Thesis for the Master's degree in Molecular Biosciences

A role of MRF4 and Myf-5 in regulation of adult muscle fiber type?

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Abstract

The myogenic regulatory factors (MRFs) are a group of muscle specific transcription factors important during determination and differentiation of muscle fibers. In adult muscle two of the members, MyoD and myogenin, are expressed preferentially in fast and slow muscles, respectively, and have been suggested to play a role in fiber type specific gene regulation. Overexpression of MyoD in the slow soleus muscle has been shown to cause a shift towards a faster phenotype, while overexpression of myogenin in the fast extensor digitorum longus (EDL) causes an increase in oxidative capacity. Less is known about the two last members of this family, MRF4 and Myf-5. In adult muscle MRF4 is expressed at very high levels and Myf-5 at very low levels, but their function is unknown.

The expression of MRF4 has only been investigated at the transcript level, so we wanted to map the normal expression of MRF4 protein. In order to investigate whether MRF4 and Myf-5 play a role in regulation of fiber type specific genes, we wanted to knock down the expression of MRF4 and to overexpress Myf-5 in muscles of adult rats.

Mapping of MRF4 protein expression showed that there was a significantly higher expression level in the slow soleus compared to the faster muscles. For the knockdown study, six siRNA was tested in cell culture, but since none of them gave a measurable knockdown, further studies *in vivo* were abolished. Overexpression of Myf-5 in the fast EDL resulted in an increase of 2b fibers, indicating that Myf-5 activates expression of MyHC 2b.

Abbreviations

bHLH	Basic helix-loop-helix
BSA	Bovine serum albumin
cDNA	Complementary DNA
cAMP	Cyclic adenosine monophosphate
CMV	Cytomegalovirus
DMEM	Dulbecco's Modified Eagle Medium
E	Embryonic day
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
Gastroc.	Gastrocnemius
GL	Gastrocnemius Lateralis
GM	Gastrocnemius Medialis
HEK-cells	Human embryonic kidney cells
MEF	Myocyte enhancer factor
MRF	Myogenic regulatory factor
MRF4	Myogenic regulatory factor 4
Myf-5	Myogenic factor 5
MyHC	Myosin heavy chain
PBS	Phosphate buffered saline
RSV	Rous sarcoma virus
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SV40	Simian virus 40 / Simian vacuolating virus 40
TA	Tibialis anterior
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

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1. Introduction

Skeletal musculature is made up of long, cylindrical cells with multiple nuclei, called muscle fibers or myofibers. The main internal structure of muscle fibers is myofibrils composed mainly of actin and myosin organized into sarcomeres. These sarcomeres are responsible for a process called the cross-bridge cycle. Excitation is mediated by motor neurons and leads to activation of the cross-bridge cycle, causing the muscle to contract. In absence of electrical stimuli, the muscle relaxes.

Skeletal muscle fibers are classified based on their speed of contraction and their resistance to fatigue with repeated stimulation. These properties are determined by the expression of proteins involved in the excitation-contraction-relaxation cycle.

1.1 Fiber types

In the 19th century muscles were categorized as ”red” and ”white” (Ranvier, 1874), the former with slow muscle contraction and the latter with fast muscle contraction. Later, muscle fibers were classified as type 1, type 2a and type 2b according to their ATPase activity and pH liability (Brooke and Kaiser, 1970). Myosin ATPase (mATPase) hydrolyzes ATP in the crossbridge cycle. The variation of mATPase activity was shown to correlate with variation in contraction speed and expression of myosin heavy chain (MyHC) isoforms in different fiber types (Reiser et al., 1985).

MyHC is a part of the contractile apparatus, and four different isoforms are expressed in adult rodent muscle. With help of immunohistochemistry using antibodies against the MyHC isoforms (Schiaffino et al., 1989), they are now classified as MyHC 1, MyHC 2a, MyHC 2x (also called MyHC 2d) and MyHC 2b. Muscle fibers expressing MyHC 1 have the slowest speed of contraction and are therefore called slow type 1 fibers. These have high oxidative capacity, primarily relying on oxidative phosphorylation for production of ATP. The MyHC 2a, 2x and 2b are expressed in type 2a, 2x and 2b fibers, respectively. In the listed order these have increasing speed of contraction, decreasing oxidative capacity, and increasing glycolytic capacity. Glycolytic fibers rely primarily on anaerobic glycolysis to produce ATP. The properties of the different fiber types are listed in table 1.1. The expression of the different isoforms of MyHC is now often used to identify the different fiber types (Spangenburg and Booth, 2003).

Table 1.1. An overview of fiber types in skeletal muscle of rodents defined by MyHC isoform and physiological properties.

Fiber type	MyHC	Speed of contraction	Metabolic profile	Endurance
1	MyHC 1	Slow	Oxidative	Good
2a	MyHC 2a	Fast	Oxidative-glycolytic	Good-medium
2x	MyHC 2x	Faster	Glycolytic-oxidative	Medium-poor
2b	MyHC 2b	Fastest	Glycolytic	Poor

The fiber type composition of a muscle determines the muscle's properties. The fast glycolytic extensor digitorum longus (EDL) muscle is composed primarily of type 2 fibers, while the slow oxidative soleus muscle is composed primarily of type 1 fibers. Due to their extreme phenotype they are often used to study transcription factors and what properties they determine. Normal distribution of the different fiber types in soleus and EDL, as well as three other hind limb muscles; plantaris, tibialis anterior (TA) and gastrocnemius (gastroc.), are listed in table 1.2. The gastroc. consists of a medial (GM) and a lateral part (GL). While most of gastroc. is mixed with respect to fiber type, there is also a deep red portion in GL and a superficial white portion in GM (Armstrong and Phelps, 1984) into which the muscle often is separated when studied.

Table 1.2. Fiber type frequency (%) in hind limb muscles of adult rats.

Fiber type	1	1/2a	2a	2a/2x	2x	2x/2b	2b	Reference
Soleus	87	1	12	0	0	0	0	(Ekmark et al., 2007)
	82	9	9	0	0	0	0	(Staron et al., 1999)
EDL	3	-	23	-	29	-	45	(Windisch et al., 1998)
	4	2	16	7	36	5	30	(Staron et al., 1999)
Plantaris	2	-	13	-	37	-	48	(Caiozzo et al., 1996)
	6	-	14	-	33	-	47	(Delp and Duan, 1996)
TA	3	-	9	-	16	-	72	(Caiozzo et al., 1996)
	2	0.5	12	5	27	4.5	49	(Staron et al., 1999)
Gastroc.	21-25	*	*	*	*	75-79	*	(Voytik et al., 1993)
-Red	51	-	35	-	13	-	1	(Delp and Duan, 1996)
-White	0	-	0	-	8	-	92	
-Mixed	3	-	6	-	34	-	57	

Note that different strains of rats were used, and that fiber types were classified by three different methods. Delp and Duan (1996) did classification based on the activity of mATPase and metabolic properties. Staron et al. (1999) did fiber typing according to the mATPase activity. Caiozzo et al. (1996) determined fiber type by doing immunoblotting with MyHC specific antibodies, while both Windisch et al.(1998) and Ekmark et al. (2007) did immunohistochemistry with MyHC specific antibodies. *Voytik et al. (1993) classified fibers as type 1 or 2 with immunohistochemistry with an antibody specific for MyHC 2. They could therefore not make distinctions between the different isoforms and hybrids of MyHC 2, only determine that 75-79 % of all fibers expressed MyHC 2. - = not measured.

1.2 Fiber type transitions

Muscle fibers have the capacity to alter their physical and functional properties, a feature often referred to as muscle plasticity. During such transition states, hybrid fibers expressing two or more MyHC isoforms are common (Pette and Staron, 1990). It should also be noted that 11-67 % of the

fibers from various limb muscles express more than one MyHC isoform even under steady state activity conditions (reviewed by Stephenson, 2001). The role of such hybrid fibers are not known, but the prevailing view is that they have intermediate properties so that the muscle can fine-tune its output to the physiological demands (Bottinelli et al., 1994, Staron et al., 1999, Pette and Staron, 2000).

Transitions in expression of MyHC isoforms occurs in a sequential and reversible order (Windisch et al., 1998, Pette and Staron, 2000):

$$1 \leftrightarrow 1/2a \leftrightarrow 2a \leftrightarrow 2a/2x \leftrightarrow 2x \leftrightarrow 2x/2b \leftrightarrow 2b$$

The transitions can be induced by changes in electrical stimulation, mechanical loading and unloading, hormones and aging. Cell lineage does however to some extent seem to limit the changes (Hoh and Hughes, 1991, Rosenblatt et al., 1996).

The nerve and electrical activity has an important influence on muscle contraction and fiber type composition (reviewed by Gundersen, 1998). Cross-innervating a slow motor neuron to a fast muscle and a fast motor neuron to a slow muscle change the muscles according to the nerve activity (Buller et al., 1960). The fast muscle gain properties typical of slow muscles and the slow muscle gain properties typical of fast muscles. The same results are obtained when soleus is denervated and subsequently stimulated with an electrical pattern normal for fast muscle (Lømo et al., 1974). When the fast EDL is stimulated with electrical patterns typical for a slow muscle, it become more like a slow muscle (Eken and Gundersen, 1988). Total loss of electrical stimulation, for example by denervation, makes slow muscles become faster and fast muscles becoming slower (Huey and Bodine, 1998, Windisch et al., 1998, Loughna and Morgan, 1999).

It has been discussed whether the influence of the nerve is caused by release of neurotrophic substances secreted from the nerve terminal. But since stimulation by electrodes give the same results as cross-innervating nerves, it seems likely that it is the electrical activity *per se* that influences the muscles (Gundersen, 1998).

Endurance training induces changes in the fast-to-slow direction, both in fiber type (Green et al., 1984) and oxidative metabolism (Baldwin et al., 1972). Stretch overload causes the same transition (Pattullo et al., 1992). Aging (Larsson and Ansved, 1995) and reduced levels of thyroid hormones (Ianuzzo et al., 1977) have also been shown to induce fast-to-slow conversion in muscle fiber type. Immobilization (Jänkälä et al., 1997), unloading and hyperthyroidism (Pette and Staron, 2000) on the other hand, causes slow-to-fast transitions.

Several molecules have been studied in order to understand how skeletal muscle fiber types are regulated. The myogenic regulatory factors are some of the implicated factors in the regulation of fiber type specific genes.

1.3 Myogenic regulatory factors

The four myogenic regulatory factors (MRFs) MyoD (Davis et al., 1987), myogenin (Edmondson and Olson, 1989, Wright et al., 1989) MRF4 (myogenic regulatory factor 4), also called Myf-6 and herculin (Rhodes and Konieczny, 1989, Braun et al., 1990a, Miner and Wold, 1990) and Myf-5 (myogenic factor 5; Braun et al., 1989) are members of the superfamily of basic helix-loop-helix (bHLH) transcription factors. They are specific for skeletal muscle and are expressed at distinct times during myogenesis. Each of the factors can, when expressed in non-muscle cells like fibroblasts, convert these cells to differentiated muscle fibers that express muscle specific genes (reviewed by Buckingham, 1992).

The bHLH domain consists of a helix-loop-helix (HLH) motif and a basic DNA binding region (Puri and Sartorelli, 2000). The bHLH motif is required for heterodimerization of the MRFs with E-proteins, another class of bHLH proteins. These heterodimers bind to DNA in a sequence specific manner at sites known as E-boxes with a consensus sequence of CANNTG. E-boxes are found in both promoter and enhancer areas of muscle specific genes (Olson, 1990). The myocyte enhancer factor (MEF)-2 family is a second group of transcription factors which are important in myogenesis. They belong to the MADS box family and bind to a consensus A/T-rich sequence found in the promoters of many muscle specific genes. Interactions mediated by the basic region of MRFs and the MADS domain of MEF-2 stimulate MRF-driven transcription (Black and Olson, 1998).

The basic region is required for binding to DNA, but additional sequences are required for transcriptional activation. Each of the four factors has slightly different activational domains. Myf-5 contains an activation domain in the carboxyl region (Braun et al., 1990b), while both MyoD (Weintraub et al., 1991) and MRF4 (Mak et al., 1992) have an activation domain located in the amino terminus. MyoD and MRF4 can therefore to some extent substitute for each other, though they also have some activational specificity (Mak et al., 1992). Myogenin on the other hand contains two transcription activation domains, one in the amino terminal and one in the carboxyl terminal of the bHLH region, which act in conjunction with the DNA binding domain to activate muscle-specific transcription (Schwarz et al., 1992). Common to all the MRF activation domains is an overall negative charge positioned on the hydrophobic face of the peptides (Mak et al., 1992). They all inhabit the

ability to positively autoregulate their own expression and to cross-activate each other (Braun et al., 1989, Thayer et al., 1989, Miner and Wold, 1990).

All the MRFs contain a conserved threonine (T) which is subject to phosphorylation. In MyoD it corresponds to T115, in myogenin T87, in MRF4 T99 and in Myf-5 T89, and phosphorylation of this threonine reduces their activity by inhibiting their ability to interact with DNA (Li et al., 1992b, Hardy et al., 1993, Liu et al., 1998). The serines (S) in MRFs can also be phosphorylated. The cAMP dependent protein kinase (PKA) and casein kinase II (CKII) can phosphorylate serines in MRFs, although these phosphorylations does not seem to influence their transcriptional activity (Li et al., 1992a, Johnson et al., 1996). The proto-onco gene Mos, on the other hand, can activate MyoD by phosphorylating its S237 (Pelpel et al., 2000). MRFs can also be modified by acetylation and ubiquitination (Puri and Sartorelli, 2000).

1.4 The roles of MRFs during myogenesis

Myogenesis is the differentiation of somite progenitor cells into skeletal muscle fibers. During myogenesis the four MRFs are expressed at distinct time points. Myf-5 is the first member of the family to be expressed in mouse and is detected at embryonic day 8 (E8) (Ott et al., 1991). Expression of myogenin and MyoD are observed at E8.5 and E10.0, respectively (Sassoon et al., 1989). MRF4 is expressed transiently between E9.0 and E11.5 and is then down-regulated until it is later expressed in differentiated muscle fibers (Bober et al., 1991, Hinterberger et al., 1991).

Several studies on inactivation of the myogenic regulatory factors have been conducted to investigate their role in myogenesis. Mice lacking a functional MyoD gene are viable and fertile, and exhibit no morphological or physiological abnormalities in skeletal muscle (Rudnicki et al., 1992). MRF4 null-mice are viable and have normal muscle phenotype, but show defects in rib development (Zhang et al., 1995). Myf-5 mutants have normal muscles, but die immediately after birth due to severe rib defects (Braun et al., 1992). Myogenin null-mice show severe reduction of skeletal muscle, abnormalities in spine and ribs, and die immediately after birth (Hasty et al., 1993). It should be kept in mind that there might be some compensating mechanisms in transgenic animals since the protein then is absent right from the beginning.

Double mutants of MyoD and Myf-5 have shown that these two are essential determination factors in muscle development (Rudnicki et al., 1993). However, a study by Kassar-Duchossoy et al. (2004) indicates that also MRF4 may have a role in determination. MRF4, MyoD and myogenin acts as differentiation factors during myogenesis (Buckingham et al., 2006).

1.5 MRFs and fiber type specificity

MyoD and myogenin are found to be differentially expressed in fast and slow muscles. It has therefore been proposed that MyoD and myogenin are possible links between electrical activity and phenotypic gene expression of fast and slow genes, respectively (Hughes et al., 1993, Voytik et al., 1993).

Cross-innervation of a fast nerve to soleus reduces myogenin expression and elevates MyoD expression in areas responding to the activity change by formation of fast fibers at the expense of slow fibers (Hughes et al., 1993). Ekmark et al. (2007) showed that both mRNA and protein levels of myogenin increases when the fast EDL is stimulated with a pattern mimicking the activity in slow motor units. The level of myogenin is also found to be elevated in response to endurance training (Siu et al., 2004).

Overexpression of myogenin in transgenic mice show a 2-3 fold elevation of oxidative mitochondrial enzymes and a reduced level of glycolytic enzymes in fast muscles. This reveals a myogenin-induced shift in enzyme activity from glycolytic to oxidative metabolism (Hughes et al., 1999). Overexpression of myogenin in pre-existing adult fibers in the fast EDL muscle, show an increase in oxidative enzyme levels and a reduction in the fiber cross sectional area, without changes in the major MyHC composition (Ekmark et al., 2003).

Overexpression of MyoD in denervated soleus causes a change in MyHC gene expression towards a faster fiber type, while overexpression in innervated soleus show small or no effect (Ekmark et al., 2007). Phosphorylation of the conserved threonine, T115, is shown to prevent MyoD from binding to DNA and thereby abolish its function (Liu et al., 1998). Overexpression of MyoD mutated at T115 induces fast fiber types in innervated soleus (Ekmark et al., 2007). These results indicate that electrical activity regulates phosphorylation and hence activity of MyoD and that MyoD influences the expression of fast MyHC isoforms.

1.6 A role of MRF4 and Myf-5 in determination of fiber type?

Extensive research on MyoD and myogenin in relation to fiber type specificity has been done, but less is known about the role of MRF4 and Myf-5 in adult muscle. These two factors exhibit characteristic expression levels; MRF4 has the highest expression level of the MRFs, while Myf-5 has a very low expression level. Yet there has been little evidence implicating their role in the regulation of adult muscle phenotype.

Transcripts of MRF4 start to accumulate between E15 and the first postnatal week, a period that coincides with nerve innervation. During the same period, the transcript levels of the other MRFs decline (Buonanno et al., 1992). This, and the fact that MRF4 is the MRF with highest expression level in adult muscles (Rhodes and Konieczny, 1989, Hughes et al., 1993, Voytik et al., 1993), suggests that MRF4 has an important role in adult skeletal muscle.

Several researchers have found that the level of MRF4 mRNA is the same throughout every muscle of the leg (Rhodes and Konieczny, 1989, Hughes et al., 1993, Voytik et al., 1993). On the other hand, Walters et al. (2000a) found that the transcript level of MRF4 was higher in soleus than in EDL. In another study (Walters et al., 2000b) they found that the transcript level was higher in slow fibers than in fast fibers in the gastroc. muscle, though no differences were evident between fiber types in soleus. This may indicate that MRF4 has a fiber type specific expression pattern.

Overexpression of MRF4 in adult EDL (Sjåland, 2005) do not have any effect on fiber type, oxidative capacity or cross sectional area. If the transcript level of MRF4 is equally high in all adult muscles, overexpression may not make much of a difference in the overall level of MRF4. A reduced level of MRF4 would perhaps be more effective in order to reveal a possible role in fiber type specific gene regulation.

Myf-5 mRNA is expressed at equally low levels in all adult muscles (Hughes et al., 1993, Voytik et al., 1993). The expression is restricted to satellite cells and muscle spindles (Beauchamp et al., 2000, Zammit et al., 2004), but denervation causes reactivation in myonuclei (Zammit et al., 2004). However, Sakuma et al. (1999) find that Myf-5 at the protein level is preferentially expressed in fast muscles. They also show that denervation causes a decrease in MyoD and Myf-5 protein levels in the fast plantaris and the mixed gastroc. muscles, while they are not detected either in normal or denervated soleus. This suggests a role for Myf-5 in regulation of the fast gene program.

Myf-5 and MyoD have high sequence similarity (Braun et al., 1989), and during determination in myogenesis they also have a similar role (Braun and Arnold, 1996). MyoD has been shown to influence the expression of the fast MyHC isoforms (Ekmark et al., 2007), and it is possible that Myf-5 could have a similar role in fiber type determination.

Since the transcript level of Myf-5 in adult muscles is so low, an overexpression of this MRF would be interesting in order to determine a possible effect on fiber type.

1.7 Aims of the study

The expression of MRF mRNA has been mapped, but the results regarding MRF4 are conflicting (Rhodes and Konieczny, 1989, Voytik et al., 1993, Walters et al., 2000a, Walters et al., 2000b). No one has so far mapped the normal expression of MRF4 protein, and doing this would help us come closer to find the function of this transcription factor.

Our group has earlier overexpressed MRF4 without seeing any effect on either fiber type, oxidative metabolism or cross sectional area (Sjåland, 2005). Since MRF4 is the MRF with the highest transcription level in adult muscle, it would be interesting to investigate whether a knockdown of MRF4 has any effect on fiber type and oxidative capacity.

Since Myf-5 and MyoD are so similar both regarding sequence (Braun et al., 1989) and role during myogenesis (Braun and Arnold, 1996), we wanted to investigate if Myf-5 also has a similar role as MyoD in adult muscles. The expression level of Myf-5 is very low in adult muscle, so we wanted to overexpress it in order to investigate possible effects on fiber type.

The aims of this study were therefore:

- Map normal expression of MRF4 protein in hind limb muscles of adult rats
- Investigate possible effects of siRNA knockdown of MRF4
- Investigate possible effects of overexpression of Myf-5 in the fast EDL and the slow soleus muscles of adult rats

2. Materials and methods

The material and methods used to attain the three aims of the study are discussed in three different sections; the mapping of MRF4 in 2.1, siRNA knockdown in 2.2 and overexpression of Myf-5 in 2.3.

All animal experiments were approved by the Norwegian Animal Research Authority, and conducted in accordance to the Norwegian Animal Welfare Act of December 20th, 1974, no.37, chapter VI, sections 20-22, and the Regulation of Animal Experimentation of January 15th 1996.

The animal experiments were performed on male Wistar rats (200-400 g), delivered by Harlan Laboratories BV (Netherlands), and kept in the animal facilities at the University of Oslo. The animals were kept in cages in rooms with a regulated temperature of 22 °C and with air humidity at 50-60 %. Food and water were given *ad libitum*.

2.1 Mapping of MRF4 expression

2.1.1 Isolation of muscle proteins

For mapping MRF4 protein expression, hind limb muscles of adult rats were taken out. These included soleus, EDL, plantaris, TA, and gastroc. For characterization regarding the fiber type composition of the muscles, see table 1.2.

The animals were anesthetized with an intraperitoneal injection of Equithesin (704845, Ullevål Sykehusapotek, Norway), 5 µl/g body weight. Muscles were surgically removed, snap frozen in liquid nitrogen and stored at -80 °C until homogenization and further analysis. Gastroc. was cut in two; lateral and medial part, before freezing.

The muscles were first crushed using a steel mortar, and then homogenized using an electrical homogenizer (IKA Labortechnik T25 basic, Tamro lab AS). The muscles were then fractioned into cytoplasmic and nuclear fractions according to a compartmental protein extraction kit (2145, Chemicon). Only the nuclear fraction was analyzed as MRF4 is a transcription factor and therefore is located in the nucleus.

2.1.2 DNA constructs

Before mapping MRF4 expression in muscles, the specificity of the MRF4 antibody was tested against lysates from tissue culture (HEK 293) transfected with MRF4, MyoD, myogenin or Myf-5 expression vectors. The MRF4 expression vector, pAP-MRF4, was made by cutting the pAP-lacZ vector (see section 2.3.1) at the BamHI and HindIII restriction sites, thereby cutting out the lacZ gene, and

ligating the cDNA sequence of rat MRF4 into the same site. The expression is driven by a *Rous sarcoma virus* (RSV) promoter. The MyoD expression vector, pCMS-EGFP-MyoD, was made by ligating mouse MyoD cDNA sequence into the multiple cloning site of the pCMS-EGFP vector from Clontech. The expression of MyoD is driven by the *Cytomegalovirus* (CMV) promoter. Likewise, the myogenin expression vector, pCMS-EGFP-Mg, contains the rat myogenin cDNA driven by the CMV promoter. The Myf-5 expression vector, pEMSV-Myf5, is described in section 2.3.1.

2.1.3 Transfection in tissue culture

The expression vectors for the four MRFs were transfected into human embryonic kidney cells (HEK 293). The HEK-cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; see appendix 5.2.1) at 37 °C and an atmosphere of 5 % CO_2. Every 4th day they were split with trypsin EDTA 1:6 or 1:8, dependent on the confluency. Transfection was carried out in six well plates in accordance with the Lipofectamine 2000 kit (11668, Invitrogen).

48 h after transfection, the medium was removed and the cells put on ice before being washed twice in 1 ml ice cold PBS (70011-044, GIBCO). The cells were then lysed in 500 µl lysis buffer (see appendix 5.2.2), the lysate was further centrifuged at 13 000 rpm for 20 min at 4 °C, and the supernatant stored at -80 °C until further analysis.

2.1.4 Protein measurement

Protein concentration of cell lysates and muscle fractions were measured according to the Bio-Rad Protein Assay Protocol (500-0006, Bio-Rad) and read at 595 nm by a microplate reader (Wallac Victor^2 1420, Perkin Elmer).

2.1.5 SDS-PAGE and Western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the NuPAGE Technical Guide (IM-1001, Invitrogen Instruction Manual (2003)). Electrophoresis was run with 40 µg protein samples on NuPAGE® Novex 4-12 % Bis-Tris Gels (NP0321, Invitrogen) at 200 V. Muscle fractions were run with MES running buffer for 40 min, while cell lysates were run with MOPS running buffer for 1 h. Weights of protein bands were determined using SeeBlue Plus2 Pre-Stained Standard (LC5925, Invitrogen).

Western blotting was performed according to the Trans-Blot Electrophoretic Transfer Cell Instruction Manual (170-3939, Bio-Rad). The gels were blotted onto PVDF membranes for 2 h at 1000 mA/140 V. Membranes other than those with muscle fractions were blocked in 5 % dry milk (7352F, Acumedia) in TBS-T (see appendix 5.3.2) for 1 h at room temperature. Membranes with muscle fractions were blocked in 7.5 % milk solution. Primary and secondary antibodies were diluted in 5 % milk solution, and applied over night at 4 °C and for 1 h at room temperature, respectively. After incubation with

primary and secondary antibodies, the membranes were washed three times 10 min in TBS-T. An additional 10 min wash step was included for membranes with muscle fractions.

To verify the specificity of the MRF4 antibody, lysates from cells transfected with expression vectors for MRF4, Myf-5, MyoD or myogenin were run on a gel and immunostained with rabbit monoclonal anti-MRF4 (see table 2.1 for concentrations and supplier). This was followed by an anti-rabbit IgG horseradish peroxidase (HRP)-linked whole antibody (NA934V, Amersham). Immunostaining was followed by visualization on film (28906837, Amersham) using the ECL Western Blotting Analysis System (RPN2109, Amersham). The membrane was later stripped (according to Thermo Scientific Restore Western Blotting Stripping Buffer (#21059) protocol) and immunostained with antibodies for Myf-5, MyoD and myogenin (see table 2.1). After verifying the specificity of the MRF4 antibody, detection of endogenous MRF4 in muscle fractions could be done.

To ensure even loading on the gels, staining with a mouse monoclonal anti-Vinculin antibody or a mouse monoclonal anti-β-actin antibody, followed by ECL Peroxidase labeled anti-mouse antibody (NA931VS, Amersham) was performed.

Table 2.1. List of primary and secondary antibodies with concentrations used in western blotting.

Protein	Primary antibody	Concentration	Secondary antibody	Concentration
MRF4	sc-301, Santa Cruz Biotechnology	1:250/1:500*	NA934V, Amersham (anti-rabbit)	1:2500/1:2000*
Myf-5	sc-302, Santa Cruz Biotechnology	1:500	NA934V, Amersham (anti-rabbit)	1:2000
MyoD	sc-304, Santa Cruz Biotechnology	1:1000	NA934V, Amersham (anti-rabbit)	1:2000
Myogenin	sc-12732, Santa Cruz Biotechnology	1:500	NA931VS, Amersham (anti-mouse)	1:2000
Vinculin	V9131, Sigma-Aldrich	1:80 000	NA931VS, Amersham (anti-mouse)	1:2000
β-actin	A00702, GenScript	1:2000	NA931VS, Amersham (anti-mouse)	1:2000

* 1:250 and 1:2500 was used on muscle fractions, 1:500 and 1:2000 on cell lysates

2.1.6 Statistical Methods

The films of the western blots were scanned, and the band intensities measured with ImageJ 1.43 (NIH). For statistical comparison of relative protein expression (i.e. band intensities), a Kruskal-Wallis test with Dunn's Multiple Comparison test as a post test (figure 3.2 B) or a Mann-Whitney test (figure 3.3) was performed. The level of significance was set to 0.05. For relative protein levels, the MRF4 level in protein extracts of plantaris was set to 1. These measurements were without variance and were therefore left out of the statistical analysis. Statistical analysis was carried out in GraphPad Prism 5.

2.2 siRNA knockdown

2.2.1 Principle of siRNA technology

Small interfering RNAs (siRNAs) are short RNA molecules with length of 19 to 22 nucleotides. They are generated by cleavage of double stranded RNA templates by a ribonuclease called Dicer. An RNA induced silencing complex (RISC) then binds the siRNA and separates it into single strands. The single stranded siRNA guides the RISC-complex to the target mRNA for destruction, causing RNA interference (Pratt and MacRae, 2009). Expression of the target gene can either be completely blocked or measurably suppressed. The mechanism of siRNAs is shown in figure 2.1.

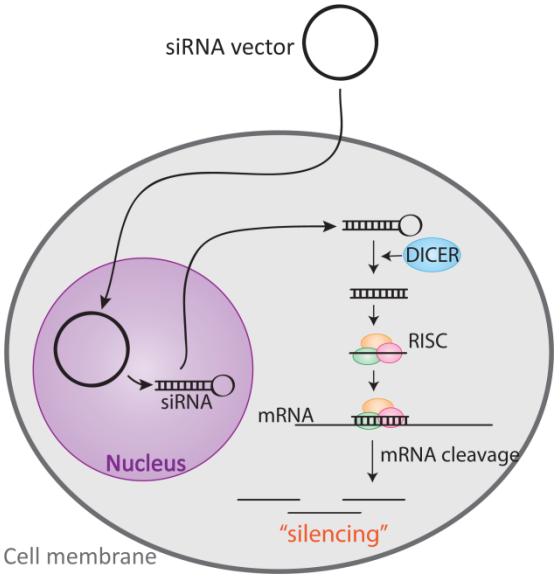

Figure 2.1. Mechanism of siRNAs. An expression vector containing the coding sequence of the siRNA is transfected into the cell. The siRNA is expressed and forms a hairpin structure which in the cytoplasm is cleaved by an enzyme called Dicer. The siRNA is then bound and separated into two strands by an RNA induced silencing complex (RISC). The RISC is guided to the target mRNA by the single stranded siRNA which base-pairs with the target mRNA. The target mRNA is then degraded by RISC causing "silencing" or knockdown of the gene of interest.

When the siRNA coding DNA is cloned into an expression vector it will cause sustained expression of the siRNA, and is therefore well suited to study the long-term effects of a protein knockdown.

2.2.2 siRNA expression vectors

The commercial company GenScript designs siRNAs from submitted cDNA sequences by using a machine learning algorithm. Optimal thermodynamic properties and GC content are taken into account, and low complexity regions and single-nucleotide polymorphisms (SNPs) are avoided. A BLAST/SmithWaterman search within the target organism is performed to exclude any siRNA that has a sequence overlap of 16 bp or more with another transcript. By submitting the rat MRF4 cDNA sequence (GI: 205522), siRNAs which were predicted to knock down the expression of MRF4 in rats were designed.

The siRNA sequences are contained in a pRNA-CMV3.1-Neo vector. The CMV promoter drives the expression of siRNA, and a *Simian virus 40* (SV40) promoter drives an ampicillin resistance gene.

siRNAs are inserted into the vector between BamHI and HindIII sites. The coding sequences of the six siRNAs used in this study are listed in table 2.2.

Table 2.2. List of siRNA coding sequences tested in this study.

	Coding sequence
siRNA 1	TCAGCGCCTTTCTTCCATCGT
siRNA 2	TTGAGGCCTTGAAGCGTAGAA
siRNA 3	TTCAGCGCCTTTCTTCCATCG
siRNA 4	TCTTCAGCGCCTTTCTTCCAT
siRNA 5	TTTGAAACTGGCTCCTATTTC
siRNA 6	TCTGAGAAGTGCCATCAACTA

2.2.3 Testing of siRNAs

To test if the siRNAs knocked down the expression of MRF4, both the siRNA vectors and the pAP-MRF4 vector were transfected into HEK-cells. These cells do not normally express MRF4 which is a muscle specific transcription factor.

To ensure that the amount of MRF4 was the same in every transfection well, an empty sham plasmid (pRNA-CMV3.1-Neo, GenScript) was co-transfected with pAP-MRF4 in the positive control well, while the same amount of pAP-MRF4 was co-transfected with every siRNA vector. Non-transfected cells were used as negative control.

Lysates of tissue culture were run on western blots in order to investigate the knockdown effect of the siRNAs. Methods for transfection and western blotting have been described earlier (section 2.1.3 trough 2.1.5).

2.3 Overexpression of Myf-5

2.3.1 Animal experiments

The animals were anesthetized with 1.5-2.5 % v/v of Isoflurane gas (506949, Forene, Abbot) with airflow of 1000 cc/min when electroporation was performed. When the experiments were terminal, the animals were anesthetized with an intraperitoneal injection of Equithesin (704845, Ullevål Sykehusapotek, Norway), 5 µl/g body weight. The effect of anesthesia was tested by pinching the metatarsal region of the foot to check for absence of withdrawal reflex. Further anesthetics were administered if necessary. Hair on the legs was removed by an electric shaver followed by application of hair removal cream (Veet). A 2 cm opening was made in the skin with a scalpel, and the muscles were exposed surgically.

2.3.2 DNA constructs

The EDL and soleus in the right leg of the rats were transfected with a DNA solution containing a mix of pEMSV-Myf5 and pAP-lacZ, while the muscles in the left leg, the sham muscles, were transfected with a mix of pEMSVscribe and pAP-lacZ (for concentrations see appendix 5.1). The use of pAP-lacZ ensured easy identification of transfected fibers. Studies have shown that two plasmids which are co-transfected most often will be co-expressed after electroporation (Rana et al., 2004).

The pAP-lacZ plasmid, which was a gift from Professor N. Gautam, contains the *Escherichia coli* β-galactosidase coding sequence between a BamHI and a HindIII restriction site. The gene is driven by the RSV promoter, and the origin of replication is driven by a SV40 promoter.

The pEMSV-Myf5 vector was made by Braun (1989) by ligating the human Myf-5 cDNA into the unique EcoRI site of pEMSVscribe. The unique EcoRI cloning site of pEMSVscribe (Davis et al., 1987) is driven by the *Maloney sarcoma virus* (MSV) long term repeat (LTR), and has a SV40 PolyA signal. Transfection with this vector served as a sham control to exclude effects on the muscle from the transfection itself.

To verify desired expression from pEMSV-Myf5 and pEMSVscribe, they were transfected into HEK-cells and the lysates run on a western blot (as described above).

2.3.3 *In vivo* electroporation

In vivo transfection of DNA was performed by electroporation as described by Mathiesen (1999). The soleus and EDL muscles were surgically exposed and 50 µl 0.5 µg/µl DNA solution (see appendix 5.1) was injected into the belly of the muscle with a syringe. Immediately after DNA injection, an electrical field was applied to the muscle using a pulse generator (Pulsar 6bp-a/s, Fredrick Haer & Co). Two silver electrodes (1 cm long and 1 mm thick) placed 2-3 mm apart were moved along the muscle while five trains of 1000 symmetrical bipolar pulses (200 µs in each direction), with amplitude of 100 V were conducted on the muscle. The electrical charge was registered using an analogue oscilloscope (OS245A, Gould Advance). Wounds were closed by suturing.

The transfected muscles were surgically removed after 14 days. They were slightly stretched, pinned out on a thin wax plate and then submerged in isopentane cooled to its freezing point. The muscles were stored at -80 °C, and later used for cryosectioning and histochemistry.

2.3.4 Histochemistry

10 µm sections from the middle towards the ends of the muscles were made on a cryostat (HM560M Microme), mounted on microscope slides and stored at -80 °C.

Staining for β-galactosidase activity

As previously mentioned, a vector with a lacZ-gene was co-transfected together with the experimental plasmid or the sham plasmid so that transfected fibers easily could be identified. LacZ encodes the enzyme β-galactosidase which breaks down β-galactosides. One such compound is X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) which is broken down to indoxyl and galactose. Two indoxyl monomers dimerize and form an insoluble blue compound, and when applying X-gal to sections, transfected fibers will therefore appear blue.

Staining for myosin heavy chain isoforms

To determine the muscle fiber types, monoclonal antibodies against the four main MyHC isoforms were used on neighboring sections. Antibodies against MyHC 1, 2a and 2b were kindly provided by Stefano Schiaffino's lab, and the antibody against the 2x MyHC isoform was a gift from Joseph F.Y.Hoh. To determine whether the electroporation had caused damage in the muscles, staining for embryonic MyHC was also performed as this is the first MyHC to be expressed in regenerating fibers. This antibody was grown in our lab in BF-45 hybridoma cells (ATCC). All antibodies are listed in table 2.3. For concentrations and staining protocol, see appendix 5.4.3.

Table 2.3. Primary and secondary antibodies for detection of MyHC isoforms.

MyHC:	Primary antibody:	Secondary antibody:
1	BA-D5	Rabbit Anti-mouse IgG, FITC conjugated (F-9137, SIGMA)
2a	SC-71	Rabbit Anti-mouse IgG, FITC conjugated (F-9137, SIGMA)
2x	6H1	Goat Anti-mouse IgM FITC conjugated (F-9259, Sigma)/ Goat anti-mouse IgM, Cy3 (J115-165-020, Jackson Immuno Research Laboratories)
2b	BF-F3	Goat Anti-mouse IgM FITC conjugated (F-9259, Sigma)/ Goat anti-mouse IgM, Cy3 (J115-165-020, Jackson Immuno Research Laboratories)
Embryonic	BF-45	Rabbit Anti-mouse IgG, FITC-conjugated (F-9137, SIGMA)

After staining and imaging, transfected fibers were identified and numbered on each section. For every transfected fiber, two non-transfected normal fibers from the same muscle were numbered and analyzed. When it was possible the two fibers to the left of the transfected one were chosen, if not, the two fibers below were chosen. Finally, fiber type was determined for each fiber by registration of which MyHC isoform it stained positive (figure 2.2).

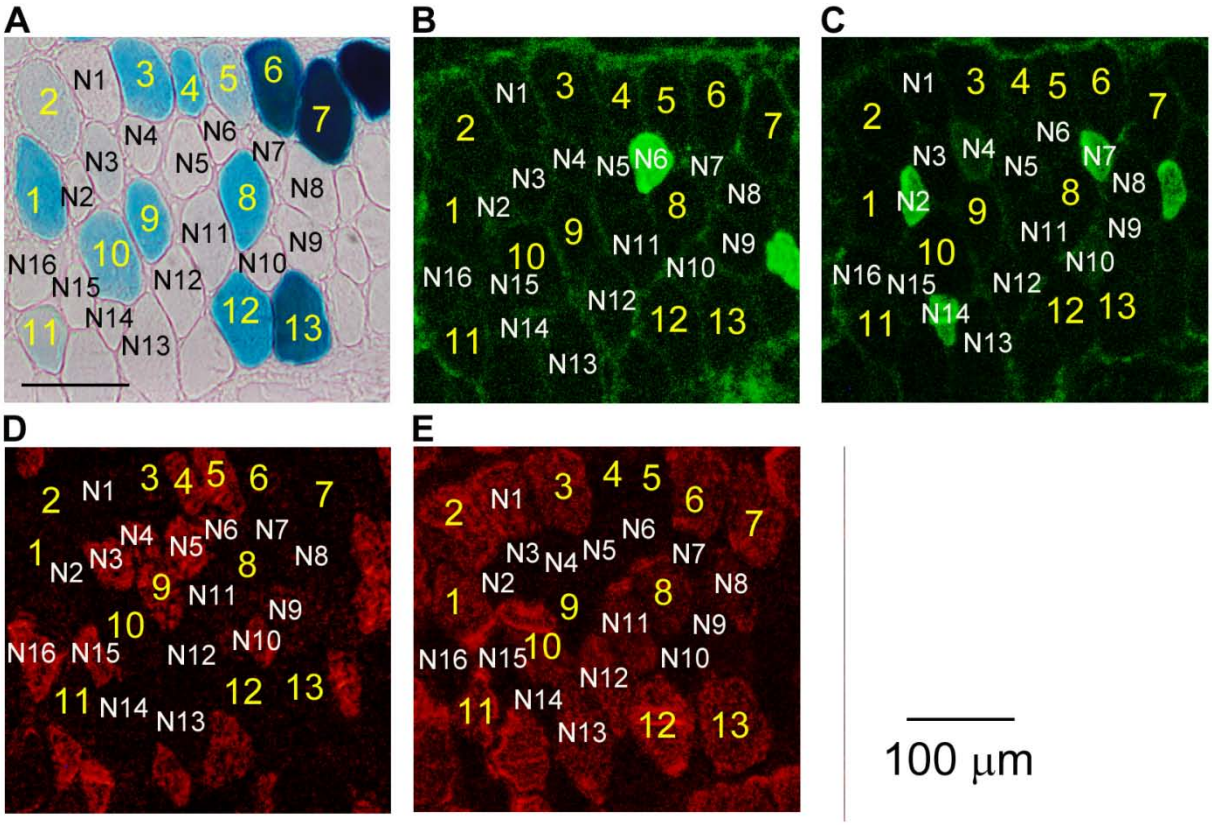

Figure 2.2. A-E Example of serial sections of an EDL muscle stained for β-galactosidase activity and myosin heavy chain (MyHC) isoforms. Serial cross sections stained for β-galactosidase activity (**A**), MyHC 1 (**B**), MyHC 2a (**C**), MyHC 2x (**D**) and MyHC 2b (**E**). **A**. Fibers positively stained for β-galactosidase appear blue (numbered 1-13 in yellow), while non-transfected normal fibers for control appear bright (numbered N1-N16). **B-E**. Positively stained fibers for the respective MyHC isoforms appear bright green or red, while negative fibers appear dark. Note that the system for numbering normal fibers described in the text does not apply to this figure, as all fibers have been given new numbers for illustrating purposes.

2.3.5 Imaging

Bright-field imaging

Images of muscle sections stained for β-galactosidase activity were taken with water immersion at 10X magnification with a ColorView camera connected to an Olympus BX50WI microscope. The microscope has an automatic stage controlled by the Olympus Cell^{B} software, which automatically takes a series of images of the whole section and aligns them. Further processing was carried out in Adobe Photoshop CS4.

Fluorescence imaging

Images of muscle sections stained with Cy-3 or FITC conjugated antibodies were photographed in a dark room with the same microscope setup as with bright-field imaging (see above). A green filter (XF37) was used to illuminate sections with the Cy-3 conjugated secondary antibody, while a blue-

green filter (WF22) was used to illuminate sections with FITC conjugated secondary antibodies. Further processing was performed as with bright-field imaging.

2.3.6 Statistical methods

For statistical comparison of fiber type distribution between the Myf-5 transfected, sham transfected and normal control fibers, a Chi-square test was performed. The level of significance was set to 0.05. The statistical analyses were performed in GraphPad Prism 5.

3. Results

3.1 Expression of MRF4 protein

3.1.1 Testing of the MRF4 antibody

Since all the MRFs show strong sequence similarities (Braun et al., 1989, Wright et al., 1989, Miner and Wold, 1990) the MRF4 antibody (sc-301) was tested for cross reactivity with MyoD, myogenin and Myf-5 (figure 3.1). The MRF4 antibody did not bind to any of the other MRFs, and neither did the MyoD and the myogenin antibody.

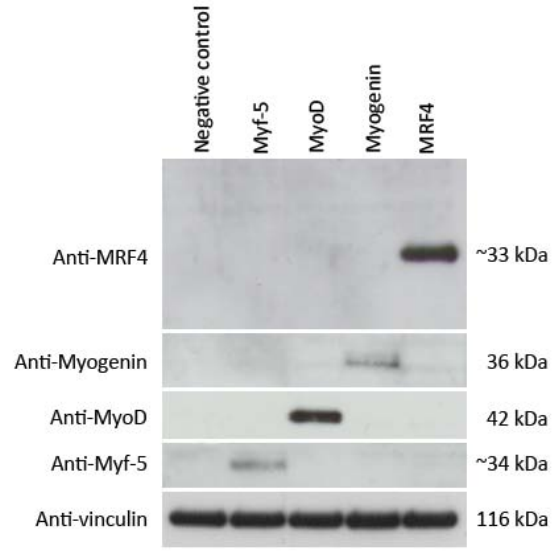

Figure 3.1. Western blot showing specificity of the MRF4 antibody. Protein extracts from HEK-cells transfected with expression vectors for Myf-5, MyoD, myogenin or MRF4 were run on the same gel and immunostained with anti-MRF4 (sc-301) to test for specificity of this antibody. No cross reactivity with the other MRFs was observed. Immunostaining with anti-myogenin, anti-MyoD and anti-Myf-5 were also performed to verify presence of these proteins. Non-transfected cells were used as a negative control. Staining with anti-Vinculin served as loading control.

The Myf-5 antibody showed strong cross-reactivity with MyoD (not shown), but since these two factors have different molecular weight (34 and 42 kDa, respectively) it was possible to tell them apart. A reason for this cross-reaction may be the sequence similarity between these two factors. The Myf-5 antibody (sc-302) is a polyclonal antibody raised against the carboxyl terminal (aa 236-255; Sakuma et al., 1999) of the human Myf-5 protein. A pairwise alignment (EMBOSS Pairwise Alignment Algorithm) show a sequence identity of 36 % and similarity of 52 % between Myf-5 and MyoD in this region.

3.1.2 MRF4 expression patterns

Mapping of MRF4 protein expression was done in hind limb muscles of adult rats. These included the slow soleus, the fast EDL, plantaris and TA, and the more mixed gastroc. which were separated in GM and GL.

Muscles from 4 untreated rats were taken out. From two of the rats, muscles of both left and right leg were used, while from the two other, only muscles of the right leg were used. The muscles were homogenized and fractioned, and the nuclear fractions run on western blot. Six blots (one per leg) were treated as independent observations and used in the measurements of MRF4 expression. A representative blot is presented in figure 3.2 A. Lysates from MRF4 and non-transfected HEK-cells were included in the blots as positive and negative controls, respectively. The positive control is shown in lane 8, and MRF4 band intensities were measured in a horizontal line from this band. In every blot, band intensities were standardized to the intensity of the band in the plantaris lane on the same blot. This means that the band intensity of plantaris was always 1. These measurements therefore showed no variance, and were left out of the statistical analysis.

Relative amounts of MRF4 protein are plotted in figure 3.2 B. Soleus showed the highest relative expression level, while the other muscles showed approximately the same expression level as plantaris. EDL had a slightly higher level, but this was largely due to one outlier in the measurements. It may seem that the overall expression level was higher in the soleus compared to the other muscles of the study. However, when comparing all the muscles, there were no significant differences in the relative protein levels, probably due to large spread in the measurements of individual blots.

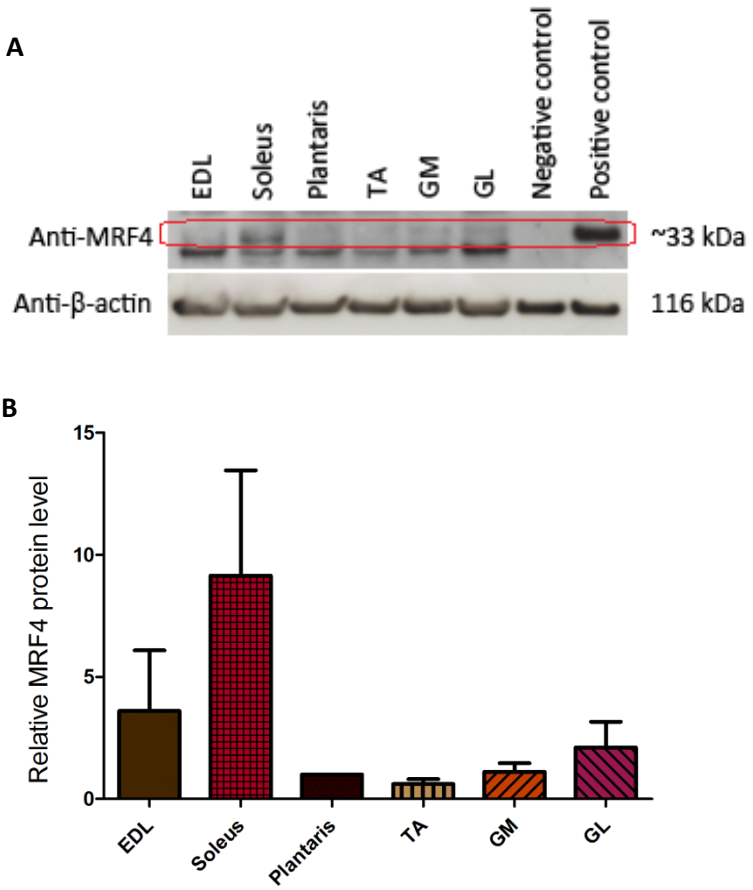

Figure 3.2. Expression level of MRF4 protein in the hind limb muscles of rat. **A.** Representative western blot showing endogenous MRF4 protein in the nuclear fraction of extensor digitorum longus (EDL), soleus, plantaris, tibialis anterior (TA) and gastrocnemius, medial (GM) and lateral (GL) part. Cell extracts from non-transfected and MRF4 transfected HEK-cells were used as a negative and positive control, respectively. **B**. A quantitative assessment showing the relative amount of MRF4 protein. n=6. Mean ± SEM. Band intensities were compared to the band in the plantaris-lane in the same blot. Band intensity of plantaris was therefore always 1.

As seen in table 1.2, soleus is the muscle with the slowest phenotype of the muscles included in this study, while the other muscles have a much faster phenotype. The expression level of MRF4 in soleus seems to be different than in these faster muscles, and the fast muscles were therefore pooled and their relative expression level compared with that of the slow soleus (figure 3.3). The difference was significant ($p = 0.021$).

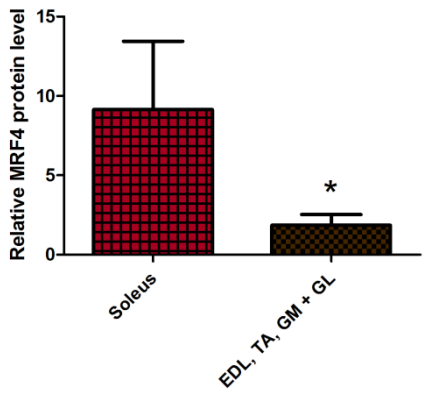

Figure 3.3. Relative level of MRF4 in soleus compared to the other, faster muscles of the study. The relative MRF4 protein levels of EDL, TA, GM and GL were pooled together and tested against the relative MRF4 protein level of soleus. Measurements of plantaris were left out as they were always 1. n = 6 for soleus, n = 24 (6x4) for the other group. Mean ± SEM. * = $p<0.05$.

To see if there was any correlation between MRF4 expression and the muscles' fiber type composition, mean values of the relative MRF4 level from each muscle were plotted against the muscles' frequency of slow type 1 fibers (figure 3.4). Approximate type 1 fiber frequencies of GM and GL were calculated using measurements of muscle mass and type 1 fiber frequency of red, white and mixed gastroc. from Delp and Duan (1996).

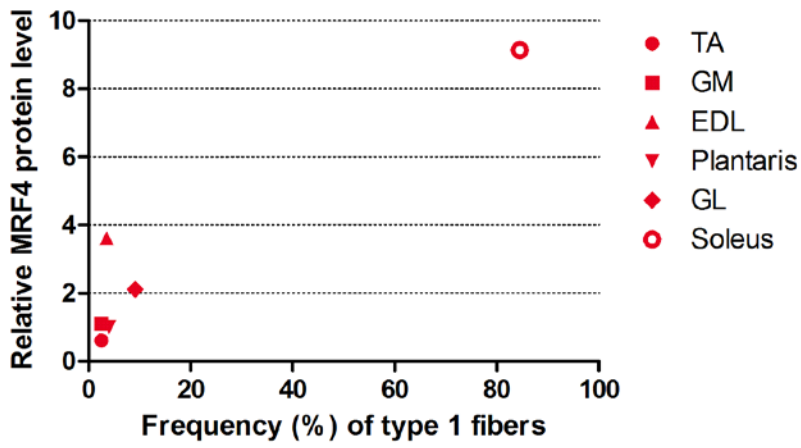

Figure 3.4. Correlation between MRF4 expression and frequency of type 1 fibers. Mean values of the relative MRF4 levels of each muscle are plotted against the muscles' frequency (%) of type 1 fibers. Frequencies of fiber types are listed in table 1.2. Frequencies of GM and GL were calculated using Delp and Duans (1996) measurements of muscle mass and fiber type frequencies of red, white and mixed gastroc.

Again it is clear that soleus is different than the other muscles both regarding relative level of MRF4 and frequency of slow type 1 fibers. This might be an indication of a correlation between fiber type and MRF4 expression. Unfortunately, no muscles with intermediate type 1 fiber frequency were included, but either way the results indicate that MRF4 is enriched in slow muscle fibers. However,

since there was large spread in the western blot measurements, more experiments are needed before any conclusions about expression of MRF4 protein can be drawn.

3.2 Testing of siRNAs

A total of six different siRNA vectors were tested in tissue culture of HEK-cells. These cells do not normally express MRF4, so a MRF4 expression vector was co-transfected together with the siRNA vectors. Figure 3.5 shows a western blot of the three first siRNA tested. None of them seemed to give a knockdown of MRF4. In fact, the bands in the siRNA lanes had an intensity that was somewhat higher than the MRF4 control band.

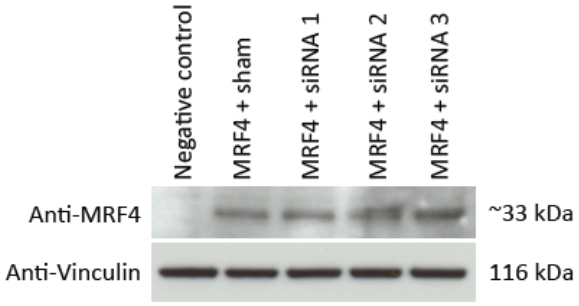

Figure 3.5. Testing of siRNAs 1, 2 and 3. Western blot with lysates of siRNA transfected HEK-cells. Co-transfection with an expression vector for MRF4 was necessary because HEK-cells do not normally express MRF4. Cells transfected with a sham plasmid and the MRF4 expression vector served as a positive control. Non-transfected cells were used as a negative control. Staining with anti-Vinculin served as loading control.

Three more siRNAs were tested in HEK-cells. Western blot of protein extracts are shown in figure 3.6. Neither of these siRNAs gave a knockdown of MRF4, and also here the bands in siRNA lanes were somewhat stronger than the band in the MRF4 control lane.

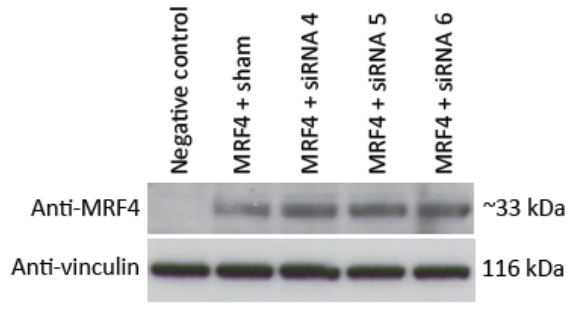

Figure 3.6. Testing of siRNAs 4, 5 and 6. Western blot with lysates of siRNA transfected HEK-cells. Because HEK-cells do not normally express MRF4, co-transfection with an expression vector for MRF4 was necessary. Transfection with a sham plasmid and the MRF4 expression vector was used as a positive control. Negative control was non-transfected cells. Staining with anti-Vinculin served as loading control.

Since none of the tested siRNAs seemed to give a knockdown of MRF4, the MRF4 *in vivo* knockdown study was abandoned for the time being.

3.3 Effects of Myf-5 overexpression on fiber type distribution

3.3.1 Verification of pEMSV-Myf5 expression vector

The Myf-5 expression vector (pEMSV-Myf5) and the sham plasmid (pEMSVscribe) used in this experiment were transfected in tissue culture (HEK-cells) to verify desired expression. Lysates of the transfected cells were run on a western blot and immunostained with a Myf-5 antibody (figure 3.7). A band around 34 kDa (according to the ladder used) was seen in the lane of Myf-5 transfected cells, but not in the lane of sham transfected cells. The band corresponds to the molecular weight of Myf-5 (32 kDa), and we concluded that pEMSV-Myf5 but not pEMSVscribe gives expression of Myf-5. *In vivo* overexpression of Myf-5 could therefore be conducted using this expression vector.

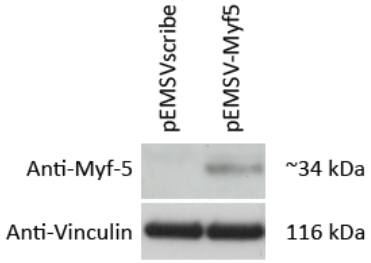

Figure 3.7. Expression of Myf-5 in tissue culture. Western blot of protein extracts from HEK-cells transfected with the sham plasmid pEMSVscribe or the Myf-5 expression vector pEMSV-Myf5. Staining with anti-Vinculin served as a loading control.

3.3.2 Fiber type distribution in EDL

A total number of 374 Myf-5 transfected fibers, 455 sham transfected fibers and 2153 normal fibers were analyzed (table 3.1/figure 3.8). There were significant differences between all three groups.

Table 3.1. Fiber type distribution (%) in EDL.

Fiber type	1	1/2a	2a	2a/2x	2x	2x/2b	2b	Total fibers
Normal	2.4	1.7	15.8	5.9	29.1	17.2	27.8	2153
Sham	4.4	1.3	19.3	4.2	20.0	18.2	32.5	455
Myf-5	1.9	1.6	9.6	6.4	18.7	17.7	44.1	374

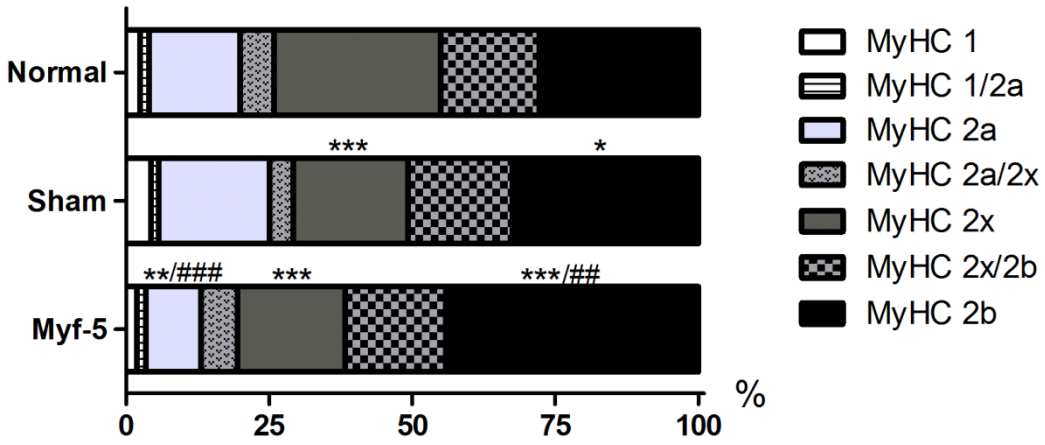

Figure 3.8. Fiber type distribution in EDL. Fiber type distribution after Myf-5 or sham transfection, and in normal controls. Total n is 2982 fibers from 8 different animals. For further information regarding fiber type distribution see table 3.1 and appendix 5.5.1. Significant differences compared to normal fibers are indicated by *, while significant differences compared to the sham transfected fibers are indicated by # (*/#=p<0.05, **/##=p<0.01, ***/###=p<0.001). The level of significance was set to 0.05.

When comparing Myf-5 transfected fibers with the normal fibers, the amount of 2a fibers have decreased from 15.8 % to 9.6 % (p=0.0016), the amount of 2x fibers decreased from 29.1 % to 18.7 % (p<0.0001), and the amount of 2b fibers increased from 27.8 % to 44.1 % (p<0.0001).

When comparing sham transfected fibers with the normal fibers the amount of 2x fibers have decreased from 29.1 % to 20.0 % (p=0.0001) and the amount of 2b fibers increased from 27.8 % to 32.5 % (p=0.0423).

Compared to sham transfected fibers, Myf-5 transfected fibers have a decrease from 19.3 % to 9.6 % (p<0.0001) in 2a fibers, and an increase from 32.5 % to 44.1 % (p=0.002) in 2b fibers.

As described, there was an expansion in the pool of 2b fibers at the expense of 2x and 2a fibers when comparing Myf-5 transfected fibers to normal fibers. The expansion of 2b fibers was also evident when comparing Myf-5 to sham transfected fibers, although the change was smaller, and was only accompanied by decrease in 2a fibers and not 2x fibers.

However, the results should be interpreted with caution as there was large animal to animal variation with regard to transfection efficiency and fiber type composition. This is not accounted for in the statistical analysis as the fibers of the same experimental group from all rats were pooled before the analysis.

3.3.3 Fiber type distribution in soleus

A total of 835 Myf-5 transfected fibers, 746 sham transfected fibers and 3974 normal fibers were analyzed (table 3.2/figure 3.9). Significant differences were observed between all three groups.

Table 3.2. Fiber type distribution (%) in soleus.

Fiber type	1	1/2a	2a	Total fibers
Normal	92.5	2.8	4.7	3974
Sham	87.0	7.0	6.0	746
Myf-5	93.8	4.0	2.3	835

There was no staining for type 2x or 2b MyHC so these are excluded from the table.

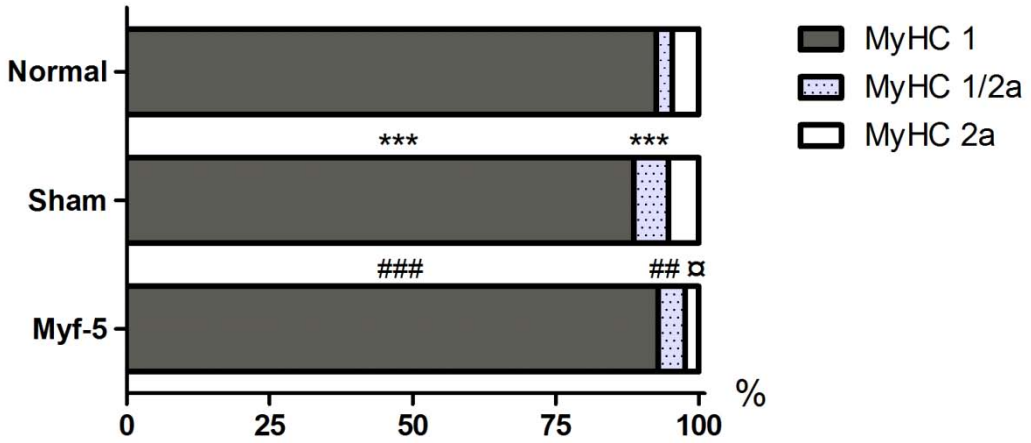

Figure 3.9. Fiber type distribution in soleus. Fiber type distribution after Myf-5 or sham transfection, and in normal controls. Total n is 5555 fibers from 10 different animals. For further information regarding fiber type distribution see table 3.2 and appendix 5.5.2. Significant differences compared to normal fibers are indicated by *, while significant differences compared to the sham transfected fibers are indicated by # (**/##=p<0.01, ***/###=p<0.001, ¤=*** and ###). The level of significance was set to 0.05.

When comparing Myf-5 transfected fibers with normal fibers, a decrease in 2a fibers from 4.7 % to 2.3 % (p=0.002) is seen. Compared to normal fibers, sham transfected fibers showed an increase from 2.8 % to 7.0 % (p<0.0001) in 1/2a fibers, and a decrease from 92.5 % to 87.0 % (p<0.0001) in type 1 fibers.

Between Myf-5 and sham transfected fibers there were significant differences in all fiber types. A decrease in both 2a (from 6.0 % to 2.3 %, p=0.0002) and 1/2a (7.0 % to 4.0 %, p=0.0099), as well as an increase from 87.0 % to 93.8 % (p<0.0001) in type 1 in Myf-5 transfected fibers was seen.

Overall, there seems to be differences between all the experimental groups. Sham transfected fibers show a shift towards a faster fiber type than normal fibers, and Myf-5 transfected fibers show a slower fiber type than both sham transfected and normal fibers.

As mentioned above, caution should be shown when interpreting these results as there was large animal to animal variation in transfection efficiency and fiber type composition.

No staining of embryonic MyHC was observed in any of the fibers analyzed in this study, and little or no such staining was evident in the muscles at all. In cases where it was observed, it was only in very small fibers in the fringe of the muscle.

4. Discussion

To our knowledge, this study is the first to map the expression of MRF4 protein in normal, adult muscle. The lack of publications on this topic could be related to difficulties with the available antibodies. In the present study we saw that the level of MRF4 was significantly higher in the slow soleus when compared to fast muscles.

We were not able to detect any knockdown effect from the six different siRNAs tested, as shown in western blot figures 3.5 and 3.6. Although the siRNAs are designed to specifically knock down their targets, failing frequently occur. Because of the cost of the siRNA vectors and the limited time remaining in my master's degree, further work with this project was put on hold.

Results from overexpression of Myf-5 in EDL indicate that Myf-5 stimulates expression of MyHC 2b, as the Myf-5 transfected fibers showed a somewhat higher percentage of 2b fibers than sham transfected and normal fibers.

4.1 Is MRF4 enriched in slow fibers?

The results indicated a correlation between the muscles' relative MRF4 expression and their frequency of slow type 1 fibers. Soleus, the slowest muscle included, shows the highest expression level of MRF4. The difference in MRF4 level of soleus compared to all the other faster muscles pooled together was significant. This finding is in agreement with Walters et al. (2000a), who found that the transcript level of MRF4 in normal muscles was higher in soleus than in the fast EDL.

Since there seems to be a correlation between MRF4 expression and fiber type composition, a natural question to ask is whether MRF4 influences fiber type. Overexpression of MRF4 in EDL do not alter the expression of MyHC isoforms, the oxidative enzyme SDH (succinate dehydrogenase), or change the fibers' cross sectional area (Sjåland, 2005). If the mRNA level of MRF4 is the same throughout every muscle as found by Rhodes and Konieczny (1989), it is possible that MRF4 is subject to fiber type specific post-transcriptional regulation since the results of the present study showed that the protein expression differs between muscles. It is also possible that its translation is regulated by the electrical activity of the muscle, leading to different protein levels in fast and slow muscles. If the mRNA levels differ between fiber types and muscles like Walters et al. have suggested (2000a, 2000b), the overexpression results of Sjåland (2005) indicate that MRF4 does not participate in the regulation of MyHC isoforms or SDH. It may never the less be involved in regulation of other

fiber type specific proteins, for example in regulation of other metabolic enzymes, regulation of proteins involved in twitch duration or calcium flux.

With the antibodies available, western blotting does not seem to be the optimal method for detection of MRF4 protein in muscle extracts, as there was high background staining and many unspecific bands. No such problems were experienced with lysates from tissue culture, probably due to a much higher protein content and diversity in muscles. For detection of endogenous MRF4 in muscle, an alternative approach could be to perform two-dimensional gel electrophoresis, where proteins are separated both according to size and isoelectric point. This means that the proteins are separated to a greater extent which might make it easier to identify the MRF4 band. Preliminary studies with this method with one of our collaborators have shown promising results for MRF4 expression in soleus and EDL. By doing mass spectrometry on the bands stained with anti-MRF4, verification of the correct protein and identification of possible post-translational modifications can be done.

4.2 Does Myf-5 have an effect on skeletal muscle fiber type?

4.2.1 The "sham-effect"

Myf-5 transfected fibers of EDL showed a significantly higher number of type 2b MyHC fibers than both sham transfected and normal fibers. But there were also a significantly higher number of type 2b expressing fibers among the sham transfected fibers when compared to normal fibers. This is probably due to the "sham-effect" which has been observed in some previous studies in our group (Grönevik 2000, Sjåland 2005, Hansen 2009, Staurseth 2009).

The sham-effect is a phenomenon where more fast fibers are found among the sham transfected fibers than the normal control fibers. The transfection of the sham plasmid should not have any effect on fiber type distribution, so in theory sham transfected and normal fibers should have an equal distribution of fiber types. This "sham- effect" is believed to be caused by selective transfection of large fibers. A fiber's conductance is proportional to its circumference (Katz, 1948) so that large fibers have greater conductance (and smaller resistance) than small fibers, and therefore will transfect more easily. 2b fibers have the largest circumference of all the different fiber types and selective transfection of these may therefore cause a "sham-effect".

In soleus there were large variations between all experimental groups, and it is difficult to say if some of the variation can be due to selective transfection. The cross sectional area of different fibers in soleus is more homogenous than in EDL, where the variation is rather large (Delp and Duan, 1996), thus making it more likely to achieve a non-selective transfection in soleus.

4.2.2 Does Myf-5 induce a fast phenotype?

Myf-5 transfected fibers of EDL have a higher percentage of 2b fibers than both sham transfected and normal fibers. This may indicate a transformation in the fast direction in fibers overexpressing Myf-5.

Overexpression of any of the MRFs in cultured myotubes greatly increases 2b promoter activity (Takeda et al., 1995), but not 2a or 2x promoter activity (Allen et al., 2001). Overexpression of MyoD *in vivo* has been shown to increase the expression of fast fiber type MyHC, but only 2a and 2x (Ekmark et al., 2007). *In vivo* overexpression of myogenin (Ekmark et al., 2003) or MRF4 (Sjåland, 2005) does not have any effect on MyHC expression. The results of the present study suggest that overexpression of Myf-5 in EDL has the ability to increase expression of MyHC 2b *in vivo*.

The transcription level of Myf-5 is found to be very low in adult muscle, and no differences are seen between different muscles (Hughes et al., 1993, Voytik et al., 1993). On the other hand, Sakuma et al. (1999) find that Myf-5 protein expression is higher in fast muscles, and this supports the hypothesis that Myf-5 is an activator of MyHC 2b expression. It is however possible that activation of the MyHC 2b promoter only is a pharmacological effect. The expression level of MyoD is highest in fast muscles (Hughes et al., 1993, Voytik et al., 1993), and MyoD induces expression of fast MyHC isoforms (Ekmark et al., 2007). Myf-5 has great sequence similarity with MyoD, and it may be that Myf-5 shares many properties with MyoD.

4.2.3 Animal-to-animal variation may influence fiber type distribution

Although the rats used in this study were of the same strain and batch, there was variation in fiber type composition between the individual animals. Since all sections were stained for a given MyHC isoform at the same time with the same antibody solution, this variation is not likely to be due to deviations during the staining procedure. The fiber type composition of muscles has been shown to vary along the proximo-distal axis (Wang and Kernell, 2000), and this may influence the analysis of the muscles. No marking of proximal and distal ends of the muscles were done before freezing, and some of the animal to animal variation may therefore be caused by differences in where in the muscle the analyzed sections are taken from.

Good transfection efficiency is dependent on many variables. The volume of DNA solution injected, where it is injected and how the solution is distributed within the muscle, the voltage of the current applied and the positioning of the electrodes during the electroporation are all factors that will influence the transfection.

The transfection efficiency varied between the Myf-5 and sham transfected muscles within the individual rats (see appendix 5.5). In some rats the number of transfected fibers were approximately the same for Myf-5 and sham muscles, but often the number of Myf-5 transfected fibers were higher than the number of sham transfected fibers, or vice versa. When there are large variations in fiber type composition between the rats, uneven transfection efficiency can lead to apparent differences between the experimental groups, when there in reality is none. For example, when one of the rats with particularly large difference in the number of Myf-5 and sham transfected fibers was left out, there were no significant differences between the Myf-5 and sham groups of soleus.

In soleus, the fiber type change in Myf-5 transfected fibers was in the slow direction compared to sham transfected and normal fibers. The sham transfected fibers showed a faster phenotype than the normal and Myf-5 transfected fibers. It is possible that much of the differences between the experimental groups are caused by variation in fiber type composition and variable transfection efficiency between rats. In EDL, the variable transfection efficiency resulted in that most of the fibers analyzed came from 3 rats (see appendix 5.5.1). Animal-to-animal variation might therefore have influenced the results.

4.3 Future experiments

Future studies of MRF4 expression should include two-dimensional gel electrophoresis to allow better separation of proteins and easier identification of the correct band. Possible modifications of the protein can be identified with mass spectrometry. It would also be interesting to investigate the level of mRNA expression by real time PCR, as there are discrepancies in the studies regarding MRF4 mRNA expression (Rhodes and Konieczny, 1989, Voytik et al., 1993, Walters et al., 2000a, Walters et al., 2000b). In order to further confirm a correlation between MRF4 expression and slow fiber type frequency, more muscles with intermediate type 1 fiber content should be investigated (se figure 3.4).

The six siRNAs tested in this study did not give a detectable knockdown of MRF4. In order to investigate knockdown effects, more siRNAs should be tested.

Since the material in the study of Myf-5 in EDL was small due to variable transfection efficiency, future studies should focus on increasing the sample size. More material is also needed before any conclusions can be made about the effects of overexpression in soleus. It would also be interesting to investigate whether Myf-5 has any effect on the oxidative or glycolytic metabolism, and also cross sectional area of the fibers.

4.4 Conclusions

- The level of MRF4 was significantly higher in the slow soleus compared to fast muscles
- Fibers overexpressing Myf-5 in EDL showed a higher percentage of 2b fibers than sham transfected and normal fibers

5. Appendices

5.1 DNA electroporation solutions

5.1.1 pEMSV-Myf5 and pAP-lacZ solution (200 µl)

Solution:	Amount:
pEMSV-Myf5 in TE buffer (2 µg/µl)	25 µl
pAP-lacZ (2 µg/µl)	25 µl
4 M NaCl	8 µl
dH_2O	142 µl

5.1.2 pEMSVscribe and pAP-lacZ solution (200 µl)

Solution:	Amount:
pEMSVscribe in TE buffer (2 µg/µl)	25 µl
pAP-lacZ (2 µg/µl)	25 µl
4 M NaCl	8 µl
dH_2O	142 µl

5.2 Cell culture

5.2.1 DMEM (555 ml)

Solution:	Amount:
DMEM (GIBCO)	500 ml
Fetal calf serum (Bio Whittaker)	50 ml
Penicillin/Streptomycin (Bio Whittaker)	5 ml

5.2.2 Cell lysis buffer 2 l

Solution:	Amount:
50 mM Trisacetate pH 7	12 g
0,27 M Sucrose	184.4 g
1 mM EDTA	0.75 g
1 mM EGTA	0.76 g
1 mM Sodium Orthovanadate	20 ml stock
10 mM B-glycerophosphate	6.3 g
50 mM Sodium Fluoride	4.2 g
5 mM Sodium Pyrophosphate	4.46 g
1 % Triton X-100	20 ml

- Make up to 2.0 l with distilled water. Before use, 50 µl each (per 50 ml buffer) of protease inhibitor phenylmethanesulphonylfluoride (PMSF), 4mM Benzamidine and β-mercapto-ethanol must be added.

5.3 Western blotting

5.3.1 10X transferbuffer

Solution:	Amount:
Glycine	288 g
Tris-(hydroxymetyl)aminomethane	60 g

- Dissolve Glycine and Tris in some dH_2O before adjusting volume to 2.0 l
- To make 1X transferbuffer to use, take 100 ml 10X transferbuffer, 100 ml methanol and 800 ml dH_2O

5.3.2 10X TBS (tris-buffered saline) and TBS-T

Solution:	Amount:
NaCl	292.2 g
Tris-(hydroxymetyl)aminomethane	24.25 g

- Dissolve NaCl and Tris in some dH_2O before adjusting volume to 1.0 l
- To make TBS-T, take 100 ml of TBS and 900 ml dH_2O, add 1 ml Tween20 (P1379, Sigma-Aldrich), mix well

5.4 Histochemistry

5.4.1 10X PBS solution

Solution:	Amount:
NaCl	80 g
KCl	2.0 g
$Na_2HPO_4 \times 2H_2O$	14.4 g
KH_2PO_4	2.0 g

- Dissolve all the chemicals in 800 ml of dH_2O
- Adjust the pH to 6.8 / 6.5, and the volume to 1 l
- For making 1X PBS with pH 7.4 / 7.1, take 100 ml 1X solution and 900 ml dH_2O

5.4.2 Staining for β-galactosidase activity

- Thaw sections and encircle the sections with a hydrophobic pen (H-400, Vector)
- Make fix-solution:

Solution:	Amount:
(Para)Formaldehyde	2 g
Glutaraldehyde	400 µl
10 X PBS (pH)	10.0 ml
dH_2O	69.2 ml

- Dissolve the formaldehyde in dH_2O (60 °C); adjust volume to 100 ml and pH to 7.1
- Fix the sections at 4 °C for 20 min
- Wash the sections 3 x 5 min in PBS (pH 7.1)
- Make staining solution:

Solution:	Amount:
10 X PBS (pH 7.1)	150 µl
0.2 M Potassium ferrocyanide	30 µl
0.2 M Potassium ferricyanide	30 µl
1 M Magnesium chloride	3 µl
dH_2O	1260 µl
X-gal in DMSO (50 mg/ml)	30 µl

- Stain over night at 37 °C in a moist chamber
- Wash sections 3 x 5 min in PBS (pH 7.1)
- Mount sections in glycerin gel:

Solution:	Amount:
Gelatin	15 g
Glycerol	100 ml
dH_2O	100 ml

5.4.3 Staining for MyHC isoforms

- Thaw sections and encircle the sections with a hydrophobic pen (H-400, Vector)
- Apply antibodies for the different MyHC isoforms:

MyHC:	Primary antibody:	Concentration:	Incubation:
1	BA-D5	1:80 in 1 % BSA in PBS	30 min at RT
2a	SC-71	1:100 in 1 % BSA in PBS	30 min at RT
2x	6H1	100 %	O.N at 4 °C
2b	BF-F3	1:7 in 0.5 % BSA in PBS	45 min at 37 °C in moist chamber
Embryonic	BF-45	100 %	30 min at RT

- Wash sections 3 x 5 min in PBS (pH 7.4)
- Use secondary antibody Rabbit anti-mouse IgG FITC conjugated (F-9137, Sigma) on primary antibodies BA-D5, SC-71 and BF-45 in a 1:200 dilution in 0.5 % BSA in PBS, and incubate for 30 min at 37 °C in a moist chamber
- On primary antibodies 6H1 and BF-F3, use secondary antibody Goat anti-mouse IgM, Cy-3 (J115-165-020, Jackson ImmunoResearch Lab) or Goat Anti-mouse IgM FITC conjugated (F-9259 SIGMA), in a 1:300 or 1:80 dilution in 0.5 % BSA in PBS, respectively. Incubate for 80-120 min for MyHC 2x staining, 45 min for MyHC 2b staining, both at 37 °C in a moist chamber
- Wash sections 3 x 5 min in PBS (pH 7.4)

5.5 Fiber type frequencies of individual rats

5.5.1 Fiber type frequencies (%) in EDL of individual rats

		1	1/2a	2a	2a/2x	2x	2x/2b	2b	Total fibers
R1	**Myf-5**	1.6	2.7	9.2	2.7	19.0	12.5	52.2	184
	Sham	3.2	0	9.7	6.5	25.8	16.1	38.7	31
	Normal	1.7	2.3	16.9	5.2	31.6	9.6	32.7	706
R2	**Myf-5**	-	-	-	-	-	-	-	0
	Sham	0	0	0	0	0	66.7	33.3	3
	Normal	0	0	20.0	20.0	40.0	20.0	0	5
R4	**Myf-5**	0	0	0	0	0	50.0	50.0	2
	Sham	3.8	0	0	0	15.4	23.1	57.7	26
	Normal	6.7	0	14.6	2.2	31.5	16.9	28.1	89
R6	**Myf-5**	0	0	0	0	25.0	50.0	25.0	4
	Sham	-	-	-	-	-	-	-	0
	Normal	0	0	0	0	62.5	12.5	25.0	8
R7	**Myf-5**	0	0	5.6	11.1	33.3	27.8	22.2	18
	Sham	0	0	0	0	0	0	100.0	1
	Normal	1.3	0	2.7	10.7	26.7	17.3	41.3	75
R8	**Myf-5**	0	0	0	5.9	5.9	29.4	58.8	17
	Sham	0	1.0	1.9	1.9	13.3	6.7	75.2	105
	Normal	2.3	1.4	7.6	6.2	30.2	13.8	38.4	354
R9	**Myf-5**	3.5	0	8.0	8.8	21.2	18.6	39.8	113
	Sham	6.9	1.2	31.4	5.7	24.9	20.4	9.4	245
	Normal	2.7	1.5	21.4	6.4	28.5	21.9	17.8	754
R10	**Myf-5**	0	2.8	25.0	16.7	8.3	25.0	22.2	36
	Sham	2.3	4.5	13.6	2.3	9.1	29.5	38.6	44
	Normal	3.1	2.5	11.1	5.6	16.7	36.4	24.7	162
Total	**Myf-5**	1.9	1.6	9.6	6.4	18.7	17.7	44.1	374
	Sham	4.4	1.3	19.3	4.2	20.0	18.2	32.5	455
	Normal	2.4	1.7	15.8	5.9	29.1	17.2	27.8	2153

Fiber type distribution within the experimental groups in EDL of different individuals. Myf-5 transfected EDL in rat 2, and sham transfected EDL in rat 6 did not have any transfected fibers. EDL muscles from rat 3 and 5 did not have any transfected fibers, and are therefore left out.

5.5.2 Fiber type frequencies (%) in soleus of individual rats

		1	1/2a	2a	Total fibers
R1	**Myf-5**	97.7	1.1	1.1	87
	Sham	89.1	10.9	0	55
	Normal	95.9	2.6	1.4	345
R2	**Myf-5**	91.4	2.9	5.7	70
	Sham	98.1	1.9	0	108
	Normal	94.9	2.5	2.5	356
R3	**Myf-5**	-	-	-	0
	Sham	76.9	23.1	0	13
	Normal	96.2	3.8	0	26
R4	**Myf-5**	67.6	23.5	8.8	34
	Sham	100.0	0	0	1
	Normal	91.3	3.2	5.6	126
R5	**Myf-5**	100.0	0	0	117
	Sham	100.0	0	0	12
	Normal	99.6	0	0.4	258
R6	**Myf-5**	100.0	0	0	2
	Sham	76.9	9.2	13.9	295
	Normal	84.3	4.3	11.4	1 191
R7	**Myf-5**	90.9	4.5	4.5	22
	Sham	96.4	3.6	0	28
	Normal	98.0	1.0	1.0	100
R8	**Myf-5**	100.0	0	0	173
	Sham	-	-	-	0
	Normal	99.7	0.3	0	346
R9	**Myf-5**	96.2	3.8	0	132
	Sham	97.1	2.9	0	139
	Normal	96.7	3.3	0	540
R10	**Myf-5**	86.9	8.1	5.1	198
	Sham	86.3	9.5	4.2	95
	Normal	93.4	2.3	4.2	686
Total	**Myf-5**	93.8	4.0	2.3	835
	Sham	87.0	7.0	6.0	746
	Normal	92.5	2.8	4.7	3974

Fiber type distribution within the experimental groups in soleus of individual rats. Myf-5 transfected soleus in rat 3, and sham transfected soleus in rat 8 did not have any transfected fibers.

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