:2 vol/vol) to which isoflurane (2.5–3.0% vol/vol) was added. The mouse was placed on a heating pad to maintain the body temperature at 37°C. The chest was opened in the third intercostal space and an 8-0 prolene suture was used to permanently ligate the left coronary artery. In sham operated animals, the suture was placed under the artery and removed without ligating the artery. Total RNA was extracted from infarcted and remaining myocardium using Tripure reagent (Roche) according to the manufacturer's instructions, converted into cDNA and subjected to quantitative reverse transcriptase polymerase chain reac-

tion (RT-PCR). Data on MI size and heart function have been published elsewhere by Timmers *et al.* (35).

P19 embryonic carcinoma (EC) cell culture

The differentiation of P19 EC cells emulates the biochemical and morphological processes that occur during embryonic development (19). P19 EC cells were maintained in a monolayer in DMEM F12 (Invitrogen, the Netherlands) supplemented with 7.5% FCS and 1% non-essential amino acids (Invitrogen, the Netherlands). For the differentiation experiments cells were plated in hanging drops containing 800 cells in a volume of 20 µl in the presence 0.8% dimethylsulfoxide (DMSO) onto the lid of bacterial grade culture dishes and incubated at 37 ºC for 4 days (21, 22). The base of each culture dish was filled with PBS to prevent the drops from evaporating. On the fifth day, the aggregates were collected from the hanging drops and plated in plastics. The aggregates were examined every day for the presence of beating cells and samples were collected daily from day 0 to 9. Cell pellets were stored at -80ºC until further use.

Thymidine incorporation

Cells were seeded at 40% confluency into a Cytostar-T scintillating micro plate as described by the manufacturers' description (Amersham Biosciences, Buckinghamshire, UK) and incubated overnight at 37°C, 5% CO_2. Cell cultures were supplemented with 0.5 µCi/ml final concentration of [^{14}C] Thymidine, diluted in 200 µl medium. Cultures were counted immediately, then incubated at 37°C and before counted again, cells were trypsinized, cell numbers were manually counted and [^{14}C]Thymidine incorporation was determined by scintillation counting for at least 30 sec per well in triplet. Samples were taken every 24h. Cells were checked regularly under the microscope for growth and morphology.

HL-1 Cell culture

Cells of HL-1, a cardiac muscle cell line derived from the AT-1 mouse atrial myocyte tumour lineage, were a gift from Dr. Marti Bierhuizen (Medical Physiology, University of Utrecht, the Netherlands) and maintained accordingly as described (7). HL-1 cells were used for experimentation after reaching ~70–80% confluence.

For every experiment cells were seeded in 6 well plates at a density of 300.000 cells per well in supplemented Claycomb medium. Cells were allowed to attach overnight and the next day cells were washed twice and experiments were started. Chemical hypoxia was performed by adding 100 µM desferrioxyamine (DFO, Sigma, St. Louis, USA) into the supplemented Claycomb media and cells were incubated for 24-72 hours

and harvested at 24, 48 and 72 hrs. For serum starvation, after washing the cells, cells were put on serum free DMEM with 0.2% FCS. Again cells were harvested after 24, 48 and 72 hrs. For harvesting, cells were trypsinized; cells were stained with tryptan blue and counted in a Bürker-Heimer cell counter. Cell viability was calculated by abstracting dead, blue cells from the total counted cells. Time point t=0 was set as 100%.

RT-PCR analysis

Total RNA was isolated from mouse hearts or myocytes cell lysates using an RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA was quantified by spectrophotometry (260 nm) and checked on a 1% agarose gel.

cDNA was synthesized using the iScript^{TM}cDNA Synthesis kit (Biorad, Luxembourg) according to the manufacturer's instructions. Samples were diluted to 10 ng/µl work stocks in RNAse free water and stored at 4°C. Real time PCR was performed on a MyiQ cycler (Biorad, Luxembourg) using SYBR Green Supermix (Biorad, Luxembourg); cDNA input was 50 ng and the concentration of the primers (forward and reverse) used was 10 µM. The sequences of primers used for real time PCR are described in table 1. Product specificity during PCR was verified by melting curve analysis of the products. For each amount of RNA tested, triplicate Ct values were obtained and averaged. Quantification was performed using a mathematical model of relative expression ratio in real-time PCR, the 2 -ΔΔCt –method (16, 26) and was calculated with help of the Genex-software (Biorad, Luxembourg). As we found no significant alterations between the different experimental variables in GAPDH expression, GAPDH was used as a reference gene (12).

Statistical analysis

Cell culture experiments were performed three times and means +/- SEM are shown. Values were expressed relatively to time point t=0. For statistical significance per time point between vehicles and the experimental group, Student's *t* tests were performed. For statistical analyses in time, multivariate analyses were used with Bonferonni *post hoc*.

For the animal experiments, each time point represents the mean values of 3 mice +/- SEM. The values of the MI groups are expressed relatively to the sham-operated group. For statistical analyses one way ANOVA were performed with a *post hoc* Dunnett. For all statistical tests statistical significance was set at $P < 0.05$.

Table I. *PCR primers sequences for real time PCR analyses*.

Marker	Primer	Sequence
Differentiation	IGF-1Ea forward	GCTTGCTCACCTTTACCAGC
	IGF-1Ea reverse	AAATGTACTTCCTTCTGAGTCT
Target gene	MGF forward	GCTTGCTCACCTTTACCAGC
	MGF reverse	AAATGTACTTCCTTTCCTTCTC
Hypoxia	VEGF forward	AGGCTGCTGTAACGATGAAG
	VEGF reverse	CTCCTATGTGCTGGCTTTGG
Heart	Nkx2.5 forward	CCAAGTGCTCTCCTGCTTTCC
	Nkx2.5 reverse	AGGGTCTTTGGCTGGGTCAG
	GATA4 forward	GGTTCCCAGGCCTCTTGCAATGCGG
	GATA4 reverse	AGTGGCATTGCTGGAGTTACCGCTG
House keeping gene	GAPDH forward	GAAGGTCGGTGTGAACGG
	GAPDH reverse	TGAAGGGGTCGTTGATGG

Results

Developmental regulation of MGF cardiac expression

Mouse P19 embryonic carcinoma cells differentiate into the cardiac lineage in response to high density and DMSO (31). To determine whether MGF expression is induced during early cardiomyocyte differentiation of stem cells, MGF as well as IGF-1Ea mRNA expression was analysed in differentiating P19 EC cells. Following the published procedure (21) P19EC cells were seeded onto bacteriological Petri dishes in the presence of DMSO allowing them to form aggregates for 5 days, and transferred the aggregates to tissue culture dishes. From day 5 onwards, the early cardiac transcription factors Nkx2.5 and GATA4 were significantly increased to 15- and 6-fold respectively at day 9 (figure 1A and 1B). During the cardiac differentiation of the P19 EC cells (after day 5), MGF and IGF-1Ea mRNA were significantly up-regulated; MGF by 500-fold and IGF-1Ea by 1800-fold on day 9 (figure 1C and 1D).

Previous studies have shown that MGF and IGF-1Ea can induce cellular proliferation (5, 8, 38). To determine cell proliferation in the P19 EC cells during MGF induction we performed [^{14}C] Thymidine incorporation assays. MGF/IGF-1 mRNA levels are low during the first 4 days where undifferentiated P19 EC cells were grown in aggregates. Here, cells started a first significant replication wave at day 2 and 3 marked by a significantly 10-fold increase in thymidine incorporation (figure 1E). Subsequently, the proliferation rate of the P19 cells slowed down (day 4-6), but in parallel

with cardiac differentiation and MGF/IGF-1 mRNA induction, P19 EC cells initiated a second replication wave (day 7-9) as marked with a significant 25-fold increase of thymidine incorporation. These findings suggest that MGF and IGF-1 expression in P19 EC cells is induced during differentiation and expansion of cardiac progenitor cells.

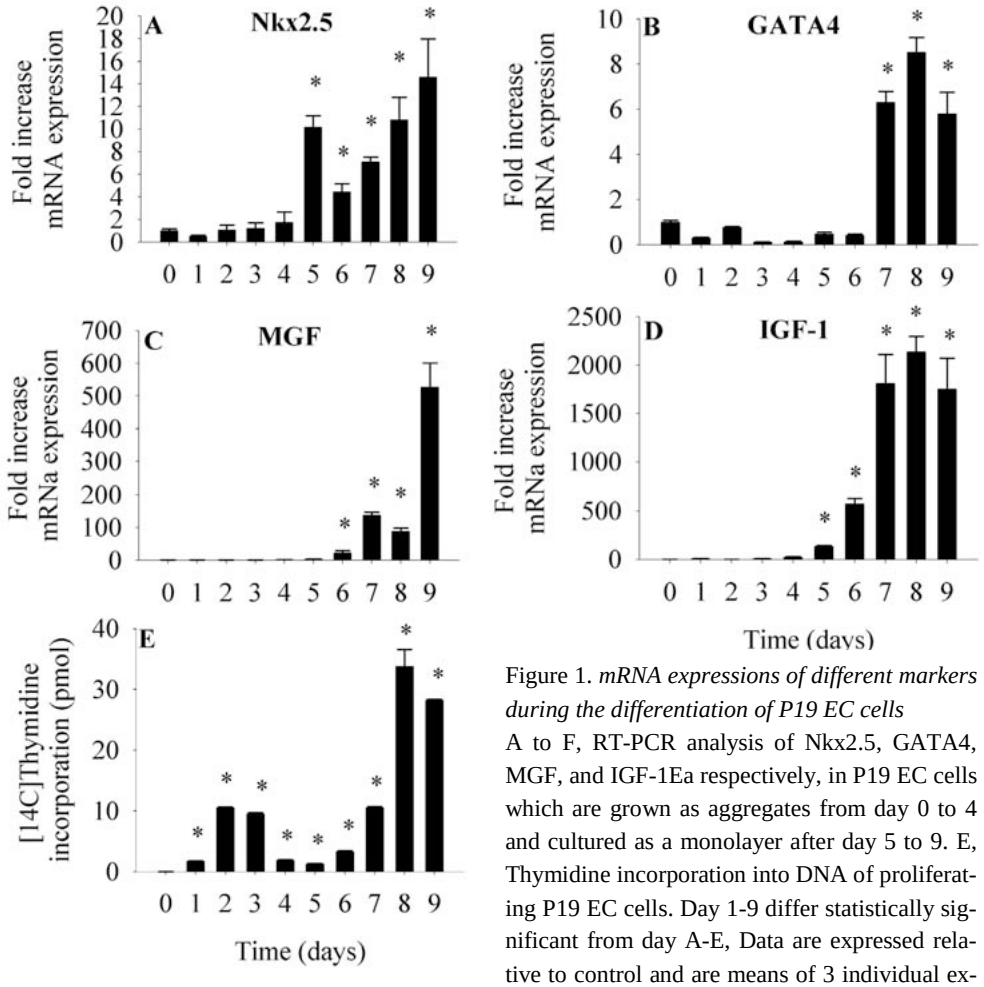

Figure 1. *mRNA expressions of different markers during the differentiation of P19 EC cells*
A to F, RT-PCR analysis of Nkx2.5, GATA4, MGF, and IGF-1Ea respectively, in P19 EC cells which are grown as aggregates from day 0 to 4 and cultured as a monolayer after day 5 to 9. E, Thymidine incorporation into DNA of proliferating P19 EC cells. Day 1-9 differ statistically significant from day A-E, Data are expressed relative to control and are means of 3 individual experiments ± SEM. Asterisks indicate significance of difference of cells of day 0 *vs.* cells on day 1-9 ($P<0.05$).

Next, we compared MGF mRNA expression between murine fetal and adult hearts. As figure 2 shows, MGF mRNA expression was endogenously expressed in both heart samples. However, MGF mRNA expression was 2-fold higher expressed in the fetal hearts than in the adult hearts, indicating that MGF levels decline during post-natal development.

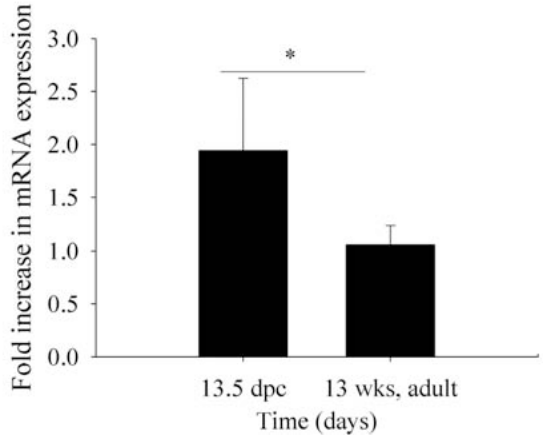

Figure 2. *mRNA expression of MGF in embryonic vs. adult hearts*
RT-PCR analysis of MGF in 3 pooled embryonic 13.5 dpc *vs.* 13 wks old postnatal hearts. Data are expressed relative to adult hearts and are means of triplets ± SD.

MGF expression is reactivated after myocardial injury

To characterize the effect of ischemic pathological injury on cardiac MGF and IGF-1Ea gene expression, their mRNA levels were determined after induction of myocardial infarction (MI) in mice between 12 hrs to 10 days post MI. As shown in figure 3A, MGF mRNA expression is significantly induced during cardiac remodelling at 24 and 72 hrs by 2-fold and by 5.5-fold respectively. IGF-1Ea mRNA expression is also significantly induced at 24, 72 and 240 hrs (figure 3B). As shown during embryonic development (see figure 1), induction of MGF and IGF-1Ea is accompanied with an increased expression of GATA 4, a cardiac specific transcription marker associated with cardiac progenitor cell differentiation (figure 3C). These findings might suggest that MGF and IGF-1Ea levels rise in the remaining myocardium during pathological cardiac remodelling, at the time when cardiac progenitor cells are activated.

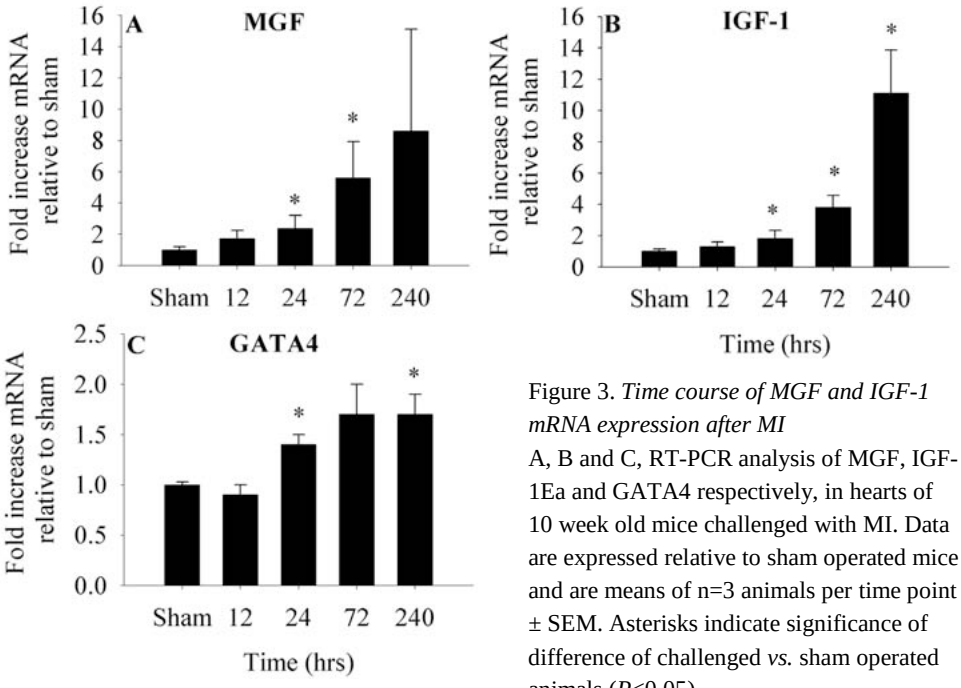

Figure 3. *Time course of MGF and IGF-1 mRNA expression after MI*
A, B and C, RT-PCR analysis of MGF, IGF-1Ea and GATA4 respectively, in hearts of 10 week old mice challenged with MI. Data are expressed relative to sham operated mice and are means of n=3 animals per time point ± SEM. Asterisks indicate significance of difference of challenged *vs.* sham operated animals ($P<0.05$).

MGF induction by hypoxia

Since, MGF and IGF-1Ea gene expression increased during cardiac ischemia, we investigated if MGF and IGF-1Ea gene expression was sensitive to hypoxia in cultured cardiomyocytes. Therefore we pharmacologically stabilized the hypoxia inducible factor (HIF) by adding desferrioxamine (DFO), a prolyl hydroxylase inhibitor, to murine cardiomyocytes (HL-1). DFO-treated cells were not proliferating; total cell numbers were significantly decreased relatively to control cells (figure 4A). Furthermore, cell viability significantly decreased with 50% after 48 and 72 hrs of DFO-treatment relatively to control cells (figure 4B). DFO-treatment of HL-1 cells resulted in significant increase of Vascular Endothelial Growth Factor (VEGF), a direct down-stream target of HIF action (figure 5A). DFO-treatment resulted in a significant up-regulation of MGF mRNA expression by 4-fold at 48 hrs (figure 5B) and of IGF-1Ea mRNA expression by 5-fold at 48 hrs (figure 5C). IGF-1Ea mRNA expression was also significantly increased 2.5-fold in control cells at 48 hrs relatively to t=0. These studies show that MGF and IGF-1Ea mRNA expression is induced after DFO-treatment, suggesting that MGF and IGF-1Ea transcript levels are regulated by hypoxia.

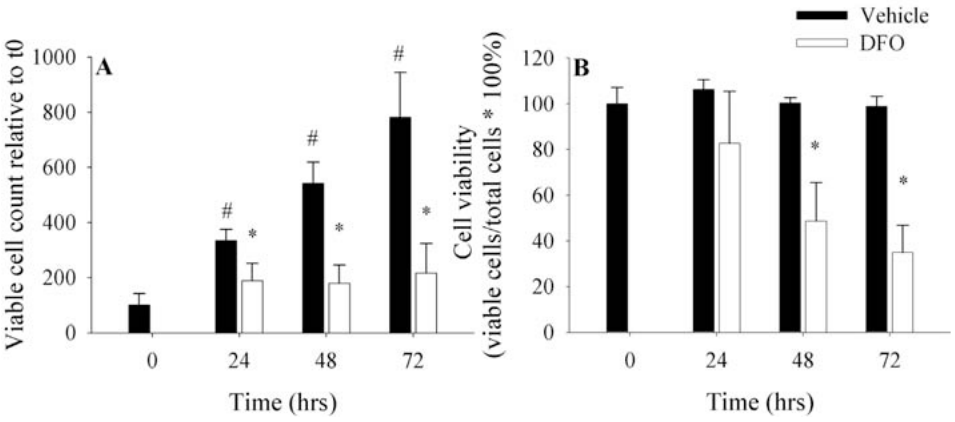

Figure 4. *Cell count and viability of HL-1 cells after DFO-induced hypoxia*
A, Viable cell count after administration of DFO to HL-1 cells. B, Cell viability of HL-1 cells after DFO-treatment. Data are expressed relative to control and are means of 3 individual experiments ± SEM. Asterisks indicate significance of difference of DFO treated *vs.* control cells ($P<0.05$).

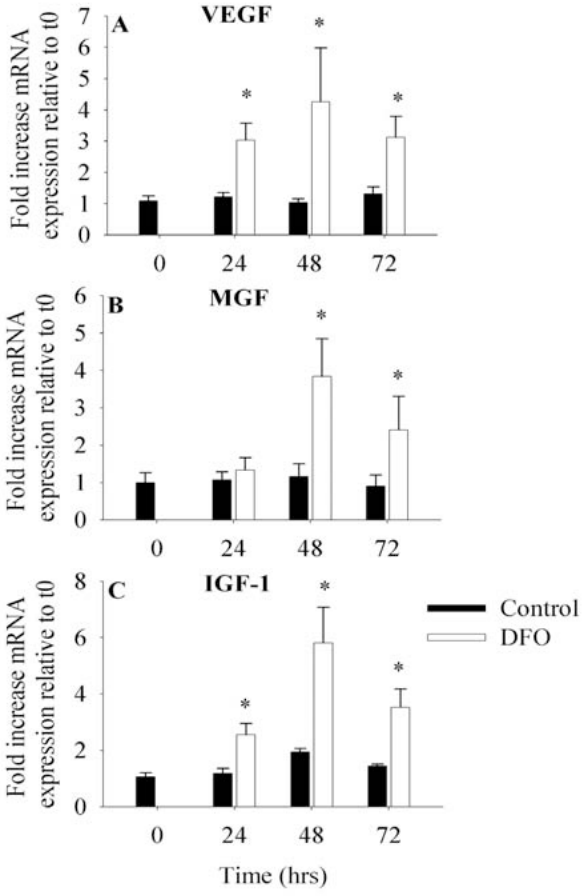

Figure 5. *mRNA expressions of different markers of HL-1 cells after DFO-induced hypoxia*
A, B and C, RT-PCR analysis of VEGF, MGF and IGF-1Ea respectively in HL-1 cells treated with DFO. Data are expressed relative to control and are means of 3 individual experiments ± SEM. Asterisks indicate significance of difference of DFO treated *vs.* control cells ($P<0.05$).

MGF reduction by starvation

Occlusion of the coronary artery results not only in lack of oxygen within the infracted areas, but also prevents the entrance of nutrients. To assess whether a shortage of nutrients after MI could also induce MGF and IGF-1A mRNA expression, HL-1 cardiomyocytes were serum starved in DMEM medium with 0.2% FCS. When HL-1 cells were serum-starved in Claycomb's medium, the cells were still proliferating, due to enriched growth factors in the Claycomb's medium (data not shown). As shown in figure 6A, total cell number did not increase over time upon serum starvation, indicating that the HL-1 were growth arrested. However, cell viability significantly decreased after 48 hrs with 60% and after 72 hrs with 30% (figure 6B). MGF and IGF-1Ea mRNA were not induced by serum starvation, but rather decreased in parallel with the cell viability (figure 7A and B).

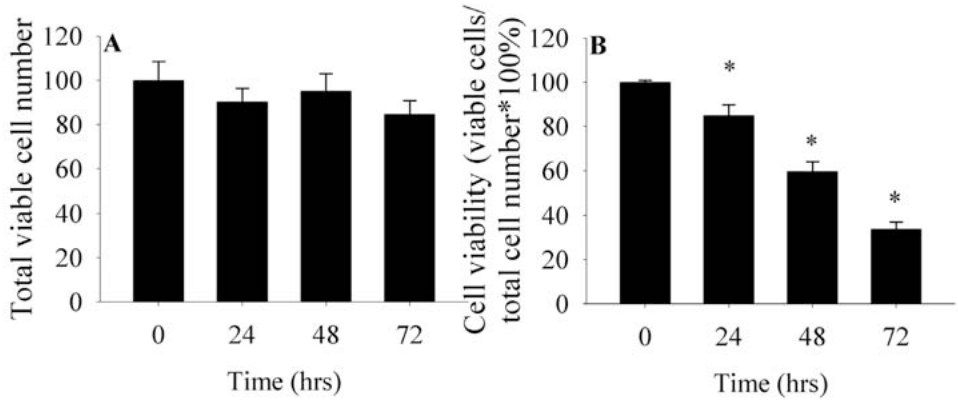

Figure 6. *Cell count and viability of HL-1 cells after serum deprivation*
A, Viable cell count after HL-1 cells are switched to medium containing 0.2% FCS. B, Cell viability of HL-1 cells after serum deprivation. Data are expressed relative to control and are means of 3 individual experiments ± SEM. Asterisks indicate significance of difference of DFO treated *vs.* control cells ($P<0.05$).

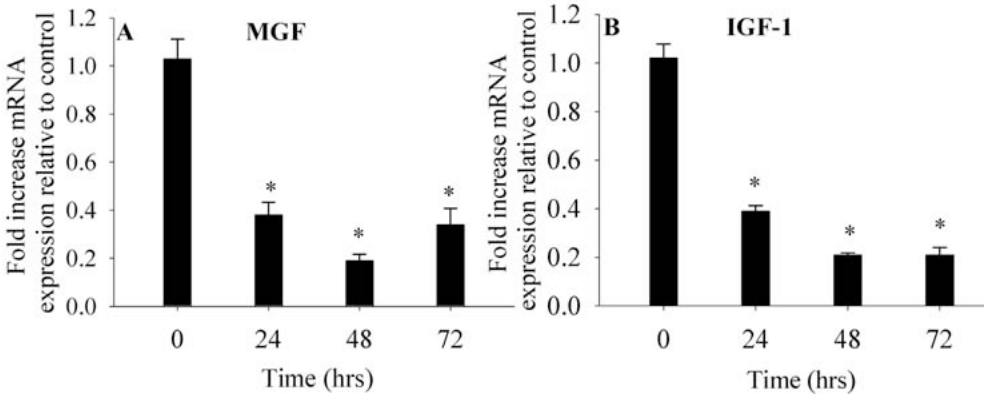

Figure 7. *MGF and IGF-1Ea mRNA expression of HL-1 cells after DFO-induced hypoxia*
A and B, RT-PCR analysis of MGF and IGF-1Ea in serum deprived HL-1. Data are expressed relative to control and are means of 3 individual experiments ± SEM. Asterisks indicate significance of difference of DFO treated *vs*.control cells ($P<0.05$).

Discussion

The discovery that MGF is involved in cardiac protection generates a need to understand the molecular regulation of MGF (6, 17, 29). The present study shows that MGF is up-regulated during cardiomyogenesis; MGF is expressed during differentiation of embryonic carcinoma cells into cardiac progenitor cells, subsequently MGF expression declined during postnatal development, and is reactivated upon myocardial ischemia in the adult mouse heart.

The P19 mouse embryonic carcinoma cell line is a well established *in vitro* model for embryonic development (19, 21). MGF and also IGF-1Ea mRNA expression were not induced during differentiation of embryonic carcinoma cells towards mesoderm, endoderm and ectoderm lineage commitment (P19 embryonic bodies at day 1-4 (21)), suggesting that neither splice variant plays a role during the first phase of embryonic development. Subsequently, 25-30% of P19 cells committed towards the cardiac lineage as marked by the increase of Nkx2.5 and GATA4 (19, 21). During this phase of early cardiac differentiation, MGF and IGF-1Ea mRNA levels were increased. Interestingly, MGF expression in adult hearts was significantly lower than in fetal hearts, indicating that down-regulation of MGF might be needed for cardiomyocyte differentiation/ homeostasis at postnatal stages.

Recent studies have suggested that endogenous cardiac regeneration via proliferation and differentiation of cardiac progenitor cells contributes to adult cardiac remodelling upon stress (4, 18, 28). The proliferation and differentiation of these cells is hypothesized to recapitulate signalling events from cardiac development (27). In this

context, we implicate that induction of MGF occurs during cardiac progenitor proliferation and differentiation in the developing and in the regenerating heart. These findings suggest that MGF might be a good candidate to activate cardiac progenitor cells after myocardial infarction and thereby improving cardiac function (17). In a previous study it has been shown that treating sheep with MGF before myocardial infarction improved cardiac function, supporting the role of MGF as a survival factor (6). MGF could play a role during the cardiac regeneration process (17), similar to its role during skeletal muscle regeneration. In damaged skeletal muscle, MGF was shown to activate the muscle stem cells, which start to proliferate and differentiate and finally fuse to the existing non-damaged fibres (9, 10, 38).

In a recent study by Stavropoulou *et al.* (33) it has been shown that MGF and IGF-1Ea levels were increased in rats 4 weeks after myocardial infarction. The present study shows that MGF and IGF-1Ea were both up-regulated as early as 24 h after myocardial infarction relatively to control mice. Although, we were not able to confirm MGF mRNA expression at the protein level due to the lack of a commercially available specific antibody, the data of Popov *et al.* showed that in myoblasts MGF mRNA measurements do exactly reflect MGF protein levels (15). The up-regulation of MGF and IGF-1Ea is similar with the study Stavropoulou (33); the reason why MGF induction upon myocardial infarction in mice occurred earlier than in rats, might be explained that the infarction inflicted in this mouse study is bigger than in the rat study. Furthermore the rat study revealed that activation of ERK1/2 in H9C2 cardiomyocytes is a potential downstream event of MGF signalling. In contrast, the present study is investigating how MGF is regulated, in order to identify upstream events that induce MGF during myocardial injury.

Myocardial infarction leads to acute necrosis and delayed cardiomyocyte apoptosis in the border zone of the infarct and the remote area (2, 34). The remaining myocardium tries to compensate the loss of contractile force resulting from this by hypertrophy of cardiomyocytes. Hypoxia has been shown to directly induce cardiac hypertrophy (3, 14). Therefore, hypoxia was investigated *in vitro* in HL-1 cardiomyocytes by adding DFO, which mimics hypoxia by stabilizing HIF (30). IGF-1Ea mRNA expression was significantly induced with the onset of hypoxia as marked by a significant up-regulation of VEGF mRNA expression. The up-regulation of IGF-1Ea is in accordance with previous studies where hypoxia was induced in various cell systems (13, 20, 23). The significant up-regulation of IGF-1Ea after 48 hrs in control cells could be explained by the role of IGF-1 in cell growth and proliferation (1, 8). Our results show for the first time that MGF mRNA expression is induced by hypoxia. Recently it has been shown that other cellular stress factors, such as hyperthermia and acidification of

culture medium, can also induce MGF levels in cultured murine myoblasts and myotubes (15), supporting the role of MGF as cellular stress response factor.

Another process occurring after myocardial infarction is blockade of the blood-supply and therefore a shortage of nutrient supply to the infarcted area. To investigate if nutrient deprivation could be a trigger to induce MGF mRNA expression, HL-1 cells were serum-starved. As figure 5A and 5B show, MGF and IGF-1 mRNA expression were not induced by serum-starvation relative to control cardiomyocytes. These results indicate that the shortage of nutrients supplies is not a trigger to induce MGF or IGF-1Ea mRNA expression.

In conclusion, this study shows first that MGF expression initiates during early embryonic cardiac development, second that MGF decreases during postnatal development, and third that it re-emerges after acute ischemic injury in the adult mouse heart. Finally we demonstrate that MGF is induced by hypoxia and not by the shortage of nutrient supply. MGF might represent a new marker of myocardial injury and further investigation on the role of MGF during cardiac remodelling using transgenic approaches *in vivo* may provide mechanistic insights and reveal therapeutic possibilities in cardiac injury.

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Acknowledgements

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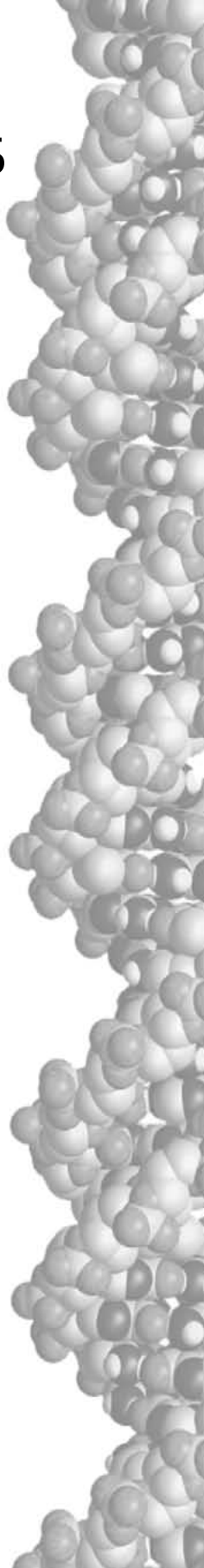
Chapter 5

General Discussion



Short summary of results

In the present thesis, regulation of the Mechano Growth Factor (MGF) in skeletal and cardiac muscle was investigated. The first experiments were performed with isolated mouse skeletal muscle, subjected *in vitro* to different types of contraction. It was shown that mouse muscles stretched during exercise, produce a specific substance which is referred to in literature as MGF (7, 23, 32, 33) (Chapter two). MGF was hardly detectable in the resting muscle. MGF could be the factor that activates muscle stem cells already present in the tissue. Once activated, these progenitor cells begin to divide (Chapter two), eventually creating additional muscle fibers and thereby increasing the size and strength of the muscle (8, 12, 24, 26, 29, 35, 45, 47).

The study continued with a series of *in vivo* experiments using thyroid hormone (T3) as a stimulus to increase the beating frequency of the heart and as an inducer of cardiac growth. The *in vivo* observations were compared with those in heart cells exposed to T3 in culture. This showed that MGF was not only mechano-sensitive in skeletal muscle, but also in cardiac muscle (Chapter three). In hyperthyroid hearts, where heart frequency was increased, MGF was up-regulated, while MGF was down-regulated in hypothyroid hearts, where heart frequency was decreased. In contrast to skeletal muscle, MGF was constitutively expressed in the heart, perhaps as a result of its continuous activity.

In agreement with literature showing that cardiac MGF expression decreases with age (2, 16, 17, 20, 21, 23, 30, 38), Chapter four also showed that MGF is expressed during embryonic development. This was a relevant finding as a pathological injured heart recapitulates the fetal gene program (25). Interestingly, Chapter four showed that MGF expression *in vivo* was up-regulated after myocardial infarction. As HL-1 cardiomyocytes expressed MGF under hypoxic conditions, it was concluded that the lack of oxygen by itself is a strong trigger for MGF induction (Chapter four).

This Chapter focuses on the possible beneficial effects of MGF on health. It addresses the issue whether MGF could be useful as a therapeutic approach in treating patients with muscle or heart diseases. Finally, the potential use of MGF to optimize physical training will be discussed as well as the misuse of MGF in the world of sports.

The Mechano Growth Factor for stronger muscles

Using MGF as a therapeutic approach in slowing down or even preventing muscle wasting is an obvious step to explore. Finding a cure for muscle wasting is an important step in Medical Sciences, because muscle wasting plays a role in many different human diseases. Some muscle pathologies are listed below:

- **Muscle atrophy** is wasting of the muscles, and may be due to disuse (immobilization), lack of exercise, loss of nerve supply, malnutrition, poor circulation or hormonal disturbances. Atrophy is a general physiological process of re-absorption and breakdown of the muscles, involving apoptosis and protein breakdown, resulting in reduced number of cells and/ or cell size.
- **Muscular dystrophies** refer to a group of hereditary muscle diseases that weaken the muscles that move the human body. Muscular dystrophies are characterized by progressive skeletal muscle weakness, defects in muscle proteins, and apoptosis of muscle cells. Examples of muscular dystrophies are Duchenne, Multiple Sclerosis (MS) and Amyloide Lateral Sclerosis (ALS) (25, 39, 42, 43).
- **Cachexia** is a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass (7). Cachexia is seen in patients with cancer, AIDS, COPD (chronic obstructive pulmonary disease) and CHF (congestive heart failure). Cachexia is distinct from starvation and is associated with increased protein breakdown of muscle (7). Once patients are diagnosed with cachexia, the chance of death from the underlying condition is increased dramatically (3, 4, 11, 18).
- **Sarcopenia** is the degenerative loss of skeletal muscle mass and strength associated with aging. Sarcopenia is characterized first by a decrease in the size of the muscle, which causes weakness and frailty. However, this loss of muscle mass may be caused by different cellular mechanisms than those which cause muscle atrophy. For example, during sarcopenia, there is a replacement of muscle fibers with fat and an increase of fibrosis (17, 20, 30, 31).

Muscle wasting diseases comprise a number of processes including: apoptosis of muscle cells, protein degradation and/or decreased protein synthesis and a less efficient repair process. These disorders affect many patients and more patients to come with an ageing population (13). Obviously, adjusted training could be an option for patients suffering from muscle atrophy due to immobilization (bed rest) to induce the endogenous release of MGF in skeletal muscle (1, 9-11). Similarly, MGF could ameliorate muscle loss resulting from long periods in zero-gravity conditions during space travel (37). However, for seriously ill patients suffering from dystrophies or cachexia, exercise may be impossible to perform.

Application of exogenous MGF either as a peptide or in a gene transfer approach could be a possible solution. The group of Goldspink *et al.* showed in a rodent study that a single intramuscular injection of a MGF-cDNA-containing plasmid resulted in a 25% increase in the mean muscle fiber cross section area of normal muscle

and a corresponding 30% increase in dystrophic mouse muscle within three weeks (12). Using a similar protocol, liver-derived IGF-1 took four months to produce a minor increase (24). In contrast Barton *et al.* also used the gene therapy approach and found that MGF indeed promoted skeletal muscle hypertrophy, but MGF was only effective in growing mice (2). IGF-1 was as effective as MGF to induce hypertrophy in this latter study.

Besides exogenous MGF, one could also target other components that influence MGF expression. One of the most marked effects of ageing is that the circulating Growth Hormone (GH) levels decline (29). Elderly patients are less able to induce sufficient stem cell activation after resistance exercise (22, 27). When GH administration was combined with resistance exercise, these patients showed a significant increase in proliferation and in parallel in MGF expression (22, 28). GH treatment combined with resistance exercise seems to be a possibility to counteract the effects of sarcopenia.

Another possible therapeutic approach for muscle wasting in dystrophies could be myogenic precursor cell transplantation. Recent work describing the transfer of the *dystrophin* gene using stem cells into dystrophic muscle indicates that dystrophin is involved in the mechano-transduction process and that the introduction of the *dystrophin* gene restores the ability to produce MGF (5, 13, 34). Unfortunately, these stem cells do not migrate well in the muscle and thus many injections have to be done to enable a good draft success. Mills *et al.* (23) demonstrated that the synthetic E-peptide of MGF has pro-migratory activity on human myogenic precursor cells and suggested therefore to combine cell transplantation with administering MGF. A more recent study of the same group showed that the MGF E-peptide improved human myogenic precursor cell transplantation (22).

One could administer MGF in different ways: one could use the total protein MGF or just the E-peptide. However, the use of a synthetic MGF as a therapeutic agent requires the peptide to be stabilized (15, 21). Delivery using mini-osmotic pumps has also proven to be effective in mice (32). The use of the viral delivery as a therapeutic approach could be risky, because engineered viruses have been found to evoke an immune response (35). Problems are also encountered as the mRNA of MGF is designed to be expressed as a pulse lasting a day or so (14, 15, 21, 38) and its peptide is also broken down within this same time scale (19). This implicates that MGF has to be administered repetitively.

MGF, the wonder drug for repair of ischemic injury?

Tissues that are sensitive to ischemic injuries include the heart and the brain. As found in Chapters three and four, the growth of the heart is associated with increased MGF expression, both in physiological as well in pathological cardiac hypertrophy. There-

fore, MGF could be a good candidate to make the diseased heart stronger by acting as a repair factor, for example after ischemic injury. Chapter three showed a clear correlation between heart beat and MGF expression. However, in many heart diseases it is not favorable to increase the heart beat to stimulate endogenous MGF production. Also intensive exercise cannot be considered, again because of the poor health condition and because it would preferably stimulate local muscular production of MGF. It has not yet been proven that exercise specifically increases cardiac MGF expression. Chapter four shows that MGF is also increased after heart infarction. Carpenter *et al.* studied MGF as therapeutic agent in sheep suffering from myocardial infarction. When those sheep were pre-treated with MGF (total protein and the E-peptide), loss of heart function was less than in sheep without pre-treatment (5). MGF was more potent in protecting cardiac function than IGF-1 (5). However the precise working mechanism and the MGF receptor still remain unknown.

Interestingly, recent studies on tissue damage and repair after a brain stroke used administration of MGF. Neurologists showed that when applied after a stroke MGF protects the vulnerable neurons and that the area of injury is smaller (1, 4, 23). Another important finding of the brain studies was that the small synthetic MGF peptide with protected C-terminus was able to cross the blood-brain-barrier (6). The studies with cells in Chapter four showed that MGF is directly induced by oxygen deprivation, but the precise mechanism remains again unknown. The important implication of the neurological studies is that MGF was effective after the stroke as pre-treatment of patients to prevent a myocardial infarction or a brain stroke is not a realistic option.

MGF: Bigger, better, faster

From the findings that MGF stimulates muscle growth, it is not difficult to consider its role as doping in sports.

MGF as well as IGF-1 and GH are on the list of prohibited substances (valid from January 2010) of the World Anti-Doping Agency (WADA). As evident from internet, MGF is already available on the black market and being used as doping amongst athletes. Many so called amateur bodybuilders/weightlifters are very happy with the results they obtained by injecting MGF peptides straight into their muscles. There is no regulation or safe use for MGF and athletes are experimenting and putting their recipes on internet. Local application by means of injections directly into the targeted muscles, theoretically diminish side effects on other tissues. The athlete will pass the doping test because the injected MGF cannot be distinguished from the compound synthesized in the body. Another negative point of injecting MGF is its local action at the site of injection which means that one has to inject every muscle separately and at different sites

to obtain the maximal result. At present new means of using muscle stimulants that cannot be detected with regular doping control are invented (6, 21).

This new form of doping generally referred to as gene doping or gene transfer raises concern. Actually, Elisabeth Barton and Geoffry Goldspink injected the MGF gene within a virus into mice and found that muscle mass increased up to 30% within 3-12 weeks (3, 12) as mentioned before. For MGF it is not a question if it is possible but rather when it is possible to apply gene transfer in human beings.

In Summary

MGF could be a very favorable candidate in treating muscle diseases associated with muscle wasting. Not only may MGF help skeletal muscle to improve, MGF could also be used in heart patients to reduce loss of cardiac function after trauma or in patients suffering from brain stroke. However, the accepted idea that MGF strengthens muscle via activation and proliferation of stem cells raises concerns about side effects like potential tumor growth. Also the way of stable and safe administration needs more attention as well as the normal availability of MGF during life.

Challenges

To better understand the mechanism of MGF and its regulation *in vivo*, more research needs to be done. There are three main challenges for MGF:

1. Availability of a specific commercially MGF-antibody to detect MGF protein *in vivo*

   Already a lot of research on MGF has been done and results were at first based on mRNA expression of MGF. Only recently three different research groups synthesized an antibody against the synthetic E-peptide of MGF. The first group raised two different polyclonal antibodies against the human and rat C-terminal MGF peptides using standard immunization protocols and affinity-purified (6). These antibodies target the specific C-terminal domain only present in MGF and therefore no cross-reaction with IGF-1 will occur. Dluzniewska *et al.* showed that these antibodies worked in samples of brains of Mongolian gerbils after ischemic injury. However, the western blots always presented two positive bands of ~30 and 25 kD (6). The identity of the larger-size band is not clear. As a positive control, synthetic MGF E-peptide was used as represented as a band with size 10 kD. Endogenous MGF protein expression was also shown with the same polyclonal antibody in ischemia-induced gerbil hippocampi (6). Because of the two bands presented in the blot, the specificity of this MGF-antibody should be questioned. The second research group raised a monoclonal antibody also against the human synthetic

MGF in mice (17). The latter group showed in various studies that this antibody of MGF is to identify endogenous MGF by ELISA (18, 19). The third group showed with immunostaining analysis that their polyclonal anti-MGF antibody was able to detect MGF in human muscle and in rat cardiomyocytes and vascular smooth muscle cells (16, 26-28). Endogenous MGF protein had a size of ~17.8 kD and IGF-1Ea of ~21 kD (33). The latter research group has shown promising results on the specificity of the MGF antibody. Unfortunately, no MGF-antibody is commercially available yet. Detection of the MGF protein *in vivo* would be the best proof of the biological relevancy of MGF, as also proposed in the recent review by Matheny et al. (20).

2. Identification of a MGF receptor

Data in literature are equivocal in respect to the receptor that mediates the action of MGF. A number of studies claim that MGF actions are mediated by an IGF-1 receptor-independent mechanism (6, 33, 39). In these studies the IGF-1R (IGF-1 receptor) was blocked and MGF could still proceed in activating satellite cells and inhibiting differentiation. However, Barton clearly showed that both MGF and IGF-1 can activate the IGF-1R in skeletal muscle (2), because both isoforms activated signaling pathways down-stream of the IGF-1R. Of course this finding does not exclude the possibility of a novel unidentified MGF receptor. Barton proposed that the change of identifying a separate MGF receptor must be bigger in young growing animals (2). The study of Gentile *et al.* showed that MGF as well as IGF-1 are capable to up-regulate β-Catenin protein and induce similar transcriptional activity in C2C12 cells, suggesting both isoforms share the same receptor in activating this specific signaling pathway (8).

Obviously more research needs to be done. A good strategy could be the use of transgenic animal models (explained below in the next paragraph).

3. Regulation of MGF

The best approach to investigate the biological function of an unknown compound is to create a transgenic animal model i.e. an over expression or knock-out animal model. The advantage over a cell system is that one could investigate the involvement of MGF during embryonic development. It could be possible that over expression or depletion of MGF could cause lethality amongst the animal pups before or shortly after delivery. One could avoid the lethality by using a conditional transgenic system. Various conditional systems have been proven to be functional (30, 31, 36). Our idea was to create a transgenic

mouse where MGF was over expressed exclusively in cardiac cells. This could be achieved by inserting the MGF cDNA sequence in a vector preceded by an alpha-Myosin Heavy Chain promoter (34). The advantage of this promoter usage is that it will be expressed after 6-8 weeks of age in mice, so the risk of lethality of the pups is limited. We already started this project and it would be very interesting to see the results in the future.

In Conclusion

In conclusion, the results presented in this thesis contribute to the characterization of the Mechano Growth Factor. Our findings show that besides mechanical loading in skeletal muscle, MGF expression is also triggered by increased heart frequency. Another interesting finding is that MGF plays a role during embryonic development and after cardiac infarction. In our daily life, this means that MGF can be induced by exercising and especially training like resistance exercise and weightlifting. So next time you put your heavy bag on your bike and cycle to work, MGF could be induced and therefore your muscles will get stronger. It is more effective if you start at younger age. Once you get older, your muscles will suffer more from stiffness and frailty. Perhaps at that time research is so far advanced that one could think of supplementation with MGF to prevent muscle wasting and simultaneously reduce the risk of a heart attack or a brain stroke.

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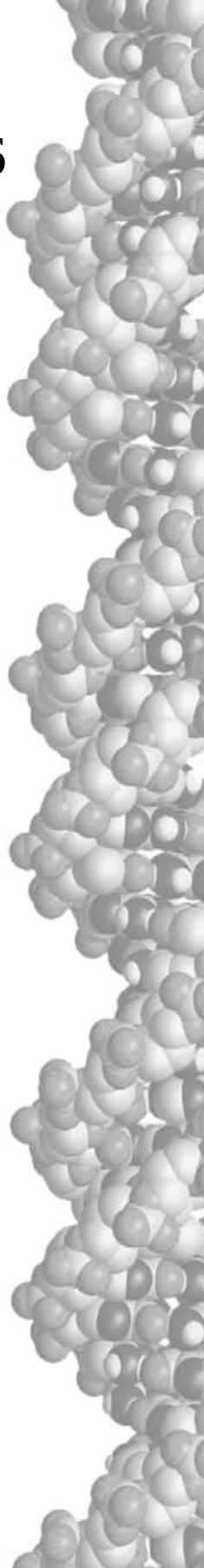
Chapter 6

Nederlandse Samenvatting



Nederlandse samenvatting

Spieren in het lichaam bestaan uit vezels. Na de geboorte delen deze vezels niet meer, ze groeien alleen. Het is opvallend dat na intensieve training alleen de getrainde spieren groter worden, en niet alle spieren in het lichaam. Dit betekent dat deze trainingsgeïnduceerde groei gereguleerd wordt door een lokaal mechanisme. We weten inmiddels dat er naast de spiervezels zogenaamde spier stamcellen liggen. Deze stamcellen worden satellietcellen genoemd. Wanneer je traint, worden deze satellietcellen tot celdeling aangezet en fuseren met de bestaande spiervezels om zo de spier te laten groeien. Dit proces heet hypertrofie. In 1996 ontdekte Prof. dr. Goldspink een eiwit dat alleen in de getrainde spieren aanwezig was. Het bleek te gaan om een isoform van “insulin-like growth factor” (IGF), en omdat deze nieuwe factor alleen tot expressie kwam in de getrainde spier werd deze de Mechano Groei Factor (MGF) genoemd. In het onderzoek beschreven in dit proefschrift staat MGF centraal. Het was namelijk nog onbekend of het soort samentrekking (overstrekt of met gelijk-blijvende lengte) van de spieren MGF kan induceren en activeren. Ook zou MGF een rol kunnen spelen in het activeren van satellietcellen. Een aantal van deze eigenschappen hebben we bestudeerd. Daarnaast hebben we onderzocht of en waardoor MGF tot expressie komt in het hart. Als diermodel werd in het onderzoek de muis gebruikt; daarnaast werden cellen gebruikt uit het hart van de muis of de rat.

Hoofdstuk één beschrijft het onderzoek naar MGF expressie in spieren van de muis. De EDL spier (spier die de tenen strekt en buigt) werd geprepareerd en werd in een opstelling gehangen tussen twee elektrodes. Door middel van elektrische impulsen kan de spier samentrekken. De studie had als doel om de expressie van MGF te bepalen na contracties waarin de spier werd overstrekt (verlengend) in vergelijking met contracties waarin de spierlengte gelijk bleef (isometrisch). De resultaten in dit hoofdstuk laten zien dat MGF tot expressie komt wanneer een spier iets uitgerekt wordt (10%) en vervolgens samentrekt, en niet na contracties waarbij de lengte gelijk blijft. Het vernieuwende aan deze resultaten was dat de spier *ex vivo* (buiten het lichaam) werd onderzocht en er geen andere factoren vanuit het hele lichaam bij de MGF inductie betrokken waren. We toonden dus aan dat MGF alleen door de contractie zelf wordt geïnduceerd. Geen van de twee soorten contracties veroorzaakte schade in de spier, waardoor ook uitgesloten werd dat factoren die vrijkomen tijdens beschadiging een rol zouden spelen bij de inductie van MGF. Omdat in de literatuur vaak trainingsprotocollen bestaande uit een aantal sessies gedurende meerdere dagen worden beschreven, is het opmerkelijk dat slechts 15 minuten contractiele activiteit voldoende is om MGF tot expressie te brengen. De satellietcellen lijken geactiveerd te worden op het moment dat

MGF wordt geïnduceerd. Samen met de resultaten van andere studies suggereert dit dat MGF een rol zou kunnen spelen bij de activatie van spier stamcellen.

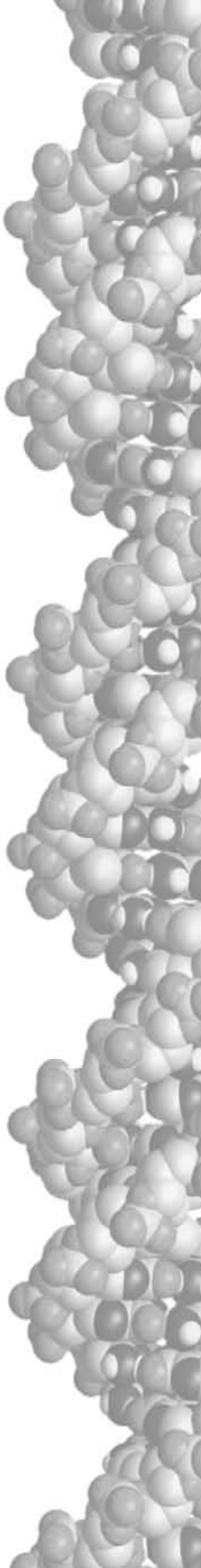
Hoofdstuk twee beschrijft resultaten van de studie van MGF in het hart van de muis en in hartcellen van de rat. Eén groep muizen kreeg per injectie schildklierhormoon toegediend, waardoor het hart sneller ging kloppen en groter werd. Een andere groep muizen werd op een speciaal dieet gezet om het schildklierhormoonniveau in het bloed te verlagen en dus ook de hartslag. De studie beschreven in hoofdstuk twee laat zien dat muizen met een versnelde hartslag een hogere MGF expressie hadden dan de controle muizen, terwijl muizen met een verlaagde hartslag een daling van MGF expressie lieten zien. De experimenten werden herhaald in hartcellen geïsoleerd uit de rat, waarin de contractiefrequentie verhoogd kan worden door schildklierhormoon aan het kweekmedium toe te voegen, en waarin het "kloppen" van de hartcellen stilgelegd kan worden door toevoeging van kaliumchloride. De MGF expressie in de niet-kloppende hartcellen was minder dan in de controle cellen of in de door schildklierhormoon gestimuleerde cellen. Het feit dat MGF aanwezig was in het hart en dat de hoeveelheid MGF gerelateerd was aan de hoogte van de hartslag was een geheel nieuw beschreven eigenschap van MGF.

De studie in hoofdstuk drie probeert de biologische rol van MGF in het hart te achterhalen. Als eerste laat hoofdstuk drie zien dat de expressie van MGF in het hart hoger is in jonge dan in oude muizen. Dit zou kunnen duiden op een rol voor MGF in de ontwikkeling van het hart. Door middel van een celkweekmodel werd de expressie van MGF onderzocht in de vroege fase van de embryonale ontwikkeling. We zagen dat tijdens de vorming van de eerste kiemlagen van een embryo, MGF expressie geen rol speelt. Wanneer de cellen gedwongen in de fase komen dat zij richting hartcellen differentiëren, komt MGF tot expressie. MGF speelt dus een rol in de ontwikkeling van het hart wanneer de bestemming van de cellen om hartcellen te worden eenmaal vastligt. Wanneer een hart beschadigd is na bijvoorbeeld een infarct, komen in het hart genen tot expressie die ook belangrijk zijn tijdens de embryonale ontwikkeling. Onze hypothese was dat MGF een rol speelt in het hart na beschadiging. Hiervoor zijn muizenharten na een infarct getest op hun MGF expressie. We zagen dat 12 uur na een infarct MGF tot expressie werd gebracht. Vervolgens werd de vraag gesteld: waardoor komt MGF dan tot expressie? Na een infarct gebeuren er meerdere dingen tegelijk: 1) het hart heeft een tekort aan zuurstof in het gebied van het infarct (hypoxie); 2) er is ook een tekort aan voedingsstoffen in die omgeving; en 3) het hart gaat hypertrofiëren (wordt groter) om het beschadigde weefsel te vervangen. Kortom, de vraag waarom MGF tot expressie wordt gebracht is niet eenvoudig te beantwoorden. Hartcellen die

zijn blootgesteld aan hypoxie door toevoeging van DFO (desferrioxamine) brengen na 24 uur MGF tot expressie, maar bij een tekort aan voedingsstoffen blijft de MGF expressie gelijk. Door de resultaten van de experimenten in hoofdstuk drie te combineren, zou je kunnen concluderen dat: a) MGF een rol speelt tijdens de ontwikkeling van het hart en dat b) na een infarct MGF door hypoxie wordt geïnduceerd (en dat MGF wel eens een factor zou kunnen zijn die een rol speelt in het herstellen van het hart.

Concluderend laten de resultaten beschreven in dit proefschrift zien, dat MGF tot expressie komt in skeletspieren na “lengthening” contracties, maar niet in een rustende spier, terwijl MGF in het hart eigenlijk altijd tot expressie komt, misschien omdat het hart altijd klopt. Het verhogen van de hartslag door schildklierhormoon leidt tot een hogere MGF expressie, en een lagere hartslag leidt tot minder MGF. De vraag is of dit ook gekoppeld is aan een groter danwel kleiner hart. Daarnaast laat dit proefschrift zien dat MGF in het hart, behalve tijdens ontwikkeling, ook een belangrijke rol speelt tijdens het proces van beschadiging en herstel. Dit alles maakt MGF misschien tot een geschikte kandidaat voor therapeutische of preventieve medicatie van patiënten met een spierziekte zoals multiple sclerose (MS) of amyotrofische laterale sclerose (ALS) of voor patiënten na een hartinfarct. Voor het zover is, zal MGF eerst nog meer en beter onderzocht dienen te worden.

Appendix



Dankwoord

Dankwoord, een woord van dank. Veel woorden zijn mij door andere promovendi al uit de mond genomen. Maar een goed woord vindt altijd een goede plaats. Daar komt bij dat een goede verstaander aan een half woord voldoende heeft. Helaas moet je altijd op je woorden passen. Het gesprokene woord vervliegt en het geschrevene blijft. Wanneer je naam dan misschien niet tussen de volgende woorden staat, weet dan wel dat ik dit niet bewust heb gedaan. Daarom wil ik ook in het vooruit iedereen bedanken die maar een zinnig woord heeft bijgedragen aan dit proefschrift. Het is niet in woorden uit te drukken, hoe dankbaar ik ben, maar het laatste woord is zeker nog niet gezegd:

Allereerst wil ik natuurlijk mijn promotor Marjanne Everts bedanken. Het was geen gemakkelijk project, wat begon als een mooi geschreven voorstel door mijn toenmalige co-promotor Paul van den Wijngaard, eindigde in een grote zoektocht naar het “lastige goedje” MGF. Het eerste jaar startten we dan ook voorspoedig en het eerste manuscript lag al snel klaar. Maar helaas kon dit beginnergeluk niet eeuwig blijven duren en door vele verschillende omstandigheden werd mijn pad tot promoveren toch heel anders dan ik in het begin had gedacht. Na Paul zijn vertrek bleven Marjanne en ik samen over met vele onbekenden en variabelen. Alain de Bruin werd toen bij mijn project betrokken als een adviserend klankbord met geweldige enthousiaste en vernieuwende ideeën. Helaas bleken sommige ideeën niet meer haalbaar binnen de tijd, maar de hele aanloop heeft me zoveel geleerd. Ik ben daarom ook heel blij dat je uiteindelijk toch promotor van mijn proefschrift wilt zijn.

Toen Ingrid Vos een Meervoud project binnen sleepte en zeer interessant onderzoek deed op het gebied van hart, hadden we het idee om MGF daar ook eens in te gaan testen en niet zonder resultaat. Ingrid, ik wil je op deze manier enorm bedanken voor de rol die jij hebt gespeeld in mijn promotietraject. Ik durf zelfs te zeggen dat wanneer jij er niet was geweest, ik het al lang een keer bekeken zou hebben. Jouw enthousiasme en ons teamwork waren altijd een enorme stimulans en inspiratie om er toch weer voor te gaan. Dit heeft er ook voor gezorgd dat we samen een geweldig stuk hebben geschreven.

Dit werk zou ik niet gedaan kunnen hebben zonder de hulp van een superteam. Peter, jij was er altijd om mij af te remmen en nog eens te laten nadenken over de vervolgstappen en ondertussen werd er druk gediscussieerd over welke films we het weekend hadden gezien. Brian, wat was ik blij dat ik nog van jouw expertise gebruik heb kunnen maken voordat je een nieuwe uitdaging aanging. Hoe precies en netjes jij je protocollen bijgehouden hebt, daar heb ik veel aangehad. Ook dat ik zo weer via via bij Medische

Fysiologie uitkwam was erg verrassend. Cellen werden over en weer aangeboden en dat was zeer motiverend. Hiervoor wil ik graag het oude team van Medische Fysiologie bedanken: Harold van Rijen, Toon van Veen en Marti Bierhuizen, maar ook de nieuwere aanwinst Leon de Windt en Kanita Salic (inmiddels alweer verhuisd naar Maastricht). En Arend, zonder jouw "klassieke" instelling en proefdierkennis was ik altijd een beetje zenuwachtig, maar gelukkig was je altijd in de buurt tijdens de eliminatie! Ook Walbert wil ik bedanken, door jouw input is er een heel interessante eigenschap van MGF naar boven gekomen, waar ik in het begin van mijn project nooit aan gedacht zou hebben. Ook de samenwerkingen die hieruit voortvloeiden waren super. Joost en Marie-Jose wil ik daarom ook graag bedanken voor hun bijdrage hieraan. Ik heb wel geleerd dat wanneer je om hulp vraagt, je eigenlijk helemaal niet alleen staat; je moet het alleen wel vragen! Tevens wil ik mijn stagiaire Lizet Opmeer bedanken voor de extra handjes met al het RNA werk. Ronald Kistjes bedankt voor de mooie kleuringen die je via Ingrid voor mij hebt gedaan. En waar zouden we zijn zonder de ondersteuning, gezelligheid en het crypto-brein van Ineke.

Siona, je was er nog net toen ik begon als AiO en het klikte meteen. Na het eerste avondje stappen met veel MAPKinases en ERKs, volgden er nog meer. Lekker dansen, wat hebben we een lol gehad. Ook het buikdansen vond ik super om te doen (Habibi!), zolang we maar tatjes vooraf aten! Al die tijd kon ik mijn verhaal kwijt en ik ben enorm blij dat je mijn paranimf bent. We gaan er een feestje van maken!

Ook zonder leuke kamergenoten kan een probleem heel groot worden of een dag heel lang. En ik heb er toch wel een aantal versleten. Om te beginnen bij Claudia: Al gauw leerde ik van jou hoe het er aan toe ging tussen anatomie en fysiologie. Dat was altijd interessant! Daarna werd er flink geschoven en kwamen de AiOs in één hok. Nancy heeft ons toen als eerste verlaten, en dat was jammer. Nancy werkte aan haar laatste loodjes van haar proefschrift en was veel aan het schrijven. Daarom zat er altijd iemand op de kamer bij wie je even je hart kon luchten als je planning in de war liep. Ook liet Nancy zien dat kinderen en een proefschrift goed te combineren zijn, zolang iedereen maar meewerkt! De volgende die vertrok was Maarten. Weer één minder. Jammer, want je had nog wel eens goede raad voor mijn moleculaire uitdagingen en ook je eeuwige positieve instelling kon me blijven verbazen. Vervolgens ging Maartje, maar ik vond het geweldig dat je nog even bleef hangen om alles af te ronden! Natuurlijk hadden we ook nog collega's die even kwamen buurten. RRRichard jij ook bedankt voor je warme kop thee. Ja, het was echt een leuke tijd. Ik wens jullie allemaal heel veel succes in de toekomst en met een gezellig dinertje op zijn tijd, blijft iedereen van elkaars doen en laten op de hoogte.

Graag zou ik nog een woord van dank willen uitspreken naar mijn nieuwe collega's van Danone Research en met name de afdeling Disease Targeted Nutrition. Bedankt voor de tijd en ruimte die me is gegeven om mijn proefschrift te kunnen voltooien, in het bijzonder mijn directe teamleden Ardy van Helvoort, Klaske van Norren, Joanna Luttikhold en Francina Dijk.

Genoeg over het echte werk, op naar de sociale kant van het promoveren.

Zonder de meiden uit Tiel zou ik niet zijn wie ik nu ben. Ik ben zo blij dat ik deel uit mag maken van jullie leven. Vanaf de middelbare school, eerste vriendjes, studies, verdriet, trouwen en nu kinderen. Wat hebben we toch al veel meegemaakt en ik hoop dat er nog veel leuke dingen gaan komen. Iedereen heeft zo zijn eigen sterke karakter en ik zou niemand van jullie willen missen. Pluis, samen zijn we nu moeder en ik ben zo blij dat we die gevoelens kunnen delen. Fijn dat we altijd overal over kunnen praten van Reiki tot paaldansen. Je bent toch wel een echte beschermengel. Romi, heerlijk zoals jij overal lekker overheen kunt walsen en voet bij stuk houdt. Je eerlijkheid heb ik altijd bewonderd. En Baranno, als ik even heerlijk wil babbelen en ik mijn file tijd wil verkorten ben je er altijd. Als ik ook maar een stukje van jullie eigenschappen heb overgenomen, dan maakt me dat een veel beter mens. Laten we jullie mannen niet vergeten, aan gekke gezelligheid heeft het nooit ontbroken!

Nog meer meiden: dan begin je aan een studie en ben je ineens weer de "kleinste uit de klas". Gelukkig heb ik jullie leren kennen: Anne, Linnea, Elly, Kim, Ingrid en Ynske. Als ik terugdenk aan hoe we toen waren en wat we nu zijn en misschien nog gaan worden, had ik nooit gedacht dat we zo een goed team zouden vormen. Na de ontgroening bij de Mac en de snackbar, kwam er alleen maar meer gezelligheid! Ik hoop dat we nog heel wat leuke avonden en weekenden in het vooruitzicht hebben. Ynske, in het tweede jaar stroomde je vanuit biologie bij ons in en toevallig kwam je ook uit Tiel. Sindsdien hebben we samen gereisd, geleerd en onze grote wisseltruc wordt helaas niet meer herhaald. Ik ben heel blij dat je mijn paranimf wilt zijn!

Save the best for lest:

Mijn lieve schoonfamilie, Gerard, Trees, Sylvia, tante Ria en ome Karel. Wat een warm onthaal heb ik van jullie gekregen. Zonder jullie steun en opbeurende bloemetjes en lieve smsjes had ik het denk ik nooit gehaald. Jullie hebben zeker een plaatsje in mijn hart verdiend!

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Pa en ma, wat ben ik blij dat jullie altijd hebben gezegd dat school voor gaat! Niet altijd heb ik het kunnen waarderen, maar kijk eens waar ik terecht ben gekomen! Zoals Erik al zei: Alles komt goed! Ik weet zeker dat jullie een fantastische opa en oma zullen zijn en van Faedra zullen genieten.

Dan natuurlijk mijn allerliefste Gert-Jan. Ik heb je leren kennen op Terschelling en het eiland heeft ons veel gegeven en toch ook ontnomen, maar ik had nergens anders willen zijn. Ik hoop dat we ons huis blijven vullen met gezelligheid en dat we samen heel oud mogen worden. Een broodje kaas is er altijd! Faedra, onze kleine lachebek, je bent de zonneschijn in mijn leven en je hoeft maar te lachen en mijn dag kan niet meer stuk.

Miriam

Curriculum Vitae

Miriam van Dijk-Ottens werd geboren op 4 maart 1980 te Tiel. Na het behalen van het VWO diploma in 1999 aan het Rijk Scholen Gemeenschap te Tiel, werd in hetzelfde jaar begonnen met de studie Medische Biologie aan de Universiteit Utrecht. Als onderdeel van deze studie werd een onderzoeksstage met als titel "*Gap junctions and lysosomal and proteasomal degradation*" voltooid bij Medische Fysiologie toen nog in het Universitair Medisch Centrum Utrecht, onder begeleiding van Dr. Harold van Rijen, Dr. Toon van Veen en Prof.dr. Habo Jongsma. Hierna heeft zij een master-programma in Biomolecuar Sciences voltooid, met een tweede onderzoeksstage bij Scheringh-Plough B.V. met als titel "*Characterization of a monoclonal antibody Mab12A*" bij de afdeling Immunologie, onder begeleiding van Dr. Marie-Jose van Lierop. In 2004 werd het master diploma Biomedical Sciences, Biomolecular Sciences behaald en ondertussen was zij werkzaam van 2004 tot 2009 als Assistent in Opleiding aan de Universiteit van Utrecht, Faculteit Diergeneeskunde, hoofdafdeling Pathobiologie, divisie Anatomie en Fysiologie, onder begeleiding van Prof. dr. Marjanne Everts en Prof. dr. Alain de Bruin. In 2009 werd dit proefschrift geschreven en onderwijl heeft zij gewerkt als stage begeleider van HLO-studenten aan de Hoge School van Utrecht. Momenteel is Miriam van Dijk-Ottens werkzaam als onderzoeker bij Danone Research bij de afdeling Metabolisme van Disease Targeted Nutrition.

Abbreviations

bpm	beats per minute
BSA	Bovine serum albumin
Ca^{2+}-ATPase	Calcium adenosinetriphosphatase
$CaCl_2$	Calcium chloride
DAPI	4',6-diamidino-2-phenylindole
DEC	Dier ethische commisie
DFO	Desferrioxamine
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulfoxide
FCS	Fetal calf's serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1α	Hypoxia induced factor-1 α
HRP	Horse radish peroxidase
IGF-1	Insulin-like growth factor
EC	Embryonic carcinoma
ECG	Electro cardiogram
EDL	Extensor digitorum longus
ISO	Isometric contractions
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrophosphate
l_0	optimal length
LC	Lengthening contractions
MI	Myocardial infarction
MGF	Mechano growth factor
$MgSO_4$	Magnesium sulfate
MRF4	Muscle regulatory factor-4
MyHC	Myosin heavy chain
MyoD	Myogenic diffentiation factor-1
NaCl	Natrium chloride
$NaHCO_3$	Natrium bicarbonate
NaH_2PO_4	Natrium dihydrophosphate
NaOH	Natriumhydroxide
NEAA	Non-essential amino acids
NFAT	Nuclear Factor of Activated T Cell

NO	Nitric oxide
NRCM	Neonatal rat cardiomyocytes
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR (rt)	Polymerase chain reaction (real time)
PI3K	Phosphoinositol-3-kinase
PTU	5-Propyl-2-thiouracil
rpm	rounds per minute
SAC	Stretch Activated Channels
SERCA	Sarco/ endoplasmic Reticulum Ca 2+-ATPase
TCA	Trichloroacetic acid
TA	Tibialis anterior
T3	Thyroid hormone
T4	Thyroxine
VEGF	Vascular endothelial growth factor

There is only one wish on my mind,
When this day is through I hope that I will find,
That tomorrow will be
just the same for you and me,
All I need will be mine if you are here.

I'm on the top of the world looking
down on creation and the only explanation
I can find
Is the love that I've found, ever since you've been around
Your love's put me at the top of the world

Carpenters, On top of the world