RNA Interference:
A New Tool to Study Gene Functions
in Adult Mammalian Muscle *in vivo*

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meine Eltern

und für

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Contents

Abbreviation

4E-BP	eIF-4E binding protein
AChR	acetylcholine receptor
ARIA	AChR inducing activity
AVO1 or 3	adheres voraciously to TOR2 no. 1 or 3
CD4	cluster of differentiation 4
dsRNA	double-stranded RNA
eIF-4E	eukaryotic initiation factor 4E
GFP	green fluorescent protein
mAVO3	mammalian adheres voraciously to TOR2 no. 3
miRNA	micro RNA
mLST8	mammalian lethal with sec-thirteen 8
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MuSK	muscle-specific receptor tyrosine kinase
N-CAM	neural cell adhesion molecule
NLS_GFP	nuclear localization signal fused to green fluorescent protein
NMJ	neuromuscular junction
nt	nucleotide
PI3K	phosphatidylinositol 3-kinase
PIKK	phosphatidylinositol kinase-related protein kinase
PKD1	3-phosphoinositide-dependent protein kinase-1
PP2A	protein phosphatase 2A
PTGS	post-transcriptional gene silencing
RdRP	RNA-dependent RNA polymerase
RISC	RNA-dependent silencing complex
Rheb	Ras homolog enriched in brain
RNAi	RNA interference

shRNA	small hairpin RNA
S6K	ribosomal S6 protein kinase
SGCA	sarcoglycan α
SIN1	sty1 interactor
siRNA	small interfering RNA
TSC1 or 2	tuberous sclerosis complex 1 (harmartin) or 2 (tuberin)

Summary

RNA interference (RNAi) is a powerful method for sequence-specific post-transcriptional gene silencing (PTGS), which allows rapid survey of gene functions using double-stranded RNA (dsRNA). At the time when we started this work, RNAi was a recently developed tool that had been successfully applied to many organisms, in particular *C. elegans* and *Drosophila*, but not to any mammalian system. It was generally doubted that RNAi would also work in mammals *in vivo*, because the introduction of dsRNA can induce general shutdown of translation and apoptosis in several mammalian cell types. One excellent model system for investigating this open question is the nerve-muscle synapse known as the neuromuscular junction (NMJ).

Characteristic for the NMJ is the precise apposition of the neurotransmitter release machinery on the nerve terminal side and the neurotransmitter receptors on the muscle fiber membrane. At least two mechanisms underlie the formation and maintenance of a postsynaptic apparatus on the muscle fiber membrane. Both mechanisms are triggered by the heparan sulfate proteoglycan agrin, which is released by the motor neuron. First, neural agrin activates all the cellular mechanisms necessary to assemble a fully functional postsynaptic structure including aggregates of acetylcholine receptors (AChRs). Besides this redistribution of preexisting molecules, agrin signaling restricts the transcription of postsynaptic proteins to myonuclei located in the NMJ. Still little is known about the agrin signaling cascade. Therefore, once RNAi could be developed for mammals system, it will in turn provide a unique tool to address the role of newly identified genes in the postsynaptic differentiation, since there are no tools available for the fast and reliable perturbation of gene function *in vivo*.

In the first part of this work, we investigated the potential of RNAi in perturbing the formation and stability of postsynaptic structures in adult muscle *in vivo* (chapter 2 and 3). First, we used the experimental paradigm where neural agrin expressed in non-junctional regions of rat soleus muscle induces formation of ectopic AChR aggregates. Knockout experiments have shown that this agrin activity requires the receptor tyrosine

kinase MuSK and the AChR-associated scaffolding molecule rapsyn, but not the cytoskeletal proteins sarcoglycan α (SGCA) and utrophin. In our experiments, we show that co-injection of dsRNAs derived from MuSK or rapsyn perturbed agrin-induced formation of ectopic AChR aggregates, while dsRNAs derived from SGCA or utrophin had no significant effect. In a further step, we used RNAi to study the role of MuSK at adult NMJs. Here, the electroporation of plasmids encoding short hairpin-based 21-bp small interfering RNAs (siRNAs) or long hairpin dsRNAs, which allow global and sustained perturbation of MuSK expression, leads to the disassembly of NMJs in adult mice. These results are consistent with the finding that auto-antibodies to MuSK, which also lower the amount of MuSK protein, cause severe forms of myasthenia gravis. In summary, these results demonstrate for the first time the effectiveness of long dsRNA as well as siRNA in silencing endogenous genes in adult mammalian muscle *in vivo* and they provide strong evidence that continuous expression MuSK is required to maintain the NMJ.

The second part of this work aimed to establishing RNAi in adult muscle to study the role of newly identified genes in the development of the NMJ and in the growth of muscle fibers (chapter 4 and appendix). First, we used RNAi to perturb neural agrin-induced formation of ectopic AChR aggregates on mouse soleus muscle. We show that electroporation of plasmids encoding short hairpin- derived siRNA for MuSK leads to the perturbation of ectopic AChR aggregation, regardless whether agrin expression vector or recombinant protein was applied to innervated or denervated muscles. These results clearly show the reliability of RNAi in adult muscle *in vivo* and therefore set the stage for experiments aimed to study the function of genes, whose expression is altered during the formation postsynaptic structures. A protocol was established to identify functional siRNA target sites in several genes. Plasmids were designed that encoded short hairpin RNAs (shRNAs) derived from different putative effectors of the mammalian target of rapamycin (mTOR) signaling pathway. For some candidate effectors, electroporation of the corresponding plasmids into mouse soleus muscle leads to altered muscle fiber size. These preliminary results are consistent with several reported findings, which indicate that the mTOR signaling pathway is a central controller of muscle fiber atrophy and hypertrophy. The efficiently induced RNAi in those experiments demonstrates that our

protocol is useful for identifying siRNA targets. In summary, these results demonstrate that we have successfully established RNAi as a fast and reliable gene knockdown method in muscle fibers of mammals. This method will be important for future investigation of gene functions in adult mammalian muscle *in vivo*.

Chapter 1

General Introduction

Gene targeting by homologous recombination is commonly used to determine gene function in mammals, but this process is costly, time-consuming and where the gene in question plays a dual role in development, it can lead to premature death of the animals and an inability to study further gene functions. In addition, many organisms are not amenable to such methods. Alternatively, the function of many genes can be determined by ribozyme and antisense technologies. Although successful in some situations, these technologies have proven difficult to apply universally (Sullenger and Gilboa, 2002; Kitabwalla and Ruprecht, 2002; Dornburg and Pomerantz, 2000). At the time this work was started, RNAi was a recently developed method for gene silencing. This tool had allowed rapid and reliable survey of gene functions in many organisms, in particular *C. elegans* and *Drosophila* (reviewed in Dykxhoorn *et al.*, 2003). These significant advantages over the current methods had made RNAi a powerful tool. However, in mice, successful use of RNAi had been only observed in oocytes and early embryos, but not in adult animals (Wianny and Zernicka-Goetz, 2000; Svoboda *et al.*, 2000). To investigate the virtue of RNAi in mammals *in vivo*, the NMJ provided an excellent model system. Next to the large size, the relative simplicity and the simple accessibility of the nerve-muscle synapse, the easy way to localize the postsynapse using a snake toxin - α-bungarotoxin - which binds specifically to AChRs, and the large size of the syncytial muscle fibers are the most appreciated experimental advantages of this system. In this work, we used these advantages of the NMJ to demonstrate for the first time the effectiveness of RNAi in silencing endogenous genes in adult mammalian muscle *in vivo.* This finding allowed us to use RNAi for knocking down gene function in muscle fiber.

The first part of this chapter will describe the gene silencing phenomenon RNAi from its discovery and mechanism to the recent developments that have made RNAi a unique tool for gene function studies. In context to the investigation of the virtue of RNAi in adult mammalian muscles, the second part will give an introduction into the structural and molecular composition of the NMJ, the gene expression at the postsynaptic part of the NMJ and the putative function of the mTOR complex in muscle fiber.

1.1 What is RNAi?

RNAi is the sequence-specific post-transcriptional gene silencing. This process is induced by double stranded RNA (dsRNA) that directs destruction to the homologous messenger RNA (mRNA), resulting in the shutdown of the target gene expression, which is also known as gene knockdown. In 1998, RNAi was first discovered in *C. elegans* as a response to injection of dsRNA (Fire *et al.*, 1998). This phenomenon has been linked to many previously described homology-dependent gene silencing mechanisms such as co-suppression, transgene-induced silencing and RNA-mediated virus resistance in plants (Napoli *et al.*, 1990; van der Krol *et al.*, 1990; Lindbo and Dougherty, 1992) and quelling in *Neurospora* (Romano and Macino, 1992). Genetic and biochemical studies have shown that all these phenomena share mechanistic similarities, and that the biological pathways underlying dsRNA-induced gene knockdown may exist in many eukaryotic organisms. Indeed, the experimental introduction of dsRNA in a variety of organisms, in particular in *Drosophila* (Kennerdell and Carthew, 1998; Kennerdell and Carthew, 2000), zebrafish (Li *et al.*, 2000), *Xenopus* embryos (Zhou *et al.*, 2002), mouse oocytes and embryos (Wianny and Zernicka-Goetz, 2000; Svoboda *et al.*, 2000), and chicken embryos (Pekarik *et al.*, 2003. Furthermore, the RNA silencing pathways in different organisms require a set of related proteins, which are absent in archea and prokaryotes, suggesting that the common aspects of the pathways are quite ancient (Zamore, 2002).

1.2. How does RNAi work?

The natural function of RNA silencing in plants is very likely the protection against virus infection and uncontrolled transposon mobilization (Ahlquist, 2002). Further, an animal virus encoding a suppressor of RNAi has been reported (Li *et al.*, 2002), and *C. elegans*, which lost function of genes required for RNAi, show activation of multiple transposable elements in the germline (Ketting *et al.*, 1999). These findings indicate that RNAi may have an anti-viral and anti-transposon function in animal as well (reviewed in Plasterk, 2002 and Zamore, 2002). Here, the 'classical' pathway of the RNAi machinery guarantees this defense function (Fig. 1): Destruction of messenger RNA (mRNA) exposed to the homologous dsRNA. In detail, cytoplasmatical long dsRNA can derived from exogenous sources, viral infection, transposon activity or RNA synthesis by endogenous RNA-dependent RNA polymerases (RdRPs) using 'aberrant' transcripts of highly expressed loci as templates (Grishok *et al.*, 2000). These dsRNAs are cleaved by Dicer, a RNase III family member, into small interfering RNA (siRNA) in an ATP-dependent reaction (Bernstein *et al.*, 2001; Nykanen *et al.*, 2001). These siRNAs are 21 to 23 nucleotide (nt) RNA duplexes with phosphorylated 5' ends and with two-nucleotides overhanging at the non-phosphorylated 3' ends (Elbashir *et al.*, 2001a; Elbashir *et al.*, 2001b). After their incorporation into the RNA-dependent silencing complex (RISC), siRNAs are unwound in an ATP-dependent step (Hammond *et al.*, 2001; Hammond *et al.*, 2000; Nykanen *et al.*, 2001). The resulting single-stranded antisense strand then guides RISC to mRNA that have a complementary sequence, and the endonuclease in the RISC cleaves the target mRNA. Alternatively, in organisms with RdRP activity, such as in *C. elegans*, an additional branch of the pathway with siRNA amplification is suggested (Sijen *et al.*, 2001). Here, the binding of the antisense strand of the siRNA to the target mRNA would lead to activation of the RdRP, which probably uses the mRNA as a transcription template to synthesize a new dsRNA. Like the initial step, this dsRNA would then be cleaved by Dicer to generate a new crop of siRNAs, resulting in amplification of the

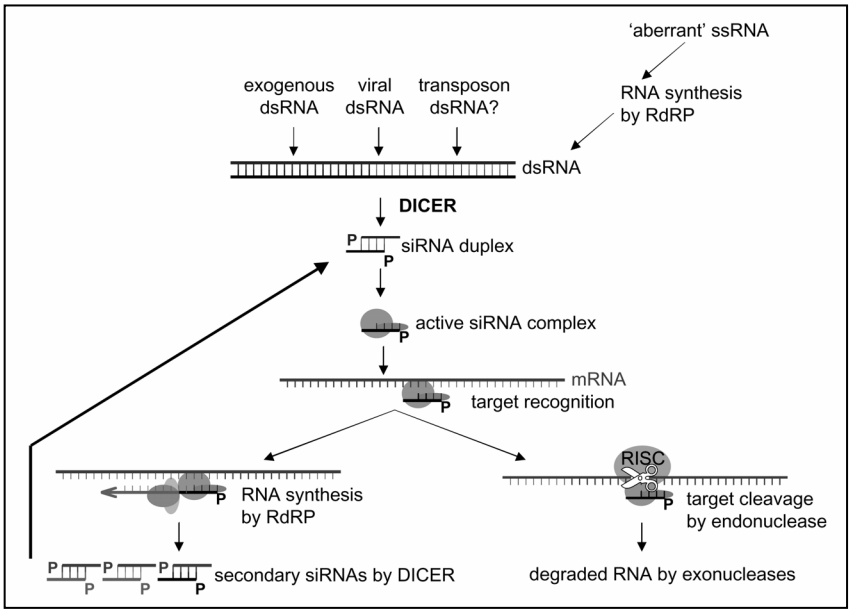

Figure 1: Overview of the molecular steps in the RNAi pathway. From Plasterk, 2002.

silencing signal. Altogether, both variants of the pathway lead to sequence-specific gene silencing mediated by destruction of the target mRNA.

Next to this dsRNA-induced defense operating at the post-transcriptional level, the RNAi machinery may modulate gene expression in animals through at least two additional mechanisms. First, several studies have suggested that in *Drosophila* and *C. elegans* the RNAi machinery may affect gene expression at the level of chromatin structure (Pal-Bhadra *et al.*, 1997; Pal-Bhadra *et al.*, 2002; Tabara *et al.*, 1999; Dudley *et al.*, 2002). Finally, in *C. elegans,* endogenously encoded inducers of the RNAi machinery, called micro RNA, operate at the level of translation (Wightman *et al.*, 1993). The conservation of at least some targets of these silencing inducers in other organisms (Reinhart *et al.*, 2000) indicates that RNAi may be a further common mechanism regulating the expression of cellular genes.

1.3. Does RNAi work in mammals?

RNAi induced by the introduction of long dsRNA has been rapidly established in various organisms. It provides a fast and reliable method to study function of genes. In vertebrates, dsRNA-mediated RNAi has been observed in a few cultured mammalian cells (Billy *et al.*, 2001; Gan *et al.*, 2002; Elbashir *et al.*, 2001a), in mouse oocytes (Svoboda *et al.*, 2000) and in mouse and chicken embryos during early development. (Wianny and Zernicka-Goetz, 2000; Pekarik *et al.*, 2003). However, its applicability is limited, since the introduction of dsRNA longer than 30 nt induces a nonspecific interferon response (Paddison *et al.*, 2002b; Stark *et al.*, 1998). Interferon triggers the general degradation of mRNA and the global shutdown of translation, leading to cell apoptosis. This non-specific response can be circumvented by introduction of siRNA that maintain their capability to induce RNAi (Elbashir *et al.*, 2001a; Dykxhoorn *et al.*, 2003). This finding has led to the widespread application of siRNA to study gene functions in cultured mammalian cells and has recently also been show to work in adult animals *in vivo* (Lewis *et al.*, 2002; McCaffrey *et al.*, 2002; Song *et al.*, 2003; reviewed in Dykxhoorn *et al.*, 2003). Unlike in *C. elegans* where siRNAs can be amplified, there is no indication of signal amplification in mammals (see above), nor have orthologous genes for RdRPs been found in the human genome (Zamore, 2002). Therefore, siRNA-induced RNAi in mammals is only of transient nature and not suited for long-term studies. To overcome this limitation, DNA-vector mediated expression of small hairpin RNA (shRNA), which is predominantly driven by the U6 or H1 RNA polymerase III promoter, has been established. shRNA is converted into siRNA *in vivo* and triggers efficiently gene silencing (Brummelkamp *et al.*, 2002; McManus *et al.*, 2002; Paddison *et al.*, 2002a; Paddison *et al.*, 2002b; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002). In a further advancement of the technique, plasmid vectors have been replaced by retrovirus vectors (reviewed in Dykxhoorn *et al.*, 2003). Using this system, siRNAs are efficiently delivered into cell lines, into which plasmid transfection is difficult or does not work at all. With the use of vectors encoding siRNA, it is now possible to generate transgenic mammals that can stably

silence gene expression, even in those animals that are not amenable to homology-directed gene targeting methods (Gordon, 1993; Carmell *et al.*, 2003; Hasuwa *et al.*, 2002; Lois *et al.*, 2002; Tiscornia *et al.*, 2003). In such knockdown animals, RNAi induced by siRNA is stable, heritable and functions in all developmental stages and in all the cell and tissue types tested so far.

2.1. The neuromuscular junction

The neuromuscular junction (NMJ) is the site of communication between nerve and muscle fiber. This nerve-muscle synapse, responsible for quick and accurate transduction of electrical signals from motor neurons to muscle fibers, consists of three cell types: motor neuron, Schwann cell and muscle fiber (reviewed in Engel, 1994; Ogata, 1988; Sanes and Lichtman, 1999; Sanes and Lichtman, 2001) (Fig. 2). At the presynapse, the nerve terminal is insulated from the environment by the capping Schwann cell. Here, the electrical signal is transformed into a chemical signal by release of the neurotransmitter acetylcholine into the synaptic cleft, the space between the nerve terminal and the muscle fiber. At the postsynaptic muscle membrane, nicotinic AChRs open upon binding of acetylcholine and allow the influx of cations. Voltage-gated Na^+ channels open in response to the resulting depolarization and generate an action potential, leading to a muscle contraction due to release of Ca^{2+} from the sarcoplasmic reticulum (reviewed in Franzini-Amstrong, 1994; Horowitz, 1994). Muscle fibers are long, tube-like syncytial cells able to contract along their longitudinal axis. To ensure movement generated by muscle tension and contraction, muscle fibers are attached at both ends to bone via tendons and the cell's cytoskeleton is linked to the surrounding basement membrane (Anastasi *et al.*, 1998; Pardo *et al.*, 1983). Perturbations of this linkage often lead to muscle dystrophies.

The synaptic portion of all three cell types are highly specialized, containing high concentrations of organelles and molecules found at low concentration extrasynaptically.

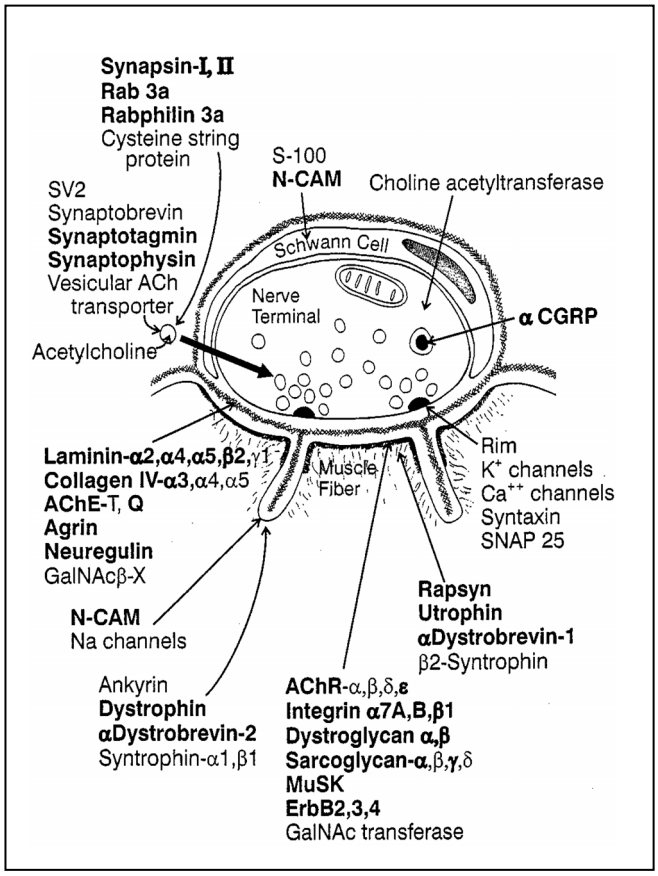

Figure 2: Structure and molecular composition of the NMJ. In boldface: proteins for which knockout mice have been generated. From Sanes and Lichtman, 1999.

In particular, the postsynaptic part of the muscle fiber membrane contains an extremely high concentration of AChRs (reviewed in Salpeter and Loring, 1985) allowing it to respond quickly and reliably to acetylcholine release from the opposed neural terminal. Additionally, signaling molecules such as neuregulin and its receptors erbB2, erbB3 and erbB4 (Moscoso *et al.*, 1995; Zhu *et al.*, 1995), the muscle-specific tyrosine kinase MuSK (Valenzuela *et al.*, 1995) as well as the α1, α7A and α7B integrins are enriched at the postsynaptic membrane (Martin *et al.*, 1996).

Another feature of the postsynaptic membrane is the junction fold: deep invaginations opposing active zones of the nerve terminal, with Na^+ channels and the neural cell adhesion molecule (N-CAM) concentrated in the depths of the folds, and AChRs at the crests of the folds. Intracellulary, rapsyn, utrophin and α-dystrobrevin-1 are colocalized with AChRs (Caldwell, 2000; Covault and Sanes, 1986; Flucher and Daniels, 1989), while ankyrin, α-dystrobrevin-2 and dystrophin are concentrated at the bottom of

the junction folds (Covault and Sanes, 1986; Flucher and Daniels, 1989; Peters *et al.*, 1998; Sealock *et al.*, 1984; Wood and Slater, 1998).

The basement membrane that runs through the synaptic cleft and invades the junction folds has the most components identical to those found outside of the NMJ. Despite this similarity, distinct isoforms of many molecules are found at the NMJ, in particular specific laminin chains like laminin-α4, α5 and β2 (Patton *et al.*, 1997), a collagen-tailed form of acetylcholinesterase (Krejci *et al.*, 1997) and as described in further detail below, agrin.

2.2. The core program agrin - MuSK - rapsyn - AChR

The formation of the NMJ requires the intricate interaction of signals derived from the innervating motor neurons and the target muscle fibers (reviewed in Sanes and Lichtman, 2001). Among the earliest signs of postsynaptic differentiation is the aggregation of AChRs beneath the innervating nerve terminal. Several lines of evidence demonstrate that the nerve-derived extracellular matrix protein agrin is necessary and sufficient to trigger a signaling cascade resulting in the assembly of the entire postsynaptic apparatus (McMahan, 1990; Bezakova and Ruegg, 2003). In particular, in agrin-deficient mice, postsynaptic differentiation is profoundly impaired (Gautam *et al.*, 1996). Conversely, neural agrin deposited on the basement membrane by secretion upon intracellular expression plasmids injection or by intramuscular recombinant protein injection induces ectopic postsynaptic differentiation in extrasynaptic region of fully innervated muscle fibers (Bezakova *et al.*, 2001a; Jones *et al.*, 1997; Meier *et al.*, 1997; Cohen *et al.*, 1997; Rimer *et al.*, 1997).

Agrin is a heparan sulfate proteoglycan (Denzer *et al.*, 1995; Tsen *et al.*, 1995) that is released from the nerve terminal and incorporated in the basement membrane of the NMJ by binding to laminin (Cohen and Godfrey, 1992; Denzer *et al.*, 1997; Reist *et al.*, 1992). Alternative mRNA splicing at the A/y and the B/z sites close to the 3' end

results in agrin isoforms that differ in tissue distribution and postsynapse induction activity (reviewed in Bezakova and Ruegg, 2003). Nerve-derived isoforms contain additional amino acids at the B/z sites and are active in triggering postsynaptic differentiation, whereas those derived from non-neuronal tissue lacking the essential inserts at the B/z sites have modest activity. Currently, the function of this so called muscle agrin is unknown.

Downstream of the agrin signaling pathway, two molecules have been identified: the transmembrane muscle-specific receptor tyrosine kinase, MuSK and the cytoplasmic adaptor molecule, rapsyn (Gautam *et al.*, 1995). Both MuSK and rapsyn are essential for the formation of postsynaptic specialization. The findings that MuSK colocalizes with AChRs in the postsynaptic membrane (Valenzuela *et al.*, 1995), that MuSK becomes rapidly phosphorylated upon addition of neural agrin to cultured myotubes (Glass *et al.*, 1996) and that MuSK-deficient mice are phenotypically similar to agrin knockout mice (DeChiara *et al.*, 1996) indicate that MuSK is very likely part of the agrin receptor, even though it does not directly interact with agrin. In addition, based on the discovery that auto-antibodies to MuSK cause myasthenia gravis, it has been suggested that MuSK is necessary to maintain the integrity of postsynaptic structures (Hoch *et al.*, 2001). Downstream of MuSK, the function of rapsyn is critical for the aggregation of postsynaptic proteins. Rapsyn and AChRs are exactly colocalized at the crests of the synaptic fold and are present in a 1:1stoichiometry (Burden *et al.*, 1983; Noakes *et al.*, 1993; Sealock *et al.*, 1984). In rapsyn knockout mice, all synaptic proteins except MuSK fail to assemble in the postsynaptic membrane (Apel *et al.*, 1997; Gautam *et al.*, 1995), indicating that MuSK is activated and clustered by agrin in a primary scaffold and rapsyn is important to recruit other synaptic components like AChR to that scaffold.

2.3. Gene expression at the neuromuscular junction

In addition to the aggregation of AChRs and other postsynaptic proteins, a second important process contributes to NMJ formation: the targeting gene expression to synaptic sites. In innervated muscle, transcription of AChR subunits and other synaptic genes is far higher in the myonuclei directly beneath the NMJ than all other extrasynaptic myonuclei (Duclert and Changeux, 1995). At early non-innervated stages of development, AChR subunits transcripts are distributed over the entire muscle fiber. Soon after the initial contact between nerve terminal and muscle fibers, transcription of AChR subunits is restricted to the postsynaptic site and repressed in extrajunctional regions (Piette *et al.*, 1993). The expression of the AChRε subunit follows a different pattern. It is switched on only around birth (Mishina *et al.*, 1986; Witzemann *et al.*, 1987) and is restricted to synaptic sites from the onset (Brenner *et al.*, 1990). In addition, ectopic expression of neural agrin in extrasynaptic regions of innervated muscle was shown to induce the transcription of the AChRε unit (Jones *et al.*, 1997).

The transcription of AChR subunits is the first example for reinforced expression at the NMJ. Expression of additional synaptic genes may be similarly regulated. Neuregulin, which was isolated as factor with AChR inducing activity (ARIA) from brain tissues, induces expression of AChRs, but failed to cluster them (Usdin and Fischbach, 1986). Neuregulin and its receptors, erbB2, erbB3 and erbB4 are accumulated at the NMJ (Goodearl *et al.*, 1995; Jo *et al.*, 1995; Moscoso *et al.*, 1995; Zhu *et al.*, 1995). Thus, alterations in postsynaptic gene expression are suggested to be an indirect effect of agrin affecting the activation of the neuregulin/erbB receptor signaling pathway (Meier *et al.*, 1998; Rimer *et al.*, 1998; Fischbach and Rosen, 1997).

Electrical activity plays a central role in the repression of extrasynaptic AChR expression during development and in the maintenance of the repressed state in the adult. This repression is overcome by denervation, resulting in restoration of the transcription of AChR subunit genes in extrasynaptic myonuclei (Merlie *et al.*, 1984; Tsay and Schmidt, 1989). Moreover, direct electrical stimulation of denervated muscle prevents

or reverses the effects of denervation (Goldman *et al.*, 1988). However, blockade of synaptic transmission by Na^+ channel blocker tetrodotoxin (TTX) has much lower effect on AChR expression (Witzemann *et al.*, 1991). This indicates that additional regulatory mechanisms independent of electrical activity contribute to the repression of the extrasynaptic AChR genes. Thus, the transcription of postsynaptic genes in innervated muscle fibers is regulated by different mechanisms, parallely enhanced at the NMJ and repressed at extrasynaptic sites. Stimulation of postsynaptic gene expression is likely warranted by provision of agrin to muscle fibers, whereas repression can be mainly achieved by electrical activity.

In the past, evidence has been provided that the principal mechanisms of synaptic transmission are identical at the NMJ and CNS synapses. It is very likely that the molecular principals govern the formation and the maintenance of synapses are also similar. Therefore, the developmental and the preserving mechanisms identified at the NMJ could be relevant to other chemical synapses in the CNS (reviewed in Sanes and Lichtman, 1999).

2.4. The mTOR signalling pathway and muscle growth

Cell growth is the fundamental biological process whereby cells accumulate mass and is a crucial determinant of the characteristic sizes of organs and organisms (Conlon and Raff, 1999; Dixon and Fordham-Skelton, 1998; Gomer, 2001; Johnston and Gallant, 2002; Stocker and Hafen, 2000). The mTOR - mammalian ‘target of rapamycin’ - signaling pathway is emerging as a critical regulator of growth in proliferating and non-proliferating cells, such as neurons and muscle fibers, in response to nutrients, hormones and growth factors (reviewed in Jacinto and Hall, 2003).

The key component of the pathway, mTOR, was discovered in assessments of the mechanism of action of rapamycin (Brown *et al.*, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995), which is clinically applied as immunosuppressant and anti-cancer drug with

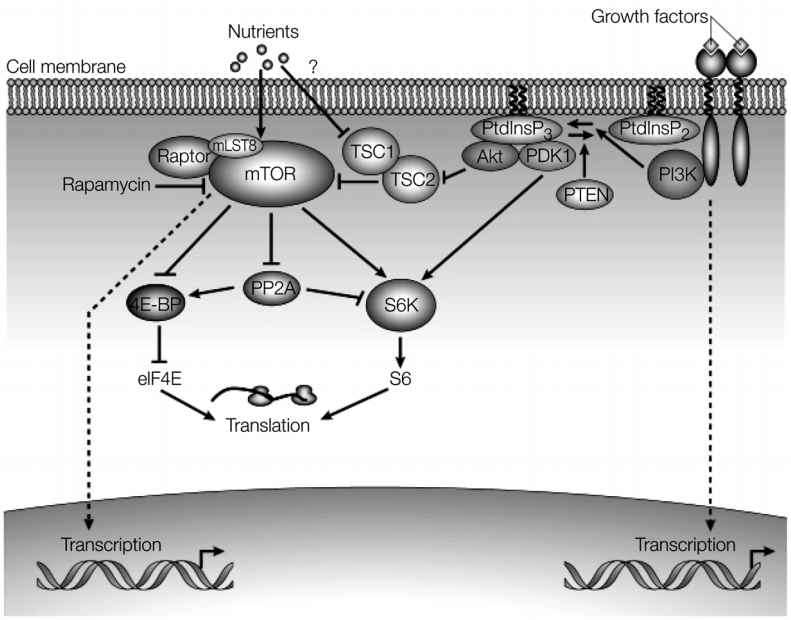

Figure 3: Cell growth initiated by signals from nutrients and growth factors via the mTOR signaling pathway. See text for details. From Jacinto and Hall, 2003.

antiproliferative and antifungal activity (Saunders *et al.*, 2001; Vogt, 2001). mTOR is a member of the phosphatidylinositol kinase-related protein kinase (PIKK) family (Keith and Schreiber, 1995) and is highly conserved in many organisms (Jacinto and Hall, 2003). It controls directly, or indirectly via inhibition of protein phosphatase 2A (PP2A), the phosphorylation of at least two regulators of protein synthesis (Figure 3): the translation activator S6 protein kinase (S6K) and the eIF-4E binding protein (4E-BP), an inhibitor of translation initiation (Brunn *et al.*, 1997; Burnett *et al.*, 1998; Isotani *et al.*, 1999). Phosphorylation of S6K and 4E-BP promotes translation via the ribosomal protein S6 and the eukaryotic initiation factor 4E (eIF-4E), respectively.

In mammalian cells, growth is stimulated by a combination of growth factors and nutrients. The mTOR pathway mediates growth factor signaling through the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B)/3-phosphoinositide-dependent protein kinase-1 (PKD1) axis, which is also part of the insulin signaling pathway (Jacinto and Hall, 2003). Activated Akt phosphorylates the tuberous sclerosis (TSC) complex and prevents it to inactivate mTOR (Gao *et al.*, 2002; Inoki *et al.*, 2002; Potter *et al.*, 2002; Tee *et al.*, 2002). The TSC complex consists of TSC1 and TSC2. Mutation in either of both leads to a tumor-prone syndrome (Sparagana and Roach, 2000). Recently, several reports showed that the inhibitory effect of the TSC complex on mTOR is mediated by the

small G protein, Rheb (Ras homolog enriched in brain) (e.g. Garami *et al.*, 2003; Zhang *et al.*, 2003; Saucedo *et al.*, 2003). The mTOR pathway is highly sensitive to the levels of nutrients, such as amino acids (Hara *et al.*, 1998) and glucose (Dennis *et al.*, 2001; Kim *et al.*, 2002), but the mechanisms of how nutrients activate the mTOR complex are still unknown. Raptor (regulator associated protein of TOR) and mLST8 (mammalian lethal with sec-thirteen 8) are stimulators of mTOR kinase activity (Kim *et al.*, 2003; Kim *et al.*, 2002). It has been proposed that raptor functions as a scaffold protein that links mTOR to S6K and 4E-BP (Hara *et al.*, 2002), and that the interaction between mTOR and raptor is nutrient sensitive (Kim *et al.*, 2002) and its formation requires mLST8 (Kim *et al.*, 2003). Further investigations are necessary to precisely determine the role of raptor and mLST8 and probably TSC1 and TSC2 in regulating mTOR activity in response to nutrients. In summary, as phosphorylation of S6K and 4E-BP in response to both insulin and nutrients is mediated by mTOR, it integrates nutrients and insulin signaling to control cell growth.

Skeletal muscle mass is controlled by several factors including insulin, amino acids and the degree of muscle activity (Baar and Esser, 1999; Bodine *et al.*, 2001; Hornberger *et al.*, 2001; Shah *et al.*, 2000). Loss of muscle mass is a hallmark of diabetes and is stopped by insulin restoration, which increases muscle protein synthesis (Charlton and Nair, 1998; Price *et al.*, 1996; Flaim *et al.*, 1980; Grzelkowska *et al.*, 1999; Pain and Garlick, 1974). Certain amino acids, especially branched chain amino acids stimulate protein synthesis in muscles (Shah *et al.*, 2000). The effects of both insulin and amino acids involve enhanced translation mediated by increasing the mTOR-controlled phosphorylation of S6K and 4E-BP (Azpiazu *et al.*, 1996). Increasing the workload on a muscle promotes hypertrophy (Roy *et al.*, 1997), and unloading a muscle leads to atrophy (Thomason *et al.*, 1987). Muscle hypertrophy requires an increase in the rate of protein synthesis and signaling by mTOR and the insulin-growth factor 1 (IGF1) pathways (Rommel *et al.*, 2001; Bodine *et al.*, 2001; Musaro *et al.*, 1999; Semsarian *et al.*, 1999). In addition to the prominent role of the calcineurin-nuclear factor of activated T cells (NFAT) pathway, Akt and mTOR play an important role in muscle hypertrophy. Upon stimulation by IGF1, Akt promotes translation by the phosphorylation of the mTOR targets S6K and 4E-BP (Rommel *et al.*, 2001; Bodine *et al.*, 2001; Pallafacchina *et al.*, 2002) and directly

phosphorylates mTOR (Reynolds *et al.*, 2002). Specific overexpression of activated Akt in the heart of transgenic mice results in enlarged cardiomyocytes, which are rapamycin-sensitive, indicating that the effect is mediated by mTOR (Shioi *et al.*, 2002). Taken together, the muscle cell growth is critically regulated by the mTOR signaling pathway.

Chapter 2

Inhibition of synapse assembly in mammalian muscle *in vivo* by RNA interference

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All work presented here was done by Xian Chu Kong except taking confocal pictures and preparation of expression constructs for electroporation experiments, which were done by Patrizia Barzaghi.

Abstract

The formation of the vertebrate neuromuscular junction (NMJ) requires the receptor tyrosine kinase MuSK and the adaptor molecule rapsyn. Here, we report that the phenotypes of mice deficient of these two molecules can be reproduced by RNA interference (RNAi) in rat muscle *in vivo*. Specifically, double-stranded RNA (dsRNA) targeting MuSK and rapsyn inhibited the formation of the NMJ in rat muscle fibers *in vivo*, while dsRNA targeting non-essential proteins did not have any effect. Moreover, plasmids encoding short hairpin RNA (shRNA) corresponding to MuSK induced the disassembly of existing NMJs. These results thus demonstrate for the first time the functionality of dsRNA in silencing endogenous genes in adult mammalian muscle *in vivo.* Moreover, they show that MuSK is also required for the maintenance of the NMJ, offering a mechanistic explanation for the myasthenia gravis caused by auto-antibodies to MuSK.

Introduction

The molecular mechanisms responsible for the formation of synapses are best understood at the NMJ (reviewed in Sanes and Lichtman, 2001). Several lines of evidence demonstrate that nerve-derived agrin is required and sufficient for the assembly of the entire postsynaptic apparatus (Bezakova and Ruegg, 2003). Moreover, NMJ formation requires the muscle-specific receptor tyrosine kinase MuSK (DeChiara *et al.*, 1996) and the cytoplasmic adaptor molecule rapsyn (Gautam *et al.*, 1995). All these data were generated by genetically engineering mice that are deficient of the particular protein.

A promising technique that may allow a more straightforward and faster assessment of gene function *in vivo* than current knockout techniques might be RNAi. This technique employs long dsRNA or short, 21-23 bp-long short interfering RNA (siRNA) that trigger specific silencing of gene expression (Tijsterman *et al.*, 2002). However, long dsRNA has not successfully been applied to mammals because it seems to induce a general shutdown of translation and apoptosis of the cell (Paddison *et al.*, 2002b). This unspecific reaction is not observed with siRNA (Elbashir *et al.*, 2001a; McCaffrey *et al.*, 2002; Lewis *et al.*, 2002). The disadvantage of siRNA is that only some siRNAs are efficient in silencing gene transcription (McManus and Sharp, 2002) and that the effect of siRNA lasts only for a few days. In a further advancement of the technique, siRNA has been replaced by plasmids encoding shRNA, what enabled prolonged and stable suppression of gene expression *in vivo* (Brummelkamp *et al.*, 2002; Yu *et al.*, 2002; Rubinson *et al.*, 2003 McCaffrey *et al.*, 2002). Here we demonstrate that long dsRNA can be used in adult muscle to perturb the function of endogenous genes and that prolonged exposure of muscle fibers to plasmids encoding shRNA for MuSK induces the disassembly of existing NMJs.

Results

Here we investigated whether dsRNA-mediated RNAi could be used to study the function of genes in the formation of postsynaptic structures in muscle fibers *in vivo*. We injected 591- to 686 bp-long dsRNA derived from different genes in conjunction with plasmids encoding neural agrin and green fluorescent protein fused to a nuclear localization signal (NLS_GFP) into non-synaptic regions of rat soleus muscle (Fig. 1A). As described previously (Cohen *et al.*, 1997; Meier *et al.*, 1997), injection of expression plasmids coding for neural agrin was sufficient to induce postsynaptic specializations in non-synaptic regions (Fig. 1A, right). These specializations are characterized by the accumulation of AChRs (red in Fig. 1A) on the surface of injected (GFP-positive; green in Fig. 1A) and neighboring non-injected muscle fibers (Fig. 1B). To test whether co-injection of dsRNA exerts any unspecific effect on protein synthesis, we injected dsRNA derived from cDNA encoding CD4 (Benoist and Mathis, 1999). As shown in Figure 1C, postsynaptic structures formed in the presence of $dsRNA_{CD4}$ were indistinguishable from controls and AChR clusters were found on injected and on neighboring muscle fibers. To test whether we could observe specific RNAi, we next co-injected dsRNA corresponding to MuSK ($dsRNA_{MuSK}$). MuSK is an essential signaling component for NMJ formation that is activated by neural agrin (Glass *et al.*, 1996). Thus, effective $dsRNA_{MuSK}$ should prevent the formation of postsynaptic specializations in response to neural agrin in the injected muscle fiber. Indeed, AChR clusters were only rarely detected on injected muscle fibers while they were readily detected on neighboring, non-injected muscle fibers (Fig. 1D). Thus, the effect of dsRNA remains restricted to the injected muscle fibers indicating that dsRNA does not cross cell boundaries in mammalian muscle, a phenomenon that has been reported in *C. elegans* (Winston *et al.*, 2002). As a further test for the specificity of the inhibitory activity of $dsRNA_{MuSK}$, we also co-injected $dsRNA_{SGCA}$ derived from α-sarcoglycan, a protein that is highly expressed in muscle fibers but is not necessary for the formation of NMJs (Duclos *et al.*, 1998). In this case, AChR clusters were formed both on injected and neighboring muscle fibers (Fig. 1E). To test the universality of the

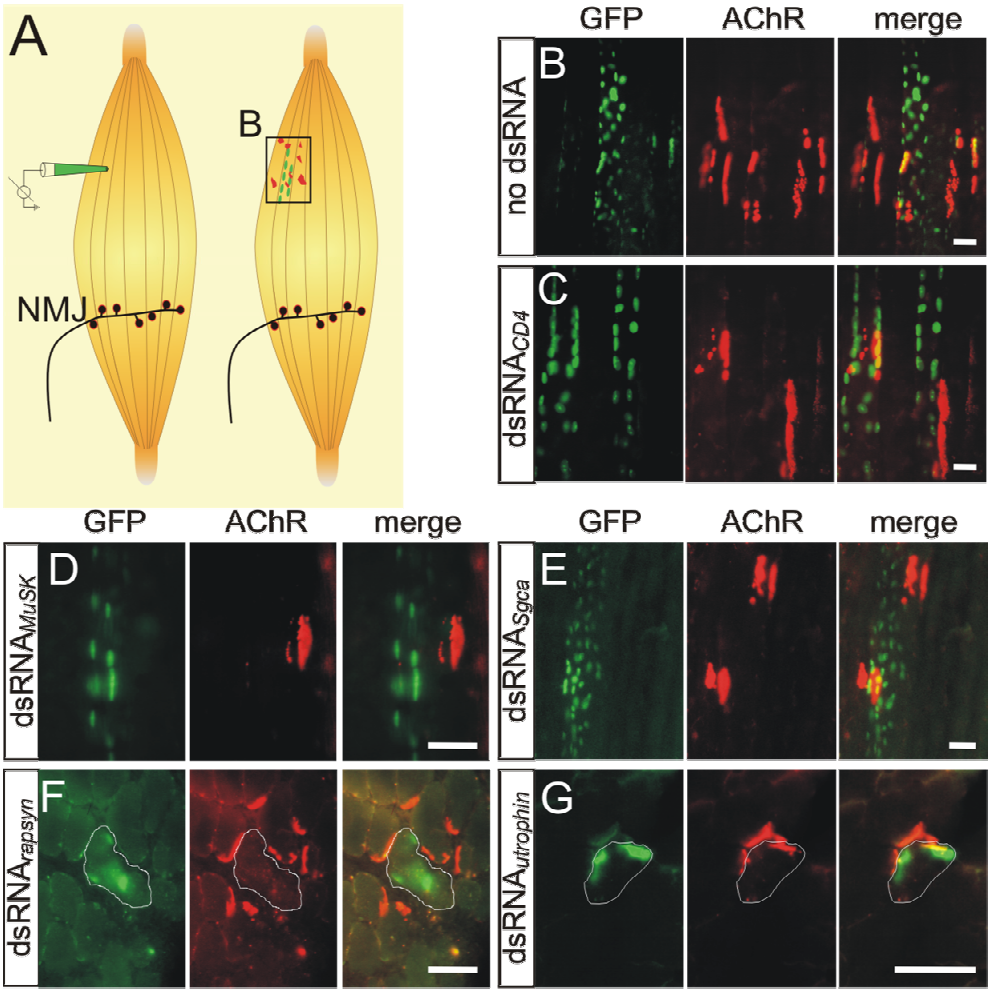

Figure 1: Inhibition of the formation of postsynaptic structures by dsRNA. **(A)** Schematic representation of the injection of cDNA constructs at non-synaptic regions of single muscle fibers of rat soleus muscle (left). Injection pipette containing expression plasmids NLS_GFP and neural agrin. dsRNA was added in RNAi experiments. Injected muscle fibers contain GFP-positive myonuclei (green) and aggregates of postsynaptic proteins including AChRs (red). The frame symbolizes the view shown in B. Postsynaptic structures formed on the surface of the injected and on nearby muscle fibers when no dsRNA **(B)** or dsRNA$_{CD4}$ **(C)** was included. Injection of dsRNA$_{MuSK}$ **(D)** prevents the formation of postsynaptic structures on injected muscle fibers but not on nearby fibers. Postsynaptic structures formed on muscle fibers injected with dsRNA$_{SGCA}$ **(E)**. Cross-section through ectopic postsynaptic structures formed in the presence of dsRNA$_{rapsyn}$ **(F)** and dsRNA$_{utrophin}$ **(G)**. Outlines in **(F)** and **(G)** indicate circumference of injected muscle fibers. Scale bars = 50 μm.

method, we also examined the effect of dsRNA$_{rapsyn}$, which was derived from rapsyn, an adaptor molecule essential for the clustering of AChRs (Gautam *et al.*, 1995). No AChR clusters were detected in dsRNA$_{rapsyn}$-containing muscle fibers while such clusters were

found on neighboring fibers (Fig. 1F). Finally, we injected dsRNA$_{utrophin}$ because utrophin is highly concentrated at the postsynaptic site of NMJs and at ectopic postsynaptic structures (Meier *et al.*, 1997), but its inactivation in mice does not impinge on the initial formation of the NMJ (Grady *et al.*, 1997; Deconinck *et al.*, 1997). As expected, AChR clusters were still formed on injected muscle fibers (Fig. 1G).

For quantification, we counted the number of AChR clusters on injected and on neighboring, non-injected muscle fibers of at least three independently injected animals. For each injection site, the number of AChR clusters on the individual cross-sections was added to yield a total number of AChR clusters per injection site (Fig. 2A). To account for variations between individual experiments, the number of AChR clusters in the neighboring, non-injected muscle fibers was normalized to 100%. As shown in Fig. 2B, this quantification demonstrates that injection of dsRNA$_{MuSK}$ or dsRNA$_{rapsyn}$ resulted in a

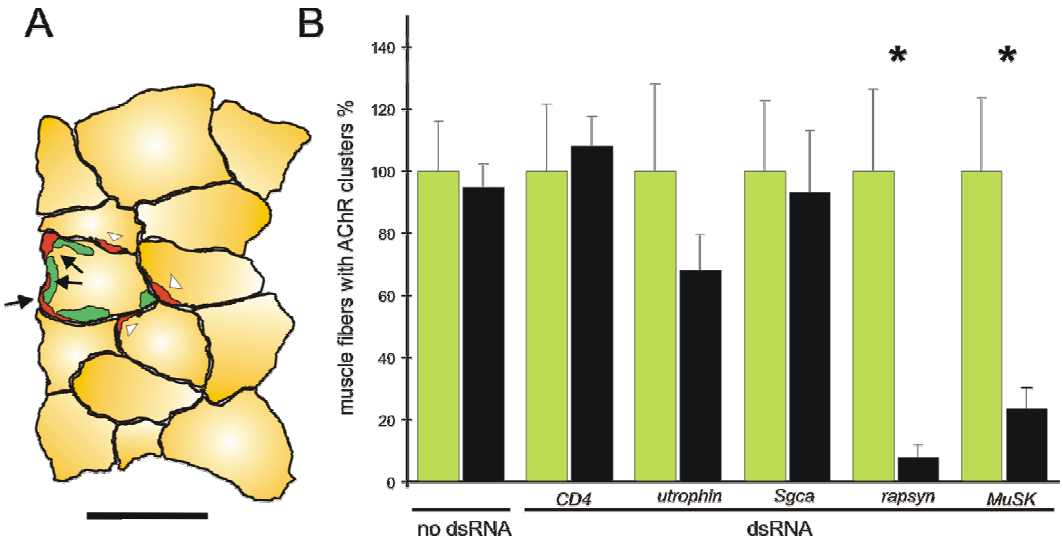

Figure 2: Quantification of the number of AChR clusters formed on injected and neighboring muscle fibers. **(A)** Camera Lucida drawing of a cross-section through an injection site, two weeks after injection. In this particular case, injection cocktail contained expression constructs for neural agrin and NLS_GFP, and dsRNA$_{utrophin}$. In the cross-section shown, AChR clusters (red) were found along the circumference of the injected (GFP-positive nuclei) and of the neighboring, non-injected muscle fibers. AChR clusters on injected fiber are marked with solid arrows, AChR clusters on neighboring fibers are marked with open arrowheads. Bar = 50 μm. **(B)** Quantification of three independent experiments. Significant differences ($p < 0.01$; Wilcoxon test) between the number of AChR clusters on injected and neighboring muscle fibers are indicated by asterisks.

highly significant reduction of AChR clusters in the injected muscle fibers while "no dsRNA", dsRNA$_{CD4}$, dsRNA$_{SGCA}$ and dsRNA$_{utrophin}$ did not inhibit AChR cluster formation. In summary, these results demonstrate that dsRNA, when injected into single, adult muscle fibers *in vivo*, knocks the expression of the targeted protein down to the extent that it mimics the phenotype of the corresponding knockout mouse.

To measure the effect of dsRNA-induced RNAi on a particular gene directly, we next quantified the amount of utrophin and dystrophin found at ectopic postsynapses. As shown earlier, agrin-induced formation of postsynaptic specializations at ectopic sites requires local transcription of synaptic proteins in the myonuclei underlying these sites (Briguet and Ruegg, 2000; Moore *et al.*, 2001). Moreover, agrin-induced ectopic postsynaptic structures form *de novo*, which makes the protein levels at the ectopic sites grossly independent of protein turnover. In controls, all postsynaptic structures induced by neural agrin contained a high concentration of utrophin (Fig. 3A) while the staining was less intense at AChR clusters of muscle fibers injected with dsRNA$_{utrophin}$ (Fig. 3B). When we stained for dystrophin, which may compensate for utrophin (Grady *et al.*, 1997; Deconinck *et al.*, 1997), we found that staining for dystrophin was very similar in injected and neighboring muscle fibers (Fig. 3C & D). Quantification of the fluorescence intensity for utrophin and dystrophin in the different experimental paradigms is shown in Fig. 3E. No difference between the staining intensity for utrophin at AChR clusters formed on injected and on neighboring muscle fibers was observed in controls (no dsRNA). Levels of utrophin at AChR clusters of dsRNA$_{utrophin}$- injected muscle were significantly lower (<40%) than at AChR clusters in neighboring fibers. The low level of utrophin that is still detected at the ectopic postsynaptic sites of dsRNA$_{utrophin}$-containing muscle fibers might be due to either some utrophin expressed in non-synaptic regions and/or the aggregation of utrophin diffused from the NMJ. Note that the level of dystrophin is slightly increased in the muscle fibers injected with dsRNA$_{utrophin}$ compared to neighboring fibers. This lends support to the idea that dystrophin compensates for the lack of utrophin in the knockout mice.

In a further step, we asked whether RNAi could also be used to study the requirement of genes for the stability of the nerve-muscle synapse. To address this

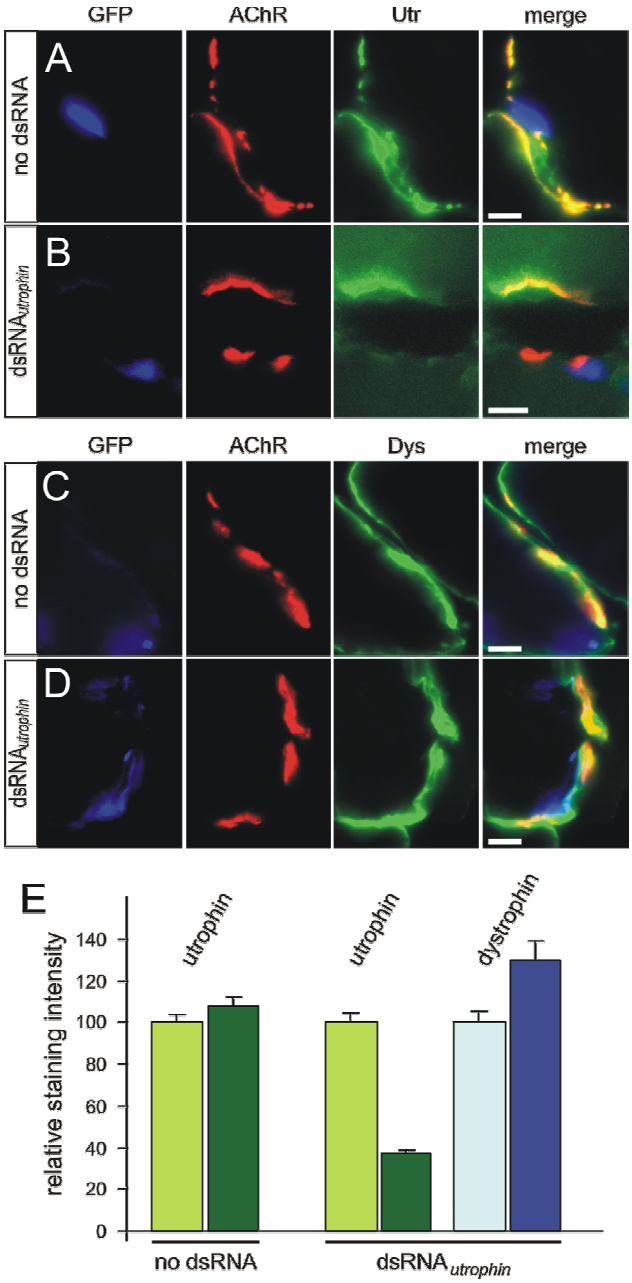

Figure 3: Staining of ectopic postsynaptic structures for utrophin and dystrophin. **(A)** No difference in the staining intensity for utrophin was seen when dsRNA was omitted. **(B)** Although AChR clusters were formed on the muscle that contained $dsRNA_{utrophin}$ (GFP-positive), these clusters were often devoid of any utrophin. **(C)** Dystrophin was more uniformly distributed along the entire plasmalemmal membrane with only some enrichment at AChR clusters. **(D)** In $dsRNA_{utrophin}$-expressing muscle fibers, dystrophin was found at sites of AChR accumulations. **(E)** Quantification of the amount of utrophin (green bars) and dystrophin (blue bars) in neighboring (light colors) and injected muscle fibers (dark colors). Bars in A-D = 5 μm.

question, we chose to target MuSK because (i) it is required for the formation of postsynapses and (ii) auto-antibodies to MuSK cause myasthenia gravis (Hoch *et al.*, 2001). This disease is characterized by muscle weakness and loss of AChRs suggesting that NMJs might disassemble. Because NMJs represent only 0.1% of the total surface of a muscle fiber, dsRNA injection into single muscle fibers did not allow a global and

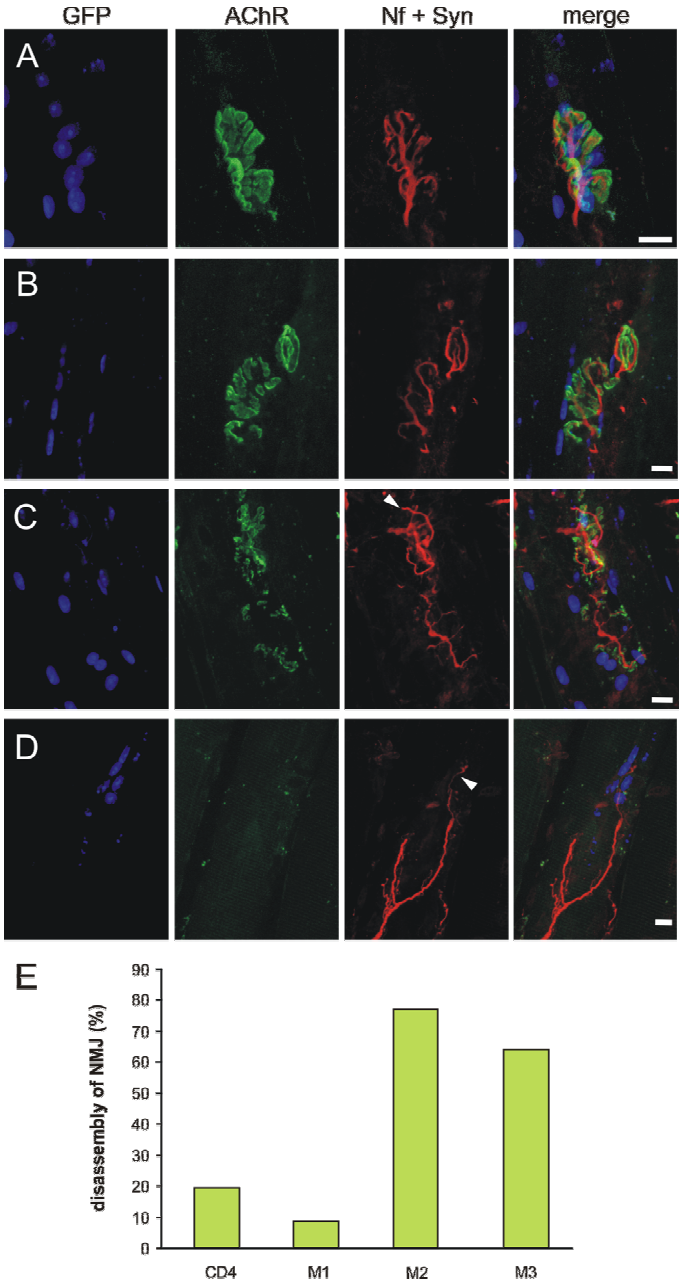

Figure 4: Whole mounts of NMJs, six weeks after *in vivo* electroporation of mouse soleus muscle with siRNA-plasmids. Electroporated muscle fibers are marked by NLS_GFP (GFP), AChRs were stained to visualize postsynapses and a mixture of antibodies to neurofilament (Nf) and synaptophysin (Syn) was used to label the presynaptic motor neurons. **(A)** NMJs are not altered by a siRNA-plasmid to CD4. **(B-D)** Disassembly of NMJs by siRNA-plasmids to MuSK. See text for details. Scale bars = 15 μm. **(E)** Quantification of the effect of plasmid-mediated siRNA (see Methods). CD4, siRNA-plasmid targeting CD4; M1, M2 and M3, three different siRNA-plasmids targeting MuSK. Note that M1 does not show any effect, which is consistent with observations by others that most but not all siRNA constructs are functional (McManus and Sharp 2002).

sustained perturbation of gene expression at NMJs. We therefore used plasmids that express functional siRNA (Brummelkamp *et al.*, 2002; Yu *et al.*, 2002) and electroporated mouse hindlimb muscles with three different MuSK-shRNA plasmids in conjunction with expression plasmids for NLS_GFP. As a control, plasmids derived from CD4 were transfected. When examining the electroporated muscles after two weeks, we could not detect a clear effect on the structure of their NMJs (data not shown) and we therefore concentrated our examination to six weeks. In CD4-targeted muscle fibers, NMJs overlying GFP-positive myonuclei were indistinguishable from NMJs on muscle fibers that were not transfected (Fig. 4A). The alterations of the postsynaptic structures after applying MuSK shRNA plasmids ranged from fragmentation (Fig. 4B) to severe disassembly of postsynaptic AChR clusters (Fig. 4C). In response to the abrogation of postsynapse integrity, presynaptic nerve terminals began to sprout (arrowheads in Fig. 4C & D). In some severe cases (Fig. 4D), the entire postsynaptic structure was lost and only the remaining motor nerve terminal indicated that a NMJ had been present before. In all GFP-negative muscle fibers, NMJs were not different from non-treated animals (data not shown). Quantification (see Methods) revealed that NMJs were not altered by electroporation of shRNA plasmids directed to CD4 and to one sequence of MuSK (M1). Two other shRNA plasmids to MuSK (M2 and M3) showed a clear effect on NMJ structure (Fig. 4E).

Discussion

Although dsRNA can mediate RNAi in cultured mammalian cells (Elbashir *et al.*, 2001a), its specific effect is overwritten by the activation of the dsRNA-dependent interferon response, which triggers general inhibition of protein translation and induces apoptosis of cells (Paddison *et al.*, 2002b). Here, we used dsRNA and show for the first time that dsRNA-induced RNAi is highly reproducible and sequence-specific. For example, dsRNA directed to utrophin clearly reduced the amount of utrophin at the ectopic postsynapses but it did not affect expression levels of its homologue dystrophin. Second, the use of dsRNA did not cause inhibition of protein translation in general indicated by the fact the number of AChR clusters formed on muscle fibers injected with dsRNA directed to CD4, α-sarcoglycan or utrophin was not different from muscle fibers that were not injected with any dsRNA. Third, the effect of dsRNA was confined to the injected muscle fiber and did not spread across cell boundaries. This allowed comparing perturbed and non-affected muscle fibers in the same muscle and makes this method well controllable.

We did not investigate why we did not find any evidence for a general silencing of translation in dsRNA-injected muscle fibers. It could well be that muscle fibers do not respond to dsRNA in this unspecific way. Indeed, vector-mediated delivery of dsRNA has also been shown to induce sequence-specific RNAi in cultured C2C12 cells, a cell line that forms myotubes. Like in our case, the silencing of endogenous genes was not accompanied by a global effect on translation (Yi *et al.*, 2003). Another reason for the absence of any unspecific effects on protein translation might be the way we applied dsRNA to the muscle fibers. Whereas all other reports used transfection or electroporation to introduce dsRNA, we micro-injected dsRNA directly into muscle fibers. Such direct application of dsRNA into the cytosol of cells may not trigger dsRNA-dependent responses (Shuey *et al.*, 2002 for discussion). This method will thus facilitate the functional characterization of unknown genes in muscle *in vivo*, which is a significant

advantage over the current methods using conventional gene-targeting techniques in mice.

Another aspect of our work is that we provide direct evidence that MuSK expression is necessary to warrant the integrity of the NMJ. The recent discovery that auto-antibodies to MuSK cause myasthenia gravis (Hoch *et al.*, 2001) are suggestive of a role of MuSK in warranting the integrity of postsynaptic structures. However, no direct evidence has yet been provided. We describe here that MuSK perturbation causes pronounced disassembly of the entire NMJ. Interestingly, the fragmentation and disassembly of NMJs required several weeks to be detected. MuSK perturbation also resulted in the sprouting of the presynaptic nerve terminal, indicating that a compact postsynaptic structure is also required to maintain presynaptic integrity. Denervation of NMJs may also be secondary consequence in patients who suffer from myasthenia gravis caused by MuSK auto-antibodies.

Methods

Plasmids and injections. Full-length chick agrin cDNA and the NLS_GFP construct have been described previously (Denzer *et al.*, 1995; Jones *et al.*, 1999). Vectors encoding shRNAs were constructed according to Yu *et al.*, 2002; using the loop sequence TTCAAGAGA (Brummelkamp *et al.*, 2002). The murine 21 nt target sequences correspond to nucleotides 125-145 (M1), 352-372 (M2) and 525-545 (M3) of MuSK (NCBI accession: NM_010944) and 494-514 of CD4 (M36850). Injection into rat muscle was done as described (Meier *et al.*, 1997).

dsRNA preparation. PCR-generated transcription templates contained T7 or T3 promoter sequences on the 5' end of the sense or antisense template. RNAs were synthesized using the Megascripts kit (Ambion) and annealed as described (Wianny and Zernicka-Goetz, 2000). The target sequences in rats correspond to nucleotides 5-608 of MuSK (U34985), 44-687 of rapsyn according to murine homologue gene (NM_009023), 114-704 of CD4 (M15768), 55-740 of α-sarcoglycan according to murine homologue gene (NM_009161) and 2132-2747 of utrophin (AJ002967). PCR products of rapsyn, α-sarcoglycan and utrophin were sequenced.

Electroporation of cDNA into muscle fibers. 5-10μl mix of cDNAs (2μg/μl each constructs) were injected into soleus muscle of C57BL/6 mice (>6 months). Electroporation was done as described previously (Gehl *et al.*, 1999) using an ECM 830 electroporation system (BTX). Eight pulses were applied for 20ms and at the frequency of 1Hz. Voltage was set to 200V/cm. After two to six weeks, the electroporated muscle was analysed.

Immunohistochemistry and antibodies. AChRs on rat muscles were visualized using rhodamine_α-bungarotoxin (Molecular Probes). To stain for AChRs, synaptophysin and neurofilament, mouse muscle was fixed by 2% paraformaldehyde and stained with biotin-

xx conjugated α-bungarotoxin, followed by streptavidin-APC (Molecular Probes). After permeabilization (1% Triton X-100), the anti-synaptophysin (DAKO) and anti-neurofilament polyclonal antibodies (Sigma) were incubated for 2 days at 4°C in PBS, 1% BSA. For detection, Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) was used. For staining of dystrophin or utrophin, 12μm-thick cross-sections were fixed for 5 min with 1% paraformaldehyde, washed briefly with PBS, and preincubated for 15 min in PBS supplemented with 5% horse serum, 1% BSA and 0.01% Triton X-100. After overnight incubation with the anti-dystrophin antibody (Novocastra) or the anti-utrophin antibody (NCL-DRP2; Novocastra), primary antibodies were detected with Alexa 350-/Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes).

Quantification. Quantification of AChR clustering using at least 20 muscle fibers for each experimental paradigm was done as described (Briguet and Ruegg, 2000). Quantification of utrophin and dystrophin protein levels was essentially done as described (Eusebio *et al.*, 2003). The extent of NMJ disassembly by plasmid-mediated siRNA was quantified by four colleagues who scored each muscle in a double-blind manner for postsynaptic fragmentation and presynaptic nerve sprouting using a scale of zero (no fragmentation and no nerve sprouting) to two (strong fragmentation and pronounced nerve sprouting). For each condition, muscles from four independent experiments were analyzed. Fig. 4E shows accumulated values from each observer and each sample after normalization to the theoretical maximum value.

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Chapter 3

In vivo synapse disassembly

in mouse muscle by expression of long hairpin RNA

Xian Chu Kong and Markus A. Ruegg

Abstract

RNA interference (RNAi) is a powerful method to study gene function in adult mammals *in vivo*. Previously, by using plasmids encoding short interfering RNA (siRNA) corresponding to the muscle-specific receptor tyrosine kinase MuSK, we have shown that continuous expression of MuSK is required to maintain the neuromuscular junction (NMJ). However, choosing an efficient siRNA target sequence is empirical, and siRNAs targeting different regions of the same messenger RNA (mRNA) vary strongly in their ability to trigger RNAi. Therefore, different siRNA candidates need to be tested for their efficiency in gene silencing. This time- and work-consuming process can be overcome by the use of long double-stranded RNA (dsRNA), which induces RNAi by presenting various siRNAs to the target mRNA. Here, we report that the disassembly of preexisting NMJs can be reproduced by plasmid encoding long hairpin RNA targeting to MuSK. This result demonstrates for the first time the capability of endogenous expressed dsRNA to silence gene expression in adult mouse muscle *in vivo*. Compared to vector-mediated siRNA, long hairpin RNA provides a more straightforward tool to study function of muscle genes.

Introduction

The availability of sequence information for the entire genome from several species including human and mice demands effective reverse genetic methods to access the function of thousands identified genes. So far there has been no general method for reverse genetic other than gene targeting by homologous recombination, which is costly, time consuming and has the risk of embryonic lethality. A highly promising new technique that may allow a faster and inexpensive assessment of gene function *in vivo* is RNA interference (RNAi). This technique was first described in C. elegans employing long dsRNA, which triggered sequence-specific post-transcriptional gene silencing (Fire *et al.*, 1998). Subsequent studies showed that long dsRNA was processed into short interfering dsRNA (siRNA) of ~22 nt, which guided the degradation to target mRNA (Hamilton and Baulcombe, 1999; Zamore *et al.*, 2000; Elbashir *et al.*, 2001b; Yang *et al.*, 2000; Parrish *et al.*, 2000; Hammond *et al.*, 2000), and that the direct introduction of siRNA induced the degradation of the homologous RNA as well (Elbashir *et al.*, 2001a). These findings led to the wide application of siRNA as an inducer of silencing for studying gene function in many mammalian systems (reviewed in Dykxhoorn *et al.*, 2003).

In adult mammals, functional gene silencing is triggered by siRNA delivered into somatic tissues by different methods. Direct delivery of siRNA by hydrodynamic injection into adult mouse tails successfully silenced the reporter gene luciferase and the endogenous gene Fas receptor in several tissues including kidney liver, lung and spleen (Lewis *et al.*, 2002; McCaffrey *et al.*, 2002; Song *et al.*, 2003). However, siRNA lasts only for a few days due to the lack of siRNA amplification mechanisms. The temporal limitation of siRNA is overcome by plasmids- or virus-mediated delivery of small hairpin RNA (shRNA), which is subsequently processed into siRNA (Dykxhoorn *et al.*, 2003). This system enables prolonged and stable suppression of gene expression *in vivo* (Rubinson *et al.*, 2003; Xia *et al.*, 2002; McCaffrey *et al.*, 2003) and allows the generation of transgenic gene knockdown animals (Carmell *et al.*, 2003; Hasuwa *et al.*, 2002; Lois *et al.*, 2002; Tiscornia *et al.*, 2003; Kunath *et al.*, 2003).

To promote efficient gene silencing using a siRNA homologous to a single site of the target mRNA, the siRNA sequence is crucial. siRNAs that targets different regions of the same gene can differ strongly in their efficiency to trigger RNAi (Holen *et al.*, 2002; Miyagishi and Taira, 2002; Vickers *et al.*, 2003; Hemann *et al.*, 2003). However, choosing the siRNA target sequence is an empirical process, since the rules that govern efficient siRNA-mediated silencing are still not defined. Based on the sequence analysis of targeted genes, different guidelines have been proposed, that aimed to facilitate the choice of efficient target sequences (reviewed in Dykxhoorn *et al.*, 2003). But finally, the potential of each siRNA has to be tested in real gene silencing experiments. The reported rate of efficiency of a particular siRNA sequence can vary from 20% to 100% (McManus and Sharp, 2002).

The additional expenditure of time and work to identify efficient target sequences is not required in using long dsRNA to trigger RNAi. Long dsRNAs are intracellularly processed by Dicer, a member of the RNase III family, into various siRNAs (Bernstein *et al.*, 2001). Subsequently, these siRNAs guide the degradation to many sites of the target mRNA. Silencing by long dsRNA has been used to investigate gene function in many organisms including plants, fungi, invertebrates (reviewed in Tijsterman *et al.*, 2002) and chicken embryos (Pekarik *et al.*, 2003). The applicability of this technique is limited in mammals, as dsRNA longer than 30 nt seems also to be responsible for the induction of interferon, which triggers mRNA degradation and general shutdown of translation, resulting in cell apoptosis (Paddison *et al.*, 2002b; Stark *et al.*, 1998). However, this non-specific interferon response is probably absent in some mammalian cell types. Successful gene silencing has been observed in several cultured mammalian cell lines and in early murine embryos, into which long dsRNA has been delivered by transfection (Billy *et al.*, 2001; Gan *et al.*, 2002), by microinjection (Svoboda *et al.*, 2000; Wianny and Zernicka-Goetz, 2000), by vector- (Svoboda *et al.*, 2001; Yi *et al.*, 2003) or transgene-mediated (Stein *et al.*, 2003) long hairpin RNA expression.

Previously, we have shown that long dsRNA, when directly injected into adult mouse muscle fibers, induced sequence-specific gene silencing. The formation of the neuromuscular junction (NMJ) requires elaborate signaling between the innervating motor

neuron and the target muscle fiber (Sanes and Lichtman, 2001). Among the first signs of postsynaptic differentiation is the aggregation of acetylcholine receptors (AChRs) beneath the innervating nerve terminal. This process crucially depends on the muscle-specific receptor tyrosine kinase MuSK. No postsynaptic structure forms in MuSK-deficient mice (DeChiara *et al.*, 1996). In our previous study, long dsRNA targeting MuSK prevented the aggregation of AChRs. In addition, the discovery that auto-antibodies to MuSK cause myasthenia gravis (Hoch *et al.*, 2001) supports the assumption that continuous MuSK expression is necessary to warrant the integrity of the NMJ. Myasthenia gravis is a disease characterized by severe muscle weakness and loss of AChRs. Consistent with the phenotype of this myasthenia gravis, we have reported the pronounced disassembly of NMJs upon silencing of MuSK expression by small hairpin-mediated siRNA.

Although siRNA could be more widely applied to mammalian system than long dsRNA, the empirical process in choosing siRNA target sequences is clearly a disadvantage. Plasmid-mediated expression of long dsRNA would provide a fast and sustained method for gene silencing. In this study, we aimed to establish long hairpin RNA-mediated RNAi for the study of postsynaptic maintenance in skeletal muscle fibers of adult mouse *in vivo*. We demonstrate that long hairpin RNA constructs are an efficient and straightforward tool to study the function of genes involved in maintenance of preexisting NMJs *in vivo*.

Results

Here we investigated whether plasmid-mediated long hairpin RNA could induce RNAi to assess the requirement of genes in the maintenance of postsynaptic structures in mouse muscle *in vivo*. To address this question, we designed an expression construct for a 604 nt long hairpin RNA targeting the receptor tyrosine kinase MuSK. As by us in the previous chapter, MuSK is required for the stability of NMJs. Thus, silencing of MuSK should induce disassembly of NMJs. Because expression of long hairpin RNA in cell types other than muscle fibers could induce general shutdown of protein translation, we chose the muscle-specific creatine kinase (MCK) promoter (Sternberg *et al.*, 1988) to drive the expression of the construct. Transcription was terminated by a polyA sequence. A mix of the constructs and plasmids encoding green fluorescent protein fused to a nuclear localization signal (NLS_GFP) was transformed into mouse soleus muscle by electroporation. GFP was used to distinguish between transformed and non-transformed muscle fibers. To test whether plasmid-mediated long hairpin RNA could exert any non-specific effect on protein expression, we transfected a plasmid encoding long hairpin RNA derived from CD4. CD4 is only expressed in the immune system (Benoist and Mathis, 1999). The effect of plasmid-mediated long hairpin RNA to postsynaptic structures was examined by staining teased muscle fibers with α-bungarotoxin, a snail toxin that specifically binds to AChRs. AChRs are accumulated at the postsynaptic part of a NMJ (Sanes and Lichtman, 2001).

The electroporated muscles were analyzed after six weeks. In CD4-targeted muscles, NMJs overlaying GFP-positive myonuclei were not different from NMJs on muscle fibers that were not transfected (Fig. 1A). In contrast, the alteration of the postsynaptic structures after applying MuSK derived long hairpin RNA plasmids ranged from no obvious fragmentation to severe disassembling of postsynaptic AChR clusters (Fig. 1B-D). In addition, as a result of the abrogation of postsynapse integrity, few presynaptic nerve terminals began to sprout (data not shown). In all GFP-negative muscle fibers, NMJs were indistinguishable from those of non-treated muscles.

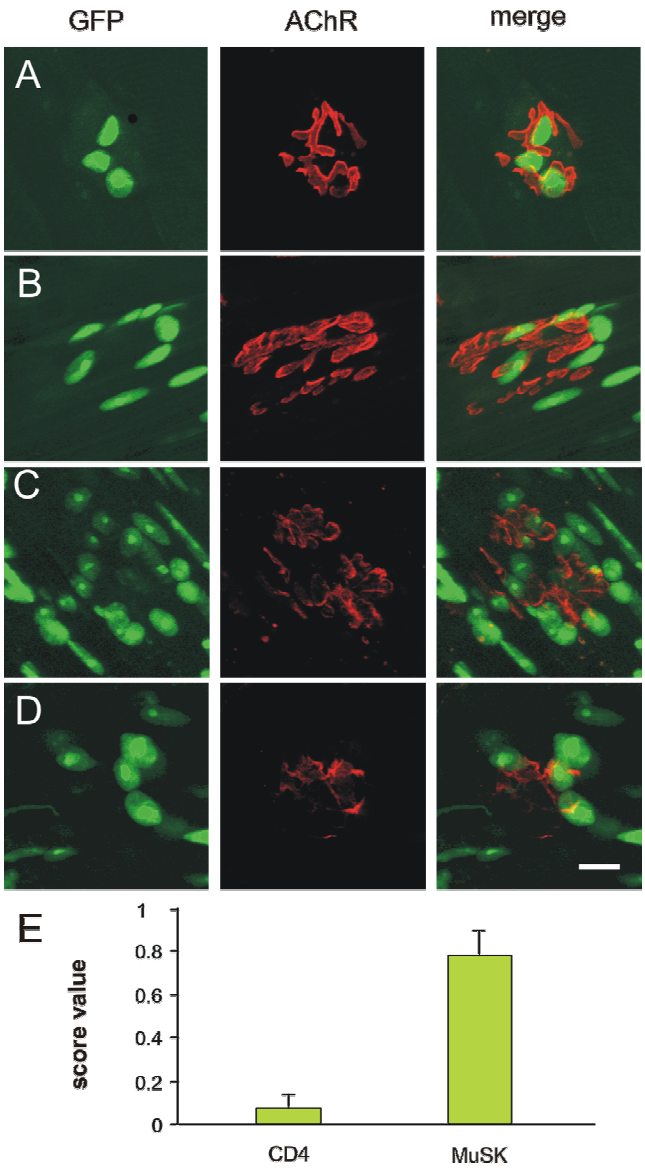

Figure 1: Postsynaptic structures, six weeks after *in vivo* electroporation of mouse soleus muscle with long hairpin RNA-plasmids. Electroporated muscle fibers are marked by NLS_GFP (GFP), AChRs were stained to visualize postsynapses (AChR). (A) Postsynapses are not altered by long hairpin RNA to CD4. **(B-D)** Disassembly of postsynapses by long hairpin RNA to MuSK. The degree of deterioration ranged from not altered postsynaptic structures **(B)** to fragmentation **(C)** and severe fragmentation **(D)**. Scale bars = 10 μm. **(E)** The extent of postsynaptic disassembly on each transfected muscle fiber was quantified using a scale of zero (no fragmentation), one (fragmentation) to two (severe fragmentation) according the postsynapses shown in A, B and C. For each condition, muscles from three independent experiments were analyzed. The graphic shows the mean value for each condition. CD4: long hairpin RNA targeting CD4; MuSK: long hairpin RNA targeting MuSK. Quantification was done with a microscope equipped with epifluorescence (Leica).

Quantification (see figure 1E legend) revealed that expression of long hairpin RNA directed to MuSK clearly altered postsynaptic structures, while long hairpin RNA targeted to CD4 did not have any effect (Fig. 1E).

Discussion

Although plasmid-mediated shRNA can be used to study the function of genes in mouse muscle *in vivo*, the selection of an efficient target sequence is a trail and error process (Dykxhoorn *et al.*, 2003). Especially in the assessment of a number of genes, this process could be very time and work intensive. Such selection of target sequence would be unnecessary in using long hairpin RNA to trigger sustained gene silencing. Here, we used plasmid-mediated delivery of long hairpin RNA and demonstrate for the first time that long hairpin RNA induced RNAi is a sequence-specific and straightforward tool. Long hairpin RNA targeted to MuSK clearly induced the fragmentation of postsynaptic structures, while postsynapses of CD4 targeted muscle fibers were indistinguishable from those of non-treated fibers.

Compared to our previous study, in which we used plasmid delivered siRNA to silence the expression of MuSK under the same conditions (see chapter 2), the fragmentation of postsynaptic structures induced by long hairpin RNA directed to MuSK was less pronounced, and fewer nerve terminal sprouting was observed, indicating that the silencing of MuSK by long hairpin RNA is less efficient. Since long hairpin RNA- and shRNA-mediated siRNA are supposed to induce gene silencing via the same pathway (Zamore, 2002), and because no interferon response was observed that could interfere in different steps of the RNAi pathway and thus inhibit its function, the reduced gene silencing effect could be well put down to a diminished number of functional RNAi in muscle fibers expressing long hairpin RNA compared to those expressing shRNA. Some of the possible explanations of limited generation of functional RNAi are: (1) The MCK promoter that governed the transcription of long hairpin constructs could be weaker than the U6 promoter, which we used to produce siRNA. This suggestion can be investigated by testing expression constructs driven by different promoters. (2) Because of steric obstacles, the folding of a long linear transcript to form dsRNA is likely more difficult that of a short haipin RNA. (3) The efficiency of processing long hairpin RNA or shRNA into siRNA could be different. (4) Finally, although siRNAs derived from processing of long

hairpin RNA targeted to a 600 nt region of the mRNA, probably only few of these are functional. It has been reported that only a limited number of regions of a mRNA provide effective target sites, and in average, three out of four tested potential siRNA were effective in inducing RNAi (McManus and Sharp, 2002). Therefore, some or even a big part of siRNAs derived from long hairpin RNA could be non-functional. In contrast, every transcript from a functional construct provides a functional siRNA.

In summary, our observation of long dsRNA induced RNAi in muscle fibers allowed us to compare the efficiency of both the long hairpin RNA and siRNA in triggering gene silencing *in vivo*. The diminished effect of long hairpin RNA-mediated gene silencing could be due to limited production of functional siRNA. Further investigations are necessary to optimize long hairpin-mediated RNAi for assessment of function of genes in adult mouse muscle *in vivo*.

Methods

Long hairpin constructs. The long hairpin constructs were generated by a two step overlapping PCR using the following primers:

5' primer of MuSK (M) or CD4 (C):

M: CCCATCGATGAGAGCTTGTCAACATTCCA

C: CCCATCGATAGCAGAACTGCCCTGCGAGA

Middle primer:

M: GGCACAGCTTACTCCAAACTTCTCTTGAAAGTTTGGAGTAAGCTGTGCC

C: AACTTTGCAGAGGAAAACGGTCTCTTGAACCGTTTTCCTCTGCAAAGTT

3' primer:

M: CCGCTCGAGGAGAGCTTGTCAACATTCCA

C: CCGCTCGAGAGCAGAACTGCCCTGCGAGA

The middle primer containing the loop sequence TTCAAGAGA (Brummelkamp *et al.*, 2002). Cloning sites Cla1 and Xho1 were introduced at the 5' and 3' ends. In the first step PCR, the forward and the reverse megaprimers for the second step PCR were produced in two separate PCRs using either the 5' or the 3' primer in combination with the middle primer. PCR products were purified using Geneclean Spin Kit (BIO 101) and mixed in an equal amount. Both megaprimers were annealed at 56°C and elongated at 72°C for 5 cycles. For the second step PCR, both the 5' and the 3' primer were added to the mix. The final PCR products were purified by gel electrophoresis and extracted with Gel Purification Kit (Qiagen). Taq polymerase (Roche) was applied in all steps. The thermo cycling condition was set the same for both PCR reactions: denaturation at 94°C for 20s, annealing at 55°C for 30s and elongation at 72°C for 30s, totally 35 cycles. The murine ~600 nt target sequences correspond to nucleotides 5-608 of MuSK (NCBI accession: NM_010944) and 150-746 of CD4 (M36850).

Plasmids. The long hairpin constructs were inserted between a MCK transcription promoter and a polyA transcription stop sequence in pBluescript II KS+ plasmids.

Insertions were verified by restriction enzyme digestions. The NLS_GFP construct encoding green fluorescent protein (GFP) with a nuclear localization sequence (NLS) has been described previously (Jones *et al.*, 1999).

Electroporation and NMJ visualization. Both were done as described in chapter 2.

Acknowledgments

We thank Dr. Hans-R. Brenner for granting us access to the electroporation equipment, Mrs. Patrizia Barzaghi for donating of the MCK-pBluescript vector, Dr. Shuo Lin and other members of the laboratory for fruitful discussions. X.C.K. is supported by a Ph.D. fellowship from Hoffmann-LaRoche Ltd. Additional support was granted by the Swiss National Science Foundation, the Kanton of Basel-Stadt and the Swiss Foundation for Research on Muscle Diseases to M.A.R.

Chapter 4

RNA interference as a new method to study gene expression during agrin induced differentiation in mouse muscle *in vivo*

Xian Chu Kong and Markus A. Ruegg

Abstract

The formation of the vertebrate neuromuscular junction (NMJ) is characterized by aggregation of acetylcholine receptors (AChRs) beneath the innervating nerve terminal and depends critically on the nerve-derived extracellular matrix protein agrin and the muscle specific receptor tyrosine kinase MuSK. Moreover, introduction of neural agrin alone into innervated or denervated muscle is sufficient to induce postsynaptic differentiation and aggregation of AChRs. The molecular mechanisms underlying the signaling induced by agrin are still poorly understood. Here, we report that the AChR clustering activity of neural agrin can be inhibited by RNA interference (RNAi) in mouse muscle *in vivo*. By electro-transfecting a plasmid encoding small hairpin RNA (shRNA) targeted to MuSK and an expression vector encoding agrin, which induces aggregation of AChRs as effective as injection of the recombinant agrin protein, we provide evidence that RNAi perturbed specifically postsynaptic differentiation in both innervated and denervated mouse muscle fibers. Thus, we established a fast, simple and reliable method as how to study the function of genes involved in agrin-induced postsynaptic differentiation in adult muscle *in vivo*.

Introduction

Formation of synaptic structures at the vertebrate neuromuscular junction (NMJ) depends on the exchange of trophic factors between the presynaptic innervating motor neuron and the postsynaptic target muscle fiber (reviewed in Sanes and Lichtman, 2001). One of the pronounced signs of postsynaptic differentiation is the aggregation of acetylcholine receptors (AChRs) beneath the nerve terminal. While transcription of postsynaptic genes like AChR subunits, which are expressed in the whole muscle fiber before innervation and after denervation, is selectively maintained in junctional myonuclei, it is repressed in the extrasynaptic myonuclei (Duclert and Changeux, 1995; Piette *et al.*, 1993; Merlie *et al.*, 1984; Tsay and Schmidt, 1989). Ample evidence has demonstrated that the heparan sulfate proteoglycan agrin, which is released from the motor neuron and binds to postsynaptic basal lamina, is essential and sufficient to trigger and maintain a signaling cascade that results in the assembly of the complete postsynaptic apparatus (McMahan, 1990; Bezakova and Ruegg, 2003). When neural agrin is introduced into muscle by single muscle fiber injection of an expression vector or by intramuscular injection of the recombinant protein itself, it elicits formation of postsynapse-like structures characterized by clustering of AChRs in extrasynaptic regions of innervated muscle fibers or in case of denervation, along muscle fibers (Bezakova *et al.*, 2001a; Cohen *et al.*, 1997; Jones *et al.*, 1997; Meier *et al.*, 1997).

The ability of neural agrin to induce postsynaptic differentiation is mediated by the activation of the muscle specific receptor tyrosine kinase MuSK, another important component in the formation of the NMJs. Postsynaptic differentiation is absent in MuSK-deficient mice (DeChiara *et al.*, 1996). Activation of MuSK results in re-organization of the cytoskeleton at the postsynaptic site and in activation of signaling pathways that are responsible for the changes of gene transcription in junctional myonuclei (Sanes and Lichtman, 2001). The molecular mechanisms underlying the formation of the NMJ downstream of agrin signaling are still not satisfactorily understood. To this end, Dr. Gabriela Bezakova in our group successfully performed microarray experiments to

analyze gene expression profiles induced by the formation of postsynaptic structures upon treatment with neural agrin *in vivo* (data not yet published). To assess the importance of these candidate genes in the NMJ formation, gene silencing based on RNA interference (RNAi) could be a suitable method for quick and reliable study of their functions in adult muscle *in vivo*.

RNAi is a powerful experimental tool for post-transcriptional gene silencing induced by double-stranded RNA (dsRNA). Experimental introduction of dsRNA triggers sequence-specific degradation of the homologous target mRNA and enables so a rapid survey of gene function (reviewed in Dykxhoorn *et al.*, 2003). First described in *C. elegans* (Fire *et al.*, 1998), RNAi has been widely applied to many organisms (Tijsterman *et al.*, 2002). In mammalian system, short dsRNA (~22bp) called small interfering RNA (siRNA) induces effectively gene silencing of transient nature (Elbashir *et al.*, 2001a). To generate long lasting suppression of gene expression *in vitro* and *in vivo*, plasmid- or virus-mediated delivery of small hairpin RNA (shRNA), which is subsequently processed into siRNA, has been developed (McManus and Sharp, 2002). Previously, we have shown that prolonged exposure of muscle fibers to an expression vector encoding shRNA for MuSK results in disassembly of existing NMJs (see chapter 2). This finding provides the first indication that plasmid-mediated shRNA can be used to induce sequence-specific and stable silencing of genes in muscle fibers *in vivo*.

In this study, we aimed to establish a fast and reliable system in adult mouse muscle *in vivo* to study the function of genes identified in the screen outlined above. Both, single fiber injection of expression vectors or intramuscular injection of recombinant protein are useful *in vivo* approaches to deliver neural agrin into muscles. However, single fiber injection allows only a limited number of fibers to be transfected and intramuscular injection of agrin requires a preceding process of protein synthesis and purification, which is time and work intensive. Here, we demonstrate that *in vivo* electro-transfection of a full-length rat neural agrin expression vector into muscle fibers can effectively induce postsynaptic differentiation. In a further step, we targeted the expression of MuSK by using the shRNA construct, whose functionality has been demonstrated in our previous study. The formation of postsynaptic structures in response

to either eletro-transfecetion of neural agrin cDNAs or injection of recombinant neural agrin protein was effectively inhibited by vector-delivered shRNAs in innervated and denervated muscles.

Results

Electroporation of agrin cDNA effectively induced AChR aggregation

To examine whether electro-transfecting agrin cDNA into fully innervated mouse soleus muscles could efficiently induce postsynaptic differentiation, we electroporated a mix of expression constructs encoding full-length rat neural agrin and green fluorescent protein, which contained a nuclear localization signal (NLS_GFP). As a control, no agrin-plasmids were included in the electroporation mix and recombinant full-length chick neural agrin was applied subsequently by intramuscular injection. Four days after electroporation, half of the transfected muscles were denervated. After two additional weeks, all muscles were analyzed. As described previously (Bezakova *et al.*, 2001a), injection of recombinant agrin protein resulted in formation of postsynaptic structures in extrasynaptic regions, closed to the myotendinous junction of innervated muscle fibers, and in the denervated muscles, numerous small postsynaptic structures were formed along muscle fibers. These findings were confirmed by electroporating agrin-plasmids (Fig. 1). Postsynaptic structures are characterized by the aggregation of AChRs. When recombinant protein has been injected into muscle, aggregates were found randomly distributed on the surface of transfected (GFP-positive myonuclei) and non-transfected muscle fibers, while electro-transfecting muscle fibers by agrin-plasmids resulted in local secretion of neural agrin and induces aggregates mainly formed on transfected and neighboring non-transfected muscle fibers. No difference in number, shape and size was observed between ectopic AChR aggregates induced either by local secretion of agrin or by injection of recombinant agrin protein.

Perturbation of AChR clustering in innervated muscle by shRNA

Next, we investigated the potential of RNAi mediated by shRNA in studying the requirement of genes for the formation of postsynaptic structures induced by application of neural agrin in fully innervated mouse muscle *in vivo*. To address this question, we chose to target the MuSK expression, because MuSK is clearly involved in neural agrin

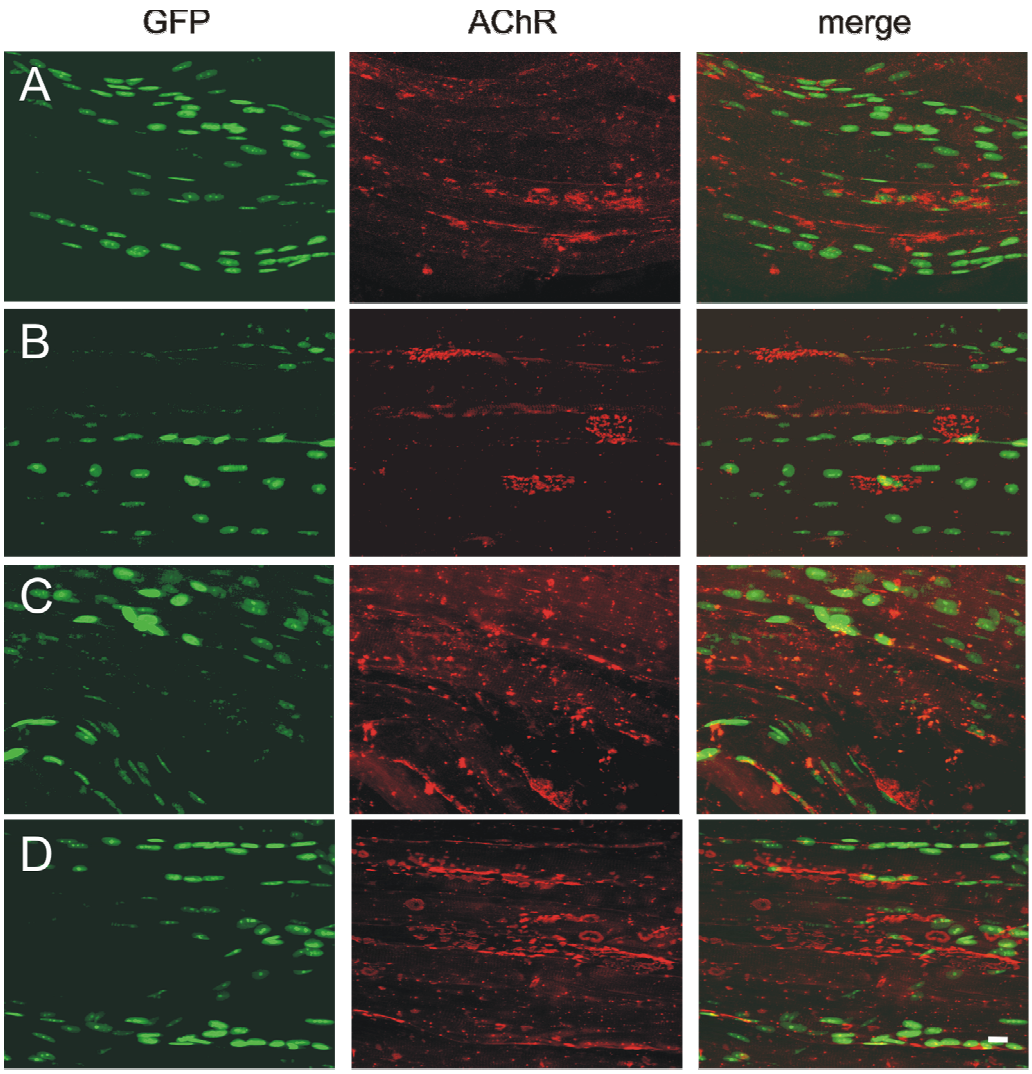

Figure 1: Staining of postsynaptic structures formed after intramuscular injection of recombinant neural agrin protein **(A&C)** or electro-transfecting an expression constructs for neural agrin **(B&D)**. To rule out effects mediated by *in vivo* electroporation, all muscles were electroporated with an expression construct for NLS_GFP. AChRs were stained to visualize postsynapses (AChR). Delivery of neural agrin either by injection of recombinant protein or by electroporation of agrin cDNA induced the formation of postsynaptic structures on the surface of electroporated and nearby muscle fibers of innervated **(A&B)** or denervated muscles **(C&D)**. Scale bars = 50 μm.

triggered postsynaptic differentiation: it is activated by agrin (Glass *et al.*, 1996) and it is required for the formation of postsynapse (DeChiara *et al.*, 1996). Thus, effective silencing of Musk expression by shRNA would inhibit agrin induced postsynaptic differentiation. As a control, we chose CD4, a protein that is not expressed in muscle (Benoist and Mathis, 1999). Targeting CD4 would test whether shRNA exerts any non-

specific interference on the general protein translation. Plasmids encoding shRNA derived from MuSK or CD4 were electroporated in conjunction with NLS_GFP plasmids into fully innervated mouse soleus muscle. The effectiveness of the MuSK-shRNA construct in silencing MuSK expression has been demonstrated in our previous study (see chapter 2). Neural agrin was delivered by local secretion upon co-transfection of neural agrin expression constructs or by injection of the recombinant neural agrin itself. Because the half-life of MuSK as well as the time between electroporation and established gene silencing is unknown, delayed delivery of neural agrin as induced by expression vector would allow silencing the basal MuSK level. Therefore, injection of recombinant neural agrin followed four days after electroporation of shRNA constructs. All muscles were analyzed two weeks after the last experimental treatment. In CD4 targeted muscle, postsynaptic structures were found on transfected and neighboring non-transfected muscle fibers (Fig. 2A) and they were indistinguishable from those formed in experiments with no shRNA-plasmids electroporated (Fig. 1A). In contrast, when the MuSK-shRNA construct was applied, postsynaptic structures were only detected on neighboring non-transfected (GFP-negative) muscle fibers, but were rarely found on transfected (GFP-positive) muscle fibers (Fig. 2B).

Global inhibition of AChR aggregation in denervated muscle

In a further step, we asked whether shRNA-mediated RNAi could also be used to perturb neural agrin induced formation of postsynaptic structures on denervated mouse muscle *in vivo*. Denervation results in generally increased synthesis of AChRs also in non-synaptic regions (Merlie *et al.*, 1984; Tsay and Schmidt, 1989). Upon delivery of neural agrin, AChRs aggregate in numerous small clusters along whole muscle fibers (Bezakova *et al.*, 2001a; Cohen *et al.*, 1997). To test whether shRNA-mediated gene silencing is sufficient to inhibit this global process of AChR aggregation, we electroporated the expression constructs encoding either MuSK or CD4 in conjunction with the NLS_GFP plasmid into mouse soleus muscle. Two days after electroporation,

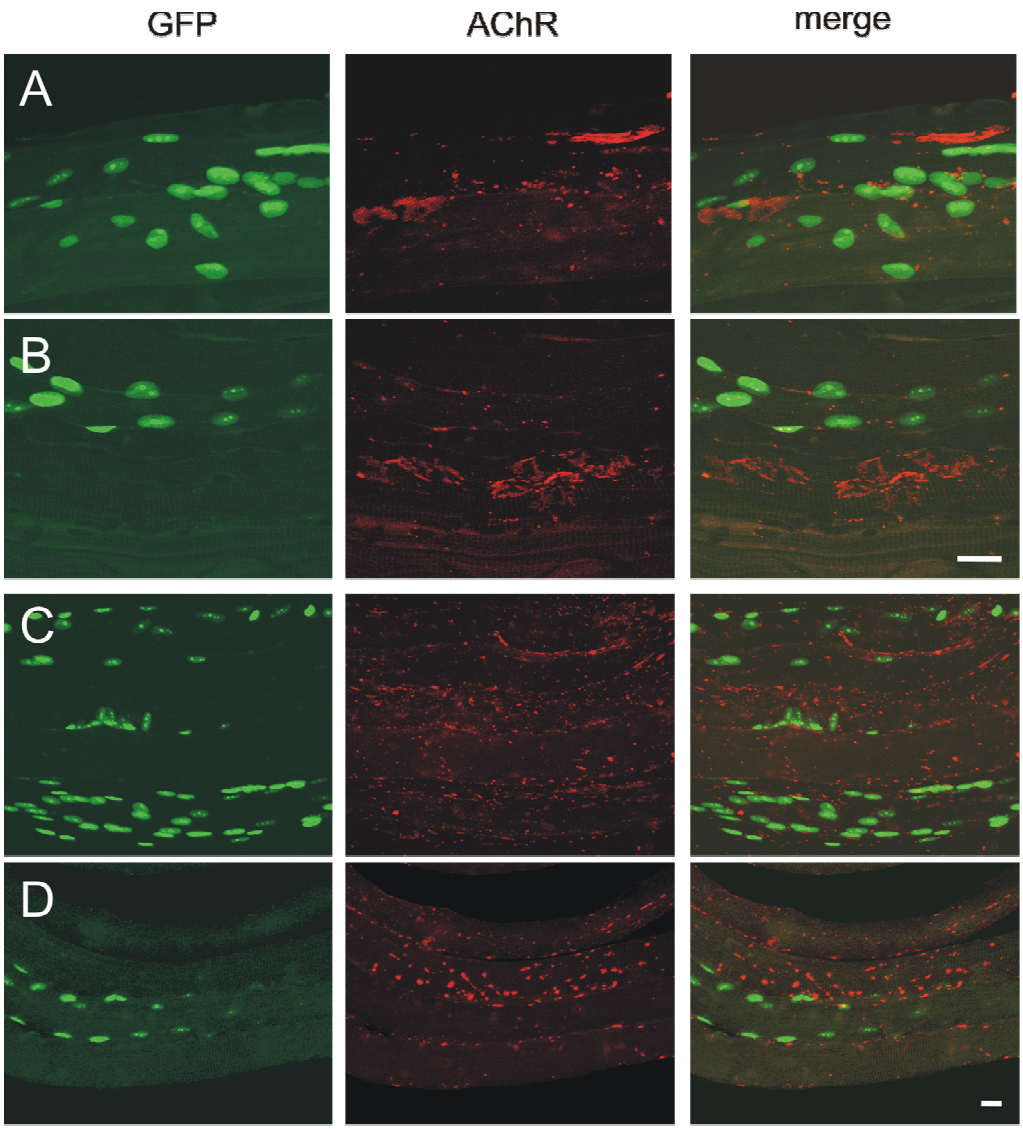

Figure 2: Staining of ectopic postsynaptic structures on innervated **(A&B)** and denervated **(C&D)** muscles. Innervated mouse soleus muscle was analyzed two weeks after *in vivo* electroporation. The electroporation cocktail contained expression constructs for neural agrin, NLS_GFP, and shRNA derived either from MuSK or CD4. In denervation experiments, soleus muscle was electro-transfected with shRNA-plasmids in combination with NLS_GFP constructs. After four days, intramuscular injection of recombinant neural agrin protein and denervation were performed. Analysis followed after two additional weeks. **(A&C)** In CD4-targeted muscle, AChR aggregates (red) are found along the surface of transfected (GFP positive nuclei) and nearby non-transfected muscle fibers. **(B&D)** Electroporation of the plasmid encoding MuSK-shRNA inhibits the formation of postsynaptic structures on transfected muscle fibers but not on nearby muscle fibers. Scale bars = 50 μm.

intracellular injection of recombinant neural agrin and denervation were performed. Muscles were analyzed after two additional weeks. In the presence of the CD4-shRNA plasmid, postsynaptic structures formed were indistinguishable from those formed in experiments with no shRNA-plasmids included (Fig. 1B) and AChR clusters were found on both transfected and neighboring non-transfected muscle fibers (Fig. 2C). In MuSK targeted muscle, no postsynaptic structures were found on transfected muscle fibers, while such structures were readily detected on neighboring non-transfected muscle fibers (Fig. 2D).

Discussion

Agrin is a nerve-released factor that can induce the formation of NMJs, but the molecular mechanisms underlying signaling by agrin are still poorly understood. Using microarray technique to analyze gene expression profiles, which were induced by the formation of postsynaptic structures upon neural agrin delivery, candidate genes have been identified (data not yet published), whose function at the NMJ are unknown. Here, we establish two ways as how to assess the requirement of these candidate genes for the formation of NMJ.

First, although single fiber injection of agrin cDNA or intramuscular injection of recombinant protein are useful methods to deliver neural agrin into muscle (Bezakova *et al.*, 2001a; Cohen *et al.*, 1997; Jones *et al.*, 1997; Meier *et al.*, 1997), the one approach is limited to a few muscle fibers per muscle and the other is combined with the time- and work-consuming process of protein synthesis and purification. Here, we overcome these disadvantages by *in vivo* electro-transfecting agrin cDNA and show that this new approach is as effective as injecting recombinant protein in the induction of global AChR aggregation.

Second, we delivered shRNA via plasmid and demonstrate that RNAi induced by shRNA is a fast and specific tool to perturb agrin-mediated formation of postsynaptic structures *in vivo*. In particular, targeting shRNA to MuSK efficiently inhibited the formation of numerous small AChRs aggregates, which were induced by delivery of neural agrin to denervated muscles. This effect is limited to transfected muscle fibers, while no inhibition was observed on non-transfected muscle fibers, indicating that shRNA-mediated gene silencing in adult muscle is strong and reliable.

In summary, using electro-transfection of neural agrin expression constructs and RNAi mediated by shRNA, we established a rapid, simple and reliable system that is suitable for studying the function of genes involved in the formation of postsynaptic structures in response to agrin signaling. This experimental paradigm will be important in

future studies aimed at investigating the function of candidate genes identified in the screen outlined above.

Methods

Plasmids. The expression construct encoding full-length rat neural agrin and the NLS_GFP construct encoding green fluorescent protein (GFP) with a nuclear localization sequence (NLS) has been described elsewhere (Hashemolhosseini *et al.*, 2000; Jones *et al.*, 1999). Vectors encoding shRNAs were described in chapter 2. The murine 21 nt target sequences correspond to nucleotides 525-545 (construct M2 in chapter 2) of MuSK (NCBI accession: NM_010944) and 494-514 of CD4 (M36850).

Electroporation of cDNA into muscle fibers. Electroporation was done as described in chapter 2. All experimental procedures *in vivo* were performed under general anesthesia by a mix of 0.5ml Ketalar (PARKE-DAVIS), 0.25ml Rompun (BAYER) and 2ml 0.9% NaCl (0.1ml/10g body weight) injected i.p. Every 12 hours in the following two days, animals were treated with a mix of 1ml Temgesic (ESSEX) and 0.65ml 0.9% NaCl (50μl/mouse) by i.p. injection.

Injection of recombinant agrin. Recombinant full-length chick neural agrin was provided by Dr. G. Bezakova (Bezakova *et al.*, 2001b; Gesemann *et al.*, 1995). Four days after electroporation, ~20μl of recombinant agrin were injected into the transfected soleus muscle.

Denervation. Immediately after the last experimental manipulation, denervation was performed as described previously (Bezakova *et al.*, 2001b). Briefly, denervation of the soleus muscle was carried out in the surgically opened thigh by removing a piece of ~5mm of the sciatic nerve.

AChR visualization. AChR staining with was done as described in chapter 2.

Acknowledgments

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Chapter 5

General Discussion

RNA interference (RNAi) is the sequence-specific posttranscriptional gene silencing mediated by double stranded RNA (dsRNA) homologue to the target gene (McManus and Sharp, 2002; Dykxhoorn *et al.*, 2003). This natural process precedes gene silencing in a two-step mechanism (Zamore *et al.*, 2000). First, long dsRNAs are cleaved by the ribonuclease Dicer, generating small interfering RNAs (siRNA) of ~22 nucleotides (nt), the key effector of the RNAi pathway (Ketting *et al.*, 2001; Bernstein *et al.*, 2001). Subsequently, the antisense strand of these siRNAs associates with the RNA-inducing silencing complex (RISC), a nuclease complex, and directs degradation to the target mRNA by homologous recognition (Hammond *et al.*, 2000; Martinez *et al.*, 2002). RNAi is highly conserved among eukaryotes and has been suggested to act as the immune system of the genome by defending against viral pathogens or uncontrolled transposon mobilization (Plasterk, 2002). The advantages of the remarkable potency and efficiency of RNAi led to its experimental establishment as a powerful gene knockdown tool in many organisms (e.g. Baulcombe, 1999; Sanchez Alvarado and Newmark, 1999; Lohmann *et al.*, 1999; Ngo *et al.*, 1998; Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999; Caplen *et al.*, 2002; Dykxhoorn *et al.*, 2003; Pekarik *et al.*, 2003; Tijsterman *et al.*, 2002).

Gene profiling – and then?

In light of the availability of sequence information for the entire genome from several species including human and mice and the technological advances in genomics, the identification of large number of candidate genes with unknown functions requires efficient methods for reverse genetic assessment. Currently, our group is analyzing gene expression profiles induced by the formation of postsynaptic structures responding to

delivery of neural agrin in adult mammalian muscle *in vivo*. We are now on the stage of devising experimental paradigms that allow us to study the function of candidate genes *in vivo*. There are number of technologies that may offer the potential for specific gene targeting in mammals including gene knockout technologies, antisense technologies, ribozymes or DNAzymes and RNAi.

Conventional or tissue-specific gene targeting by homologous recombination is commonly used to determine gene function in mice, but this is a time-consuming, expensive and labor-intensive process. Furthermore, the function of targeted genes may not be determined by this approach owing to embryonic lethality or redundant phenotypes.

Alternatively, the function of genes can be determined by gene silencing. A common strategy is the use of homologous antisense RNA, DNA or chemically modified nucleic acids that inhibit gene expression by translational blockade and induction of target mRNA degradation, primarily through the action of RNase H ribonuclease (Kurreck, 2003). Although this approach is useful in certain experimental contexts, it is rarely sufficiently efficient and specific in mammalian systems (Bernstein *et al.*, 2001; Hammond *et al.*, 2000; Martinez *et al.*, 2002; Elbashir *et al.*, 2001a). Recently, by genome-wide investigation of antisense oligonucleotide treatment, “off-target effects” have been clearly demonstrated (Cho-Chung and Becker, 2003).

Another gene silencing approach is the exploitation of a number of naturally occurring RNA-based enzymes, known as ribozymes that regulate gene expression through sequence-specific cleavage of the target mRNA (Freelove and Zheng, 2002; Khan and Lal, 2003). The advantages of ribozymes for gene silencing are their catalytic property to cleave many mRNAs per enzyme molecule and their high specificity. The vulnerability of ribozymes to ribonuclease degradation can be overcome by synthetic DNAzymes, DNA counterparts to ribozymes with similar structural and functional properties (Breaker and Joyce, 1994). Successfully use of nucleic acid enzymes has been reported (reviewed by Khan and Lal, 2003 and Kurreck, 2003), however, diverse requirements of intracellular environment are critical for an optimal function of variant enzymes types. It has been suggested that each and every cell line and tissue may be

unique in their variation in respect to structural requirements for optimal uptake, activity and stability of ribozymes (Khan and Lal, 2003). This great diversity, coupled with their use, makes ribozymes and DNAzymes difficult to be applied universally.

By contrast, a promising technique allowing a more straightforward and faster but less costly investigation of gene function *in vivo* might be dsRNA-mediated RNAi. At the time when this work was started, dsRNA had successfully been used for sequence-specific silencing of genes in many organisms as plants, fungi and invertebrates but not in adult mammals (Tijsterman *et al.*, 2002). RNAi-mediated gene silencing has the potential to allow the determination of the function of each gene that is expressed in a time-, cell-type- and pathway-specific manner. Moreover, RNAi allows the silencing of genes that are pathogenic to the host organism. A recent direct comparison of RNAi with antisense strategy attested to the superior potency and efficiency of RNAi (Hough *et al.*, 2003; Miyagishi *et al.*, 2003). To make use of these advantages in mammals, we investigated the potential of RNAi in perturbing gene expression in adult muscle *in vivo*. In our approach, we demonstrated for the first time that long dsRNA - either directly injected or delivered by expression constructs - and short hairpin RNA (shRNA) efficiently induces the silencing of endogenous gene expression in adult rodents and the mechanism underlying this process is sequence-specific. Our results thus provide a powerful method for knockdown genes in adult mammalian muscle. This method will be particularly important for large-scale investigation of candidate genes identified in gene expression profiling.

Long dsRNA and long hairpin RNA as inducer of RNAi

In the first part of this work, we show that long dsRNA can efficiently trigger gene silencing *in vivo*. Micro-injection of long dsRNAs into adult rat muscle fibers enabled to reproduce phenotypes of loss-of-functions mutation. This method is highly reproducible and sequence-specific but of transient nature. The replacement of long dsRNA by plasmids encoding long hairpin RNA enabled prolonged and stable suppression of gene

expression. The significant advantage of long dsRNA and long hairpin RNA is the possibility to bypass the necessity to validate the efficiency of each selected target site on the directed mRNA for its degradation in experimental approach, which is absolutely required for the experimental use of siRNAs. However, directly comparing long hairpin RNA to small hairpin encoded siRNAs in postsynaptic gene silencing experiments revealed the superior potency of the latter, indicating the diminished ability of those long hairpin RNA constructs to mediate RNAi. This finding is most likely due to a limited production of functional siRNAs, the key intermediate of RNAi via long hairpin RNA, though additional experiments are required to confirm this hypothesis. Therefore, further investigation is necessary to optimize the efficiency of long hairpin RNA constructs in the suppression of gene expression *in vivo*.

Small hairpin RNA-mediated RNAi *in vivo*

So far, successful use of long dsRNA or long hairpin RNA to mediate RNAi in adult mammals *in vivo* has not yet been reported. In contrast, several recent reports have demonstrated the functionality of siRNA and shRNA to silence gene expression in mice and rats by using different experimental paradigms including hydrodynamic injection of siRNA (Lewis *et al.*, 2002; McCaffrey *et al.*, 2002; Song *et al.*, 2003), viral vector-mediated delivery of shRNA (Xia *et al.*, 2002; McCaffrey *et al.*, 2003; Rubinson *et al.*, 2003) or generation of transgenic animals (Carmell *et al.*, 2003; Hasuwa *et al.*, 2002; Lois *et al.*, 2002; Tiscornia *et al.*, 2003). In our experimental approach, we used *in vivo* electroporation (Gehl *et al.*, 1999), a well established tool that enables the transfection of muscle fibers with cDNA plasmids. With the vector-based shRNA delivery, we demonstrated the sequence-specific perturbation of agrin-mediated postsynaptic differentiation in adult murine muscles, thus providing a useful system to assess the function of candidate genes involved in the formation of postsynaptic structures upon agrin signaling. As mentioned above, to promote efficient gene silencing using a shRNA to a single site in the target mRNA, the selection of potential target sites is particularly

important. A protocol containing a set of guidelines that aimed to narrow the choices of efficient target sequences and to facilitate the design of shRNA-expressing constructs is presented in the appendix of this work. The soundness of these guidelines in promoting efficient gene knockdown was preliminarily demonstrated in a large-scale study, in which the requirement of six different putative effectors of the mTOR signaling pathway in regulation of the muscle size was investigated (see appendix).

Specificity of RNA duplex-mediated gene silencing in mammalian muscle

The utility of RNA duplexes including long dsRNA, long or short hairpin RNA depends critically on its specificity, i.e., the ability to specifically silence the target gene without interfering the expression or function of other genes or proteins. In cultured cell-based knockout experiments, reports of dsRNA-mediated effects are still very controversial. Studies aimed to characterize the specificity of siRNA by using gene expression profiling have revealed either high specificity of siRNA-mediated gene silencing (Semizarov *et al.*, 2003; Chi *et al.*, 2003) or off-target gene regulation by siRNA (Sledz *et al.*, 2003; Jackson *et al.*, 2003). In addition to other possible explanations, the various ability of different cultured cell lines to elicit interferon response to dsRNA (Stojdl *et al.*, 2000) and the variable specificity of different siRNAs (Semizarov *et al.*, 2003) may critically contribute to these contradictory observations. In adult mammalian muscle, there are several non-specific effects that could be induced by the application of long dsRNA or expression constructs encoding long or short hairpin RNA, including interferon response, cross-hybridization, aptamer effect and micro RNA (miRNA) effect. In the following part, the possible occurrence of such non-specific effects in muscle is discussed.

Unlike in many organisms including plants (Baulcombe, 1999), *C. elegans* (Tijsterman *et al.*, 2002) and *Drosophila* (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999), where RNAi mediated by the introduction of long dsRNA allows the study of gene functions, long dsRNA has been replaced by siRNA in many mammalian systems (Elbashir *et al.*, 2001a) because the introduction of dsRNA longer than 30 nt induces non-specific interferon responses (Stark *et al.*, 1998), which overrides the

specific effect of long dsRNA by triggering general inhibition of mRNA translation (Paddison *et al.*, 2002b). However, recent reports have shown that introduction of siRNA (Sledz *et al.*, 2003) or vectors encoding shRNA (Bridge *et al.*, 2003) into cultured mammalian cells can induce interferon responses as well. In this work, we used the experimental system of transfecting only a limited number of muscle fibers, which allows the direct comparison between the experimental and the non-transfected, therefore control muscle fibers within the same muscle. This advantage is particularly important to assure that the observed silencing effect is selective for the target gene. We observed no general or gradual inhibition of protein synthesis accompanied with the use of long dsRNA and constructs encoding long or short hairpin RNA, indicating that muscle fibers may not respond to dsRNA and DNA plasmids in this sequence independent way. Like in our case, vector encoding long hairpin RNA has been shown to induce sequence-specific gene silencing but does not interfere the global translation in C2C12 cells (Yi *et al.*, 2003), a cultured cell lines that can fuse to myotubes upon induction (Yaffe and Saxel, 1977).

Cross-hybridization with transcripts containing partial identity to the sequence of siRNAs, the final processing product of all applied RNA duplexes, may elicit phenotypes reflecting silencing of unintended transcripts in addition to the target gene. The aptamer effect is the binding of siRNA duplex or its single strands to cellular proteins in a sequence-specific manner, which may influence the gene knockdown phenotype as it has been reported for gene silencing experiments mediated by antisense oligonucleotides (Brukner and Tremblay, 2000). In addition, siRNA may behave like miRNA by binding to transcripts with as many as 3-4 base mismatches (Saxena *et al.*, 2003) and causing translational repression of off-target mRNAs (Doench *et al.*, 2003). The use of long dsRNA or long hairpin RNA might reduce the intensity of off-target effects, because long RNA duplexes are processed into numerous subsets of siRNAs with identical sequence. Each subset is lowly concentrated and therefore may elicit less pronouced non-specific effects. However, the various siRNAs could elicit wide-spread non-specific effects. In contrast, shRNAs to a single site in the target mRNA give only identical siRNAs that may elicit limited but pronounced non-specificity. On the other hand, the use of shRNA provides the possibility to control cross-hybridization as a source of non-specificity. Unlike

aptamer and miRNA effects, off-target silencing mediated by cross-hybridization can be minimized by following stringent shRNA design rules as we did in our experiments. Especially the limitation of unwanted match was particularly important. A recent report has shown that siRNAs sharing 15 continuous nucleotide sequence identity is sufficient to silence non-targeted transcripts (Jackson *et al.*, 2003), indicating that our guideline to limit unwanted match <16 nt is useful, but still not sufficiently stringent (see appendix). The recent suggest of maximizing the difference of the melting temperature between the intended interfering RNA - target mRNA interaction and the most likely off-target interactions is plausible (Semizarov *et al.*, 2003). As siRNAs of different sequences are supposed to vary in their non-specificity, a simple way to eliminate off-target silencing is the use of two or more functional shRNAs that target different sites of the same mRNA.

In summary, this work demonstrates that RNAi is a powerful method to silence gene expression in adult mammalian muscle *in vivo*. The high specificity observed in this work increases the confidence with which phenotypes observed by dsRNA-mediated gene knockdown can be ascribed to the targeted genes. Many questions related to RNAi in adult mammalian muscle remain to be addressed. For example, the observed absence of an interferon response to long RNA duplexes is a surprising phenomenon, which requires an immunological explanation. Furthermore, improvement of the potency of long dsRNA to trigger gene silencing would provide an even more straightforward reverse genetic method compared to the use of siRNAs. Nevertheless, the functionality of RNAi demonstrated in this work established dsRNA-mediated gene silencing as a fast and valuable approach for large-scale screening of gene function in muscle *in vivo*. Moreover, this method may also be beneficial for future studies aimed at the treatment of particular muscular diseases.

Appendix

Design and analysis of small hairpin RNAs used to silence the expression of mTOR and its putative interactors in mouse muscle *in vivo*

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All work presented here was done by Xian Chu Kong except electroporation experiments and analysis of those experiments, which were done by Dr. Shuo Lin.

Abstract

RNA interference (RNAi) initiated by expression constructs encoding small hairpin RNA (shRNA) is a reliable and sustained method for sequence-specific gene silencing, which allows rapid survey of gene function in adult mammalian muscles *in vivo*. The key effector in the RNAi pathway is the ~21 nt small interfering RNA (siRNA), the processed product of shRNA, which directs degradation to mRNA by recognizing the homologous target site. The selection of the siRNA sequence is crucial, because siRNAs targeting different regions of the same mRNA vary strongly in their inability to trigger RNAi. The mechanism underlying this variation is still unknown. Here, we present a protocol that aimed at promoting the selection of effective siRNA sequences. By using this protocol, we designed shRNA expression vectors targeting the expression of mTOR and its putative interactors mLST8, SIN1, mAVO3, TSC1 and TSC2. The mTOR signaling pathway is suggested to control the growth of muscle fibers in mammals. Indeed, our preliminary results show that upon electro-transfection into denervated mouse muscles, every second shRNA constructs effectively altered the recovery of muscle fiber size. Analysis of these shRNAs shows that the efficient target sites along the mRNA coding sequence is mostly located in >300 nucleotides distance to the start codon, offering an useful guideline to narrow down the selection of site for siRNA.

Introduction

Small interfering RNAs (siRNAs) are ~21 nucleotides (nt) double-stranded RNAs (dsRNAs). The evidence that siRNA can knockdown gene expression by directing the sequence-specific degradation of the target mRNA in a process that is known as RNA interference (RNAi) (Elbashir *et al.*, 2001a), has lead to the rapid development of a powerful reverse genetic method, allowing fast and reliable assessment of gene functions in mammalian systems. Direct application of siRNA induces gene silencing of transient nature, while subsequently developed DNA-vector-based delivery systems prolong the silencing effect indefinitely (reviewed in Dykxhoorn *et al.*, 2003). Most of these expression systems take the advantage of the RNase III enzyme Dicer to process small hairpin RNA (shRNA) into siRNA, the core element in silencing the target gene (McManus and Sharp, 2002). These new technologies have enabled it to carry out stable gene silencing experiments in mammalian systems *in vitro* and *in vivo*, as well as to generate transgenic gene knockdown animals.

siRNA triggers degradation of the mRNA target by recognizing a single homologous sequence. To promote efficient gene silencing, consideration of the siRNA sequence is absolutely important, because siRNAs that target different sites of the same mRNA vary strongly in their effectiveness (Holen *et al.*, 2002; Miyagishi and Taira, 2002; Vickers *et al.*, 2003; Hemann *et al.*, 2003). Next to the base composition of a siRNA sequence, different factors including the secondary structure of the target mRNA and the presence of RNA-binding proteins could determine its effectiveness (Kretschmer-Kazemi Far and Sczakiel, 2003; Elbashir *et al.*, 2002). Significant correlation between RNase H sensitive regions and regions that provide efficient siRNA-directed mRNA degradation has been reported (Vickers *et al.*, 2003; Yang *et al.*, 2002). However, the precise rules governing the efficiency of siRNAs to silence genes are still not defined, and the selection of potential siRNAs remains a trial and error process. In mammalian cultured cell lines, 20 to 100% of selected siRNAs are functional depending on the study (reviewed in McManus and Sharp, 2002). To narrow the choices of potential siRNAs, a set of guidelines has

been proposed (Dykxhoorn *et al.*, 2003; Elbashir *et al.*, 2002). They mainly suggest that the selection of the target sequence should avoid regions of the mRNA which might bind RNA regulatory proteins, such as 5' and 3' UTR and the region proximal to the start site (<50-100 nt, with the start codon refers as 1). Between position 50-100 and the stop codon, 23 nt sequences conforming to several proposed consensus (see Dykxhoorn *et al.*, 2003) and consisting of approximately 50% GC should be selected from the mRNA sequence. Sequences of <30% or >70% GC content or sequences with stretches of any single nucleotides (especially G) should be avoided. In a further step, the selected siRNA sequences should be BLAST searched against sequence databases to ensure that a single gene is targeted.

Similar guidelines have been proposed for the selection of potential shRNA sequences (Dykxhoorn *et al.*, 2003; Paddison and Hannon, 2002). Different to the critical length of siRNAs, shRNAs with double stranded hairpin stem lengths of 19-29 nt have been shown to silence genes effectively (Brummelkamp *et al.*, 2002; McManus *et al.*, 2002; Paddison *et al.*, 2002a; Paddison *et al.*, 2002b; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002), which indicates that the stem length is not the main parameter governing effective target-gene silencing. Stems of 29 nt work 10%-40% more efficiently than stems of 19 nt, however longer stems could increase the possibility of "off-target effects" (Paddison and Hannon, 2002). In addition, the selection of the shRNA sequences is limited by the choice of the transcriptional promoter. Predominantly, two members of RNA polymerase III promoters have been used: the human and mouse U6-snRNA or the human RNase P [H1] promoter (Dykxhoorn *et al.*, 2003). The U6 promoter requires a G residue for efficient transcription initiation, whereas the H1 promoter is much more permissive. Transcriptional termination is initiated by a stretch of four to five A residues. The hairpin loop length seems unlikely to be a main parameter governing effective target-gene silencing, because loop size ranging from 2 to 23-nt have been described (reviewed in McManus and Sharp, 2002). In a direct comparison of 5-, 7- and 9-nt loops using a 19 nt duplex, the 9 nt loop (5'-UUCAAGAGA-3') was the most efficient silencer (Brummelkamp *et al.*, 2002). Finally, it has been suggested that 3-6 selected shRNA

sequences per gene should be tested to identify the most effective one (Paddison and Hannon, 2002).

Previously, by using *in vivo* electro-transfection of shRNA expression constructs, we have established RNAi as a fast and reliable method in adult rodent muscle for studying the requirement of unknown genes. Aimed to promote the efficiency of shRNA-mediated gene silencing *in vivo*, we present here a protocol for the selection of potential siRNA sequences and subsequently the incorporation of those sequences into shRNA expressing constructs. By using this protocol, we designed shRNA constructs targeting the expression of mTOR (mammalian target of rapamycin) and its putative interactors mLST8 (mammalian lethal with sec-thirteen 8), SIN1 (sty1 interactor), mAVO3 (mammalian adheres voraciously to TOR2 no.3), TSC1 and TSC2 (tuberous sclerosis complex 1 and 2) in mouse muscle. mTOR - a highly conserved member of the phosphatidylinositol kinase-related protein kinase (PIKK) family (Keith and Schreiber, 1995) – controls protein synthesis via regulating the phosphorylation of the translation activator S6K (ribosomal S6 kinase) and the translation inhibitor 4E-BP (eukaryotic initiation factor 4E-binding protein) (Brunn *et al.*, 1997; Burnett *et al.*, 1998; Isotani *et al.*, 1999). Several lines of evidence have indicated that the mTOR signaling pathway controls the growth of muscle fibers in response to nutrients, growth factors and the degree of muscle activity (Jacinto and Hall, 2003). The mechanisms through which mTOR signals and how the activity of mTOR is regulated in skeletal muscle are still unknown. Experiments carried out in mammalian cultured cells and in yeast have demonstrated that mLST8 interacts directly with mTOR and positively stimulates its kinase activity (Kim *et al.*, 2003). In contrast, binding of the TSC1-TSC2 complex to mTOR has an inhibition effect (Gao *et al.*, 2002; Inoki *et al.*, 2002; Tee *et al.*, 2002). SIN1 and mAVO3 are mammalian homologues of yeast AVO1 and AVO3; respectively, the functions of which in mammals is unknown (Jacinto and Hall, 2003; and personal communication with Dr. M. N. Hall). In yeast, AVO1 and AVO3 are associated in the TOR complex 2 that mediates actin cytoskeleton organization. Our preliminary results show that in re-innervated muscles, recovery of muscle size was perturbed by shRNAs targeting mTOR or SIN1, while targeting mAVO3 or TSC1 or TSC2 initiates muscle

hypertrophy. Silencing mLST8 expression has no effect. Subsequent analysis show that the target sites of those shRNAs, which efficiently induced alteration of the muscle fiber size, are preferentially located in distance to the start codon along the mRNA coding sequence, providing an additional useful guideline for the selection of potential shRNAs.

Results

Considering the requirements in the selection of potential target sequences for effective gene silencing and in the insertion of those sequences into expression constructs, we designed the following protocol for the design of shRNAs destined for use in adult mouse muscle. This protocol is based on a combination of guidelines described above (Dykxhoorn *et al.*, 2003; Paddison and Hannon, 2002; Elbashir *et al.*, 2002) and experience collected during our previous RNAi experiments *in vivo* (see chapter 2 and 4). It was established for the U6 promoter driven shRNA expression system and consists of two parts:

1. Selection of the target sequence

Select for 21 nt target sequences from the mRNA coding sequence between base position 50-100 (downstream of the start codon) and the stop codon conforming to the following requirements:

- Sequences should begin with AAG and end with less than 2 T residues in a row.
- Search for sequences with balanced representation of all nucleotides, stretches of a single nucleotide, especially >3 A or T or G residues in a raw should be avoided.
- Sequences that may form high temperature-resistant loops should be avoided.

To ensure that the selected sequences target only the intended gene, BLAST-search the selected sequences against EST libraries and mRNA sequences of the respective organism using the National Center for Biotechnology Information (NCBI) website. Minimize the maximal length of the homologous region with the best unwanted match <16 nt.

From all target sequences that fulfill the above criteria, we chose three candidates per gene. Taken that one out of two randomly-selected target sequences is functional (Elbashir *et al.*, 2002), we operated with a calculated success rate of 80-90% for obtaining a functional target site. To control for the specificity of the gene silencing

experiments, we targeted the expression of CD4, which is an protein specific to the immune system (Benoist and Mathis, 1999).

2. Designing the shRNA expression construct

The mouse U6 promoter was cloned into the pBluescript II KS+ vector according to method previously described (Yu *et al.*, 2002). An introduced *Bbs1* cloning site enables the insertion of shRNA sequences at the first nucleotide of the U6 transcript. shRNA sequences were synthesized as two complementary DNA oligonucleotides, annealed and inserted between the cloning sites *Bbs1* and *Xba1*.

I. Designing the sense strand of a shRNA construct:

- replace the 2 A residues at the 5' end by 3 T (the *Bbs1* cloning site);
- add the loop sequence TTCAAGAGA, which has been found to give more potent shRNAs compared to other loop sequences (Brummelkamp *et al.*, 2002), followed by the antisense strand of the target sequence and finally 3 T residues to the 3' end; the cluster of totally 5 T bases at the new 3' end terminates the transcription.

II. Designing the antisense strand of a shRNA construct:

- add the *Xho1* cloning sequence CTAG to the 5' end of the sequence complementary to the sense strand of the shRNA construct;
- remove the sequence CAAA from the 3' end.

DNA oligonucleotides of both strands were mixed, boiled and slowly cooled down for annealing. Preparation of cDNA was done with endotoxin-free reagents. shRNA expressing constructs in conjunction with plasmids encoding green fluorescent protein fused to a nuclear localization signal (NLS-GFP), which labeled the nuclei of transfected muscle fibers (Jones *et al.*, 1999), were electroporated into adult mouse soleus muscle *in vivo* (Gehl *et al.*, 1999).

In this study, we used the protocol to design shRNA constructs targeting the expression of mTOR and its putative interactors mLST8, SIN1, mAVO3, TSC1 and TSC2

in mouse muscle (Table 1). The TOR signaling pathway is a central controller of cell growth (Jacinto and Hall, 2003). In mammalian systems, silencing the expression of mTOR or mLST8, both positive elements of the mTOR pathway, in cultured cell lines results in reduced cell size (Kim *et al.*, 2003; Kim *et al.*, 2002), while loss of function mutations in the genes encoding the negative elements TSC1 or TSC2 causes tumorous cell growth (Goncharova *et al.*, 2002; Kwiatkowski *et al.*, 2002). The function of SIN1 and mAVO3 in mammals is unknown, their homologue in yeast, AVO1 and AVO3, are involved in the organization of the actin cytoskeleton (Loewith *et al.*, 2002). In mammals, the size of muscle fibers depends critically on the contact of motor neuron. Denervation causes muscle atrophy and re-innervation leads to recovery of muscle size. The molecular mechanisms of these changes are still not understood. Here, we asked whether the translation products of these six candidate genes are required in the recovery of muscle mass upon re-innervation. To this end, we denervated mouse soleus muscles by crushing the sciatic nerve. Subsequently, electro-transfecting of shRNA expression constructs into denervated muscles was performed. After six week, re-innervated muscles were examined. Our preliminary results show that targeting the expression of CD4 as a control, the size of shRNA constructs transfected muscle fibers was indistinguishable from that of the non-transfected muscle fibers (these and the following data are shown by Dr. Shuo Lin). In contrast, the mTOR or SIN1 targeted muscle fibers had significantly reduced size, whereas the AVO1, TSC1 or TSC2 targeted muscle fibers clearly increased their size compared to that of the non-transfected muscle fibers on the same muscle. When targeting the expression of mLST8, no effect on the muscle fibers size was observed.

Subsequently, we analyzed all the target sequences that effectively mediated gene silencing concerning to their location in the appropriate mRNA coding sequence (Table 1). Excluded from this analysis were 3 target sequences, which induced either a clear effect or no effect in independent experiments, and the 3 constructs targeting to the mLST8 expression, which caused no effect because either they were non-functional or mLST8 is non-essential in controlling of the muscle fiber size. 6 out of 12 shRNA expression constructs tested were effective. Among these constructs we found that the

target sequences were located on the mRNA in minimum 361 nt and an average of 700 nt downstream of the start codon. There was no correlation observed between the location of a target site and the length of the mRNA coding sequence.

Targeted genes	NCBI accession	Function	Length of mRNA	No. of siRNA	Location on mRNA	GC	Target sequence	Effectiveness
mTOR	NM_020009	Protein kinase, nutrient signaling	7650	1 2 3	752 892 1100	9 9 7	aagagaagggtatgaatcgag aagtactgcaaagacctcatg aagagaaatttgatcaggtgt	+ unclear -
mLST8	AF237676	Permease sorting, negatively regulates retrograde signaling proteins	981	1 2 3	256 472 781	10 10 10	aagaacattgcatcagtgggc aagacagaccacaatgagcag aagagtagtaaccctggagag	- - -
SIN1	AK052045	Actin cytoskeleton, Ras signaling	1569	1 2 3	264 412 979	9 8 10	aagattagaacgactccgcaa aagcagtccatattgtctgta aagcagagtgagcctaacatc	N/A unclear +
mAVO3	AK036149	Actin cytoskeleton, sphingolipid metabolism	>2472	1 2 3	335 660 833	8 8 9	aagattccagtattctccaga aagtcgaataaatgaggctct aagcacgatttctagccagta	- + unclear
TSC1	AB047561	Negatively regulates mTOR	3483	1 2 3	361 742 815	9 6 9	aagatggacactgatgttgtg aagacattagaaactcatgat aagatggctattctgtgtcac	+ N/A +
TSC2	U39818	Negatively regulates mTOR	5445	1 2 3	46 772 1215	9 9 8	aagatactgttgggattggga aagttgatgcgtaaccttctg aagatactatgaactggtgga	- + N/A

Table 1: Summary of siRNAs used in adult mouse muscle *in vivo*. **No. of siRNA:** number of tested siRNA. **Function:** Characterized or putative functions as listed by Jacinto and Hall, 2003. **Location on mRNA:** Location of the target site along the mRNA is expressed as the number of the first nucleotide downstream form the start codon. **GC:** Number of G and C residues in the target sequence. **Effectiveness:** The property of a shRNA to induce RNAi was investigated in minimum two eletro-transfection experiments. Targeting mLST8 caused no effect, either because mLST8 is not essential in the control of muscle mass or the shRNAs were inefficient. **+:** effective; **-:** no effect; **unclear:** no effect was observed in independent experiments; **N/A:** no result available.

Discussion

RNAi is a recently developed powerful reverse genetic method based on siRNA directed cleavage of the homologue target sequence on the mRNA (Dykxhoorn *et al.*, 2003). Among other open questions concerning this new technology, the observation that different target regions on the same mRNA vary strongly in their susceptibility to siRNA-mediated degradation is still an obstacle. Therefore, the selection of potential target sequences remains an empiric process. Here, we present a protocol for the design of potential shRNA expression constructs destined to induce RNAi in adult mouse muscle *in vivo*. We used this protocol to design shRNA constructs targeting the expression of mTOR and its putative interactors. Half of the tested constructs were efficient in inducing gene silencing in all experiments and one fourth were effective in some experiments. Compared to the suggestion of testing 3-6 selected shRNAs per gene to identify potential target sites (Paddison and Hannon, 2002), our preliminary result provides a likelihood of at least 50% given a set of 3 selected target sequences, indicating that the protocol presented in this work is useful for selecting efficient shRNA candidates. Although it has been suggested that target sequences should be selected in the coding region 50-100 nt downstream of the start codon (Elbashir *et al.*, 2002), our preliminary result demonstrates that the effective target sites are localized in greater distance - in mean 700 nt and in minimum 361 nt - to the start codon, which is consistent with the report of biased location of efficient target sequences towards the 3' portion of the mRNA (Dykxhoorn *et al.*, 2003). To optimize the protocol for future experiments, this finding may provide an additional guideline for narrowing down the selection of potential target sequences. However, additional studies are required to substantiate the information about regions of mRNA for RNAi targeting.

Another aspect of this work is that our preliminary results provide direct evidence that the mTOR signaling pathway is a crucial controller of the muscle mass. The findings that hypertrophic growth of skeletal muscles upon increased workload is rapamycin sensitive and that increase of signaling by mTOR and phosphorylation of mTOR itself is

associated with muscle hypertrophy (Rommel *et al.*, 2001; Bodine *et al.*, 2001; Pallafacchina *et al.*, 2002; Reynolds *et al.*, 2002; Baar and Esser, 1999) indicate that the mTOR pathway is involved in positive regulation of the muscle size. However, no direct evidence has been yet provided. Here, we demonstrate that in re-innervating muscles, silencing the expression of mTOR results in inhibition of muscle size recovery, while silencing the expression of TSC1 or TSC2, which are negative elements of the pathway, caused muscle hypertrophy. Interestingly, targeting the expression of SIN1 or mAVO3 results in opposite growth effects. Perturbation of SIN1 leads to reduced muscle fiber size, while increased muscle fiber size was observed in response to mAVO3 silencing. This finding is notable since the yeast homologue of both proteins have no function in cell growth (Loewith *et al.*, 2002), indicating that SIN1 and mAVO3 may play a different role in the control muscle mass. Finally, in mLST8 targeted muscles, no alteration of muscle mass was observed. This finding clearly differs from the report of reduced cell size upon silencing of mLST8 in cultured cell lines (Kim *et al.*, 2003). The absence of an effect indicates that either mLST8 is non-essential for the regulation of muscle fiber growth in re-innervated muscle or the applied shRNA constructs are ineffective in inducing gene silencing. Future quantification of mLST8 transcript or translational product in transfected muscle fibers will rule out one of the possibilities.

Methods

Plasmids. The NLS_GFP expression construct encoding green fluorescent protein (GFP) with a nuclear localization sequence (NLS) has been described elsewhere (Jones *et al.*, 1999). Vectors encoding shRNAs were described in result part (Table 1). The murine CD4 siRNA target sequences corresponding to nucleotides 494-514 of CD4 (M36850) was described in chapter 2. Sense and antisense oligonucleotides were annealed by boiling at 95°C for 5min and slowly cooling down to RT. Annealing buffer: 5mM EDTA, 50mM NaCl, pH=7.4; Endotoxin free kit used for preparation of cDNA was purchased from Qiagen.

Electroporation of cDNA into muscle fibers. Electroporation was done as described in chapter 2. Experiments were carried out in denervated mouse soleus muscles. All experimental procedures *in vivo* were performed as described in chapter 4. Muscles were analyzed six weeks after electroporation.

Denervation, electroporation and quantification of muscle fiber size were done by Dr. Shuo Lin. Paper in preparation.

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References

Ahlquist, P. (2002) RNA-dependent RNA polymerases, viruses, and RNA silencing. *Science*, 296, 1270-1273.

Anastasi, G., Cutroneo, G., Santoro, G. and Trimarchi, F. (1998) The non-junctional sarcolemmal cytoskeleton: the costameres. *Ital J Anat Embryol*, 103, 1-11.

Apel, E.D., Glass, D.J., Moscoso, L.M., Yancopoulos, G.D. and Sanes, J.R. (1997) Rapsyn is required for MuSK signalling and recruits synaptic components to a MuSK-containing scaffold. *Neuron*, 18, 623-635.

Azpiazu, I., Saltiel, A.R., DePaoli-Roach, A.A. and Lawrence, J.C. (1996) Regulation of both glycogen synthase and PHAS-I by insulin in rat skeletal muscle involves mitogen-activated protein kinase-independent and rapamycin-sensitive pathways. *J Biol Chem*, 271, 5033-5039.

Baar, K. and Esser, K. (1999) Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise. *Am J Physiol*, 276, C120-127.

Baulcombe, D.C. (1999) Gene silencing: RNA makes RNA makes no protein. *Curr Biol*, 9, R599-601.

Benoist, C. and Mathis, D. (1999) T-Lymphocyte Differentiation and Biology. In Paul, W.E. (ed.), *Fundamental Immunology*. Lippincott-Raven Publishers, Philadelphia, Vol. 1, pp. 367-409.

Bernstein, E., Caudy, A.A., Hammond, S.M. and Hannon, G.J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, 409, 363-366.

Bezakova, G., Helm, J.P., Francolini, M. and Lomo, T. (2001a) Effects of purified recombinant neural and muscle agrin on skeletal muscle fibers in vivo. *J Cell Biol*, 153, 1441-1452.

Bezakova, G., Rabben, I., Sefland, I., Fumagalli, G. and Lomo, T. (2001b) Neural agrin controls acetylcholine receptor stability in skeletal muscle fibers. *Proc Natl Acad Sci U S A*, 98, 9924-9929.

Bezakova, G. and Ruegg, M.A. (2003) New insights into the roles of agrin. *Nat Rev Mol Cell Biol*, 4, 295-308.

Billy, E., Brondani, V., Zhang, H., Muller, U. and Filipowicz, W. (2001) Specific interference with gene expression induced by long, double- stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc Natl Acad Sci U S A*, 98, 14428-14433.

Bodine, S.C., Stitt, T.N., Gonzalez, M., Kline, W.O., Stover, G.L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J.C., Glass, D.J. and Yancopoulos, G.D. (2001) Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol*, 3, 1014-1019.

Breaker, R.R. and Joyce, G.F. (1994) A DNA enzyme that cleaves RNA. *Chem Biol*, 1, 223-229.

Brenner, H.R., Witzemann, V. and Sakmann, B. (1990) Imprinting of acetylcholine receptor messenger RNA accumulation in mammalian neuromuscular synapses. *Nature*, 344, 544-547.

Bridge, A.J., Pebernard, S., Ducraux, A., Nicoulaz, A.L. and Iggo, R. (2003) Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet*, 34, 263-264.

Briguet, A. and Ruegg, M.A. (2000) The ets transcription factor GABP is required for postsynaptic differentiation in vivo. *J Neurosci*, 20, 5989-5996.

Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S. and Schreiber, S.L. (1994) A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature*, 369, 756-758.

Brukner, I. and Tremblay, G.A. (2000) Cellular proteins prevent antisense phosphorothioate oligonucleotide (SdT18) to target sense RNA (rA18): development of a new in vitro assay. *Biochemistry*, 39, 11463-11466.

Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science*, 296, 550-553.

Brunn, G.J., Hudson, C.C., Sekulic, A., Williams, J.M., Hosoi, H., Houghton, P.J., Lawrence, J.C., Jr. and Abraham, R.T. (1997) Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science*, 277, 99-101.

Burden, S.J., DePalma, R.L. and Gottesman, G.S. (1983) Crosslinking of proteins in acetylcholine receptor-rich membranes: association between the beta-subunit and the 43 kd subsynaptic protein. *Cell*, 35, 687-692.

Burnett, P.E., Barrow, R.K., Cohen, N.A., Snyder, S.H. and Sabatini, D.M. (1998) RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc Natl Acad Sci U S A*, 95, 1432-1437.

Caldwell, J.H. (2000) Clustering of sodium channels at the neuromuscular junction. *Microsc Res Tech*, 49, 84-89.

Caplen, N.J., Zheng, Z., Falgout, B. and Morgan, R.A. (2002) Inhibition of viral gene expression and replication in mosquito cells by dsRNA-triggered RNA interference. *Mol Ther*, 6, 243-251.

Carmell, M.A., Zhang, L., Conklin, D.S., Hannon, G.J. and Rosenquist, T.A. (2003) Germline transmission of RNAi in mice. *Nat Struct Biol*, 10, 91-92.

Charlton, M.R. and Nair, K.S. (1998) Role of hyperglucagonemia in catabolism associated with type 1 diabetes: effects on leucine metabolism and the resting metabolic rate. *Diabetes*, 47, 1748-1756.

Chi, J.T., Chang, H.Y., Wang, N.N., Chang, D.S., Dunphy, N. and Brown, P.O. (2003) Genomewide view of gene silencing by small interfering RNAs. *Proc Natl Acad Sci U S A*, 100, 6343-6346.

Cho-Chung, Y.S. and Becker, K.G. (2003) A genome-wide view of antisense. *Nat Biotechnol*, 21, 492.

Cohen, I., Rimer, M., Lomo, T. and McMahan, U.J. (1997) Agrin-induced postsynaptic apparatus in skeletal muscle fibers in vivo. *Mol. Cell. Neurosci.*, 9, 237-253.

Cohen, M.W. and Godfrey, E.W. (1992) Early appearance of and neuronal contribution to agrin-like molecules at embryonic frog nerve-muscle synapses formed in culture. *J Neurosci*, 12, 2982-2992.

Conlon, I. and Raff, M. (1999) Size control in animal development. *Cell*, 96, 235-244.

Covault, J. and Sanes, J.R. (1986) Distribution of N-CAM in synaptic and extrasynaptic portions of developing and adult skeletal muscle. *J Cell Biol*, 102, 716-730.

DeChiara, T.M., Bowen, D.C., Valenzuela, D.M., Simmons, M.V., Poueymirou, W.T., Thomas, S., Kinetz, E., Compton, D.L., Rojas, E., Park, J.S., Smith, C., DiStefano, P.S., Glass, D.J., Burden, S.J. and Yancopoulos, G.D. (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell*, 85, 501-512.

Deconinck, A.E., Potter, A.C., Tinsley, J.M., Wood, S.J., Vater, R., Young, C., Metzinger, L., Vincent, A., Slater, C.R. and Davies, K.E. (1997) Postsynaptic abnormalities at the neuromuscular junctions of utrophin- deficient mice. *J Cell Biol*, 136, 883-894.

Dennis, P.B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S.C. and Thomas, G. (2001) Mammalian TOR: a homeostatic ATP sensor. *Science*, 294, 1102-1105.

Denzer, A.J., Brandenberger, R., Gesemann, M., Chiquet, M. and Ruegg, M.A. (1997) Agrin binds to the nerve-muscle basal lamina via laminin. *J Cell Biol*, 137, 671-683.

Denzer, A.J., Gesemann, M., Schumacher, B. and Ruegg, M.A. (1995) An amino-terminal extension is required for the secretion of chick agrin and its binding to extracellular matrix. *J Cell Biol*, 131, 1547-1560.

Dixon, D. and Fordham-Skelton, T. (1998) Growth and development. *Curr Opin Plant Biol*, 1, 1.

Doench, J.G., Petersen, C.P. and Sharp, P.A. (2003) siRNAs can function as miRNAs. *Genes Dev*, 17, 438-442.

Dornburg, R. and Pomerantz, R.J. (2000) HIV-1 gene therapy: promise for the future. *Adv Pharmacol*, 49, 229-261.

Duclert, A. and Changeux, J.P. (1995) Acetylcholine receptor gene expression at the developing neuromuscular junction. *Physiol Rev*, 75, 339-368.

Duclos, F., Straub, V., Moore, S.A., Venzke, D.P., Hrstka, R.F., Crosbie, R.H., Durbeej, M., Lebakken, C.S., Ettinger, A.J., van der Meulen, J., Holt, K.H., Lim, L.E., Sanes, J.R., Davidson, B.L., Faulkner, J.A., Williamson, R. and Campbell, K.P. (1998) Progressive muscular dystrophy in alpha-sarcoglycan-deficient mice. *J Cell Biol*, 142, 1461-1471.

Dudley, N.R., Labbe, J.C. and Goldstein, B. (2002) Using RNA interference to identify genes required for RNA interference. *Proc Natl Acad Sci U S A*, 99, 4191-4196.

Dykxhoorn, D.M., Novina, C.D. and Sharp, P.A. (2003) Killing the messenger: short RNAs that silence gene expression. *Nat Rev Mol Cell Biol*, 4, 457-467.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001a) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, 411, 494-498.

Elbashir, S.M., Harborth, J., Weber, K. and Tuschl, T. (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods*, 26, 199-213.

Elbashir, S.M., Lendeckel, W. and Tuschl, T. (2001b) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev*, 15, 188-200.

Engel, A. (1994) The neuromuscular junction. *Myology: Basic and Clinical*, 261-302.

Eusebio, A., Oliveri, F., Barzaghi, P. and Ruegg, M.A. (2003) Expression of mouse agrin in normal, denervated and dystrophic muscle. *Neuromuscul Disord*, 13, 408-415.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, 391, 806-811.

Fischbach, G.D. and Rosen, K.M. (1997) ARIA: a neuromuscular junction neuregulin. *Annu Rev Neurosci*, 20, 429-458.

Flaim, K.E., Copenhaver, M.E. and Jefferson, L.S. (1980) Effects of diabetes on protein synthesis in fast- and slow-twitch rat skeletal muscle. *Am J Physiol*, 239, E88-95.

Flucher, B.E. and Daniels, M.P. (1989) Distribution of Na+ channels and ankyrin in neuromuscular junctions is complementary to that of acetylcholine receptors and the 43 kd protein. *Neuron*, 3, 163-175.

Franzini-Amstrong, C. (1994) The sarcoplasmic reticulum and the transverse tubules. In Franzini-Amstrong, A.E.a.C. (ed.), *A. Engel and C. Franzini-Amstrong*. McGraw-Hill, New York, pp. 176-199.

Freelove, A.C. and Zheng, R. (2002) The power of ribozyme technologies: the logical way ahead for molecular medicine and gene therapy? *Curr Opin Mol Ther*, 4, 419-422.

Gan, L., Anton, K.E., Masterson, B.A., Vincent, V.A., Ye, S. and Gonzalez-Zulueta, M. (2002) Specific interference with gene expression and gene function mediated by long dsRNA in neural cells. *J Neurosci Methods*, 121, 151-157.

Gao, X., Zhang, Y., Arrazola, P., Hino, O., Kobayashi, T., Yeung, R.S., Ru, B. and Pan, D. (2002) Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. *Nat Cell Biol*, 4, 699-704.

Garami, A., Zwartkruis, F.J., Nobukuni, T., Joaquin, M., Roccio, M., Stocker, H., Kozma, S.C., Hafen, E., Bos, J.L. and Thomas, G. (2003) Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol Cell*, 11, 1457-1466.

Gautam, M., Noakes, P.G., Moscoso, L., Rupp, F., Scheller, R.H., Merlie, J.P. and Sanes, J.R. (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell*, 85, 525-535.

Gautam, M., Noakes, P.G., Mudd, J., Nichol, M., Chu, G.C., Sanes, J.R. and Merlie, J.P. (1995) Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature*, 377, 232-236.

Gehl, J., Sorensen, T.H., Nielsen, K., Raskmark, P., Nielsen, S.L., Skovsgaard, T. and Mir, L.M. (1999) In vivo electroporation of skeletal muscle: threshold, efficacy and relation to electric field distribution. *Biochim Biophys Acta*, 1428, 233-240.

Gesemann, M., Denzer, A.J. and Ruegg, M.A. (1995) Acetylcholine receptor-aggregating activity of agrin isoforms and mapping of the active site. *J Cell Biol*, 128, 625-636.

Glass, D.J., Bowen, D.C., Stitt, T.N., Radziejewski, C., Bruno, J., Ryan, T.E., Gies, D.R., Shah, S., Mattsson, K., Burden, S.J., DiStefano, P.S., Valenzuela, D.M., DeChiara, T.M. and Yancopoulos, G.D. (1996) Agrin acts via a MuSK receptor complex. *Cell*, 85, 513-523.

Goldman, D., Brenner, H.R. and Heinemann, S. (1988) Acetylcholine receptor alpha-, beta-, gamma-, and delta-subunit mRNA levels are regulated by muscle activity. *Neuron*, 1, 329-333.

Gomer, R.H. (2001) Not being the wrong size. *Nat Rev Mol Cell Biol*, 2, 48-54.

Goncharova, E.A., Goncharov, D.A., Eszterhas, A., Hunter, D.S., Glassberg, M.K., Yeung, R.S., Walker, C.L., Noonan, D., Kwiatkowski, D.J., Chou, M.M., Panettieri, R.A., Jr. and Krymskaya, V.P. (2002) Tuberin regulates p70 S6 kinase activation and ribosomal protein S6 phosphorylation. A role for the TSC2 tumor suppressor gene in pulmonary lymphangioleiomyomatosis (LAM). *J Biol Chem*, 277, 30958-30967.

Goodearl, A.D.J., Yee, A.G., Sandrock, A.W., Jr., Corfas, G. and Fischbach, G.D. (1995) ARIA is concentrated in the synaptic basal lamina of the developing chick neuromuscular junction. *J Cell Biol*, 130, 1423-1434.

Gordon, J.W. (1993) Production of transgenic mice. *Methods Enzymol*, 225, 747-771.

Grady, R.M., Merlie, J.P. and Sanes, J.R. (1997) Subtle neuromuscular defects in utrophin-deficient mice. *J Cell Biol*, 136, 871-882.

Grishok, A., Tabara, H. and Mello, C.C. (2000) Genetic requirements for inheritance of RNAi in C. elegans. *Science*, 287, 2494-2497.

Grzelkowska, K., Dardevet, D., Balage, M. and Grizard, J. (1999) Involvement of the rapamycin-sensitive pathway in the insulin regulation of muscle protein synthesis in streptozotocin-diabetic rats. *J Endocrinol*, 160, 137-145.

Hamilton, A.J. and Baulcombe, D.C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science*, 286, 950-952.

Hammond, S.M., Bernstein, E., Beach, D. and Hannon, G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature*, 404, 293-296.

Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R. and Hannon, G.J. (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science*, 293, 1146-1150.

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J. and Yonezawa, K. (2002) Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell*, 110, 177-189.

Hara, K., Yonezawa, K., Weng, Q.P., Kozlowski, M.T., Belham, C. and Avruch, J. (1998) Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J Biol Chem*, 273, 14484-14494.

Hashemolhosseini, S., Moore, C., Landmann, L., Sander, A., Schwarz, H., Witzemann, V., Sakmann, B. and Brenner, H.R. (2000) Electrical activity and postsynapse formation in adult muscle: gamma- AChRs are not required. *Mol Cell Neurosci*, 16, 697-707.

Hasuwa, H., Kaseda, K., Einarsdottir, T. and Okabe, M. (2002) Small interfering RNA and gene silencing in transgenic mice and rats. *FEBS Lett*, 532, 227-230.

Hemann, M.T., Fridman, J.S., Zilfou, J.T., Hernando, E., Paddison, P.J., Cordon-Cardo, C., Hannon, G.J. and Lowe, S.W. (2003) An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes in vivo. *Nat Genet*, 33, 396-400.

Hoch, W., McConville, J., Helms, S., Newsom-Davis, J., Melms, A. and Vincent, A. (2001) Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. *Nat Med*, 7, 365-368.

Holen, T., Amarzguioui, M., Wiiger, M.T., Babaie, E. and Prydz, H. (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. *Nucleic Acids Res*, 30, 1757-1766.

Hornberger, T.A., Hunter, R.B., Kandarian, S.C. and Esser, K.A. (2001) Regulation of translation factors during hindlimb unloading and denervation of skeletal muscle in rats. *Am J Physiol Cell Physiol*, 281, C179-187.

Horowitz, P. (1994) Excitation-contraction coupling in slcletal muscle. In Franzini-Amstrong, A.E.a.C. (ed.), *A. Engel and C. Franzini-Amstrong*. McGraw-Hill, New York, pp. 423-441.

Hough, S.R., Wiederholt, K.A., Burrier, A.C., Woolf, T.M. and Taylor, M.F. (2003) Why RNAi makes sense. *Nat Biotechnol*, 21, 731-732.

Inoki, K., Li, Y., Zhu, T., Wu, J. and Guan, K.L. (2002) TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol*, 4, 648-657.

Isotani, S., Hara, K., Tokunaga, C., Inoue, H., Avruch, J. and Yonezawa, K. (1999) Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase alpha in vitro. *J Biol Chem*, 274, 34493-34498.

Jacinto, E. and Hall, M.N. (2003) Tor signalling in bugs, brain and brawn. *Nat Rev Mol Cell Biol*, 4, 117-126.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G. and Linsley, P.S. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol*, 21, 635-637.

Jo, S.A., Zhu, X., Marchionni, M.A. and Burden, S.J. (1995) Neuregulins are concentrated at nerve-muscle synapses and activate ACh-receptor gene expression. *Nature*, 373, 158-161.

Johnston, L.A. and Gallant, P. (2002) Control of growth and organ size in Drosophila. *Bioessays*, 24, 54-64.

Jones, G., Meier, T., Lichtsteiner, M., Witzemann, V., Sakmann, B. and Brenner, H.R. (1997) Induction by agrin of ectopic and functional postsynaptic-like membrane in innervated muscle. *Proc Natl Acad Sci U S A*, 94, 2654-2659.

Jones, G., Moore, C., Hashemolhosseini, S. and Brenner, H.R. (1999) Constitutively active MuSK is clustered in the absence of agrin and induces ectopic postsynaptic-like membranes in skeletal muscle fibers. *J Neurosci*, 19, 3376-3383.

Keith, C.T. and Schreiber, S.L. (1995) PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints. *Science*, 270, 50-51.

Kennerdell, J.R. and Carthew, R.W. (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell*, 95, 1017-1026.

Kennerdell, J.R. and Carthew, R.W. (2000) Heritable gene silencing in Drosophila using double-stranded RNA. *Nat Biotechnol*, 18, 896-898.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J. and Plasterk, R.H. (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes Dev*, 15, 2654-2659.

Ketting, R.F., Haverkamp, T.H., van Luenen, H.G. and Plasterk, R.H. (1999) Mut-7 of C. elegans, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell*, 99, 133-141.

Khan, A.U. and Lal, S.K. (2003) Ribozymes: a modern tool in medicine. *J Biomed Sci*, 10, 457-467.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P. and Sabatini, D.M. (2002) mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell*, 110, 163-175.

Kim, D.H., Sarbassov dos, D., Ali, S.M., Latek, R.R., Guntur, K.V., Erdjument-Bromage, H., Tempst, P. and Sabatini, D.M. (2003) GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Mol Cell*, 11, 895-904.

Kitabwalla, M. and Ruprecht, R.M. (2002) RNA interference--a new weapon against HIV and beyond. *N Engl J Med*, 347, 1364-1367.

Krejci, E., Thomine, S., Boschetti, N., Legay, C., Sketelj, J. and Massoulie, J. (1997) The mammalian gene of acetylcholinesterase-associated collagen. *J Biol Chem*, 272, 22840-22847.

Kretschmer-Kazemi Far, R. and Sczakiel, G. (2003) The activity of siRNA in mammalian cells is related to structural target accessibility: a comparison with antisense oligonucleotides. *Nucleic Acids Res*, 31, 4417-4424.

Kunath, T., Gish, G., Lickert, H., Jones, N., Pawson, T. and Rossant, J. (2003) Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. *Nat Biotechnol*, 21, 559-561.

Kurreck, J. (2003) Antisense technologies. Improvement through novel chemical modifications. *Eur J Biochem*, 270, 1628-1644.

Kwiatkowski, D.J., Zhang, H., Bandura, J.L., Heiberger, K.M., Glogauer, M., el-Hashemite, N. and Onda, H. (2002) A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. *Hum Mol Genet*, 11, 525-534.

Lewis, D.L., Hagstrom, J.E., Loomis, A.G., Wolff, J.A. and Herweijer, H. (2002) Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat Genet*, 32, 107-108.

Li, H., Li, W.X. and Ding, S.W. (2002) Induction and suppression of RNA silencing by an animal virus. *Science*, 296, 1319-1321.

Li, Y.X., Farrell, M.J., Liu, R., Mohanty, N. and Kirby, M.L. (2000) Double-stranded RNA injection produces null phenotypes in zebrafish. *Dev Biol*, 217, 394-405.

Lindbo, J.A. and Dougherty, W.G. (1992) Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. *Virology*, 189, 725-733.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P. and Hall, M.N. (2002) Two TOR Complexes, Only One of which Is Rapamycin Sensitive, Have Distinct Roles in Cell Growth Control. *Mol Cell*, 10, 457-468.

Lohmann, J.U., Endl, I. and Bosch, T.C. (1999) Silencing of developmental genes in Hydra. *Dev Biol*, 214, 211-214.

Lois, C., Hong, E.J., Pease, S., Brown, E.J. and Baltimore, D. (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science*, 295, 868-872.

Martin, P.T., Kaufman, S.J., Kramer, R.H. and Sanes, J.R. (1996) Synaptic integrins in developing, adult, and mutant muscle: selective association of a1, a7A, and a7B integrins with the neuromuscular junction. *Dev Biol*, 174, 125-139.

Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R. and Tuschl, T. (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell*, 110, 563-574.

McCaffrey, A.P., Meuse, L., Pham, T.T., Conklin, D.S., Hannon, G.J. and Kay, M.A. (2002) RNA interference in adult mice. *Nature*, 418, 38-39.

McCaffrey, A.P., Nakai, H., Pandey, K., Huang, Z., Salazar, F.H., Xu, H., Wieland, S.F., Marion, P.L. and Kay, M.A. (2003) Inhibition of hepatitis B virus in mice by RNA interference. *Nat Biotechnol*, 21, 639-644.

McMahan, U.J. (1990) The agrin hypothesis. *Cold Spring Harb Symp Quant Biol*, 55, 407-418.

McManus, M.T., Petersen, C.P., Haines, B.B., Chen, J. and Sharp, P.A. (2002) Gene silencing using micro-RNA designed hairpins. *Rna*, 8, 842-850.

McManus, M.T. and Sharp, P.A. (2002) Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet*, 3, 737-747.

Meier, T., Hauser, D.M., Chiquet, M., Landmann, L., Ruegg, M.A. and Brenner, H.R. (1997) Neural agrin induces ectopic postsynaptic specializations in innervated muscle fibers. *J Neurosci*, 17, 6534-6544.

Meier, T., Masciulli, F., Moore, C., Schoumacher, F., Eppenberger, U., Denzer, A.J., Jones, G. and Brenner, H.R. (1998) Agrin can mediate acetylcholine receptor gene expression in muscle by aggregation of muscle-derived neuregulins. *J Cell Biol*, 141, 715-726.

Merlie, J.P., Isenberg, K.E., Russell, S.D. and Sanes, J.R. (1984) Denervation supersensitivity in skeletal muscle: analysis with a cloned cDNA probe. *J Cell Biol*, 99, 332-335.

Mishina, M., Takai, T., Imoto, K., Noda, M., Takahashi, T., Numa, S., Methfessel, C. and Sakmann, B. (1986) Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. *Nature*, 321, 406-411.

Misquitta, L. and Paterson, B.M. (1999) Targeted disruption of gene function in Drosophila by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. *Proc Natl Acad Sci U S A*, 96, 1451-1456.

Miyagishi, M., Hayashi, M. and Taira, K. (2003) Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. *Antisense Nucleic Acid Drug Dev*, 13, 1-7.

Miyagishi, M. and Taira, K. (2002) U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat Biotechnol*, 20, 497-500.

Moore, C., Leu, M., Muller, U. and Brenner, H.R. (2001) Induction of multiple signaling loops by MuSK during neuromuscular synapse formation. *Proc Natl Acad Sci U S A*, 98, 14655-14660.

Moscoso, L.M., Chu, G.C., Gautam, M., Noakes, P.G., Merlie, J.P. and Sanes, J.R. (1995) Synapse-associated expression of an acetylcholine receptor-inducing protein, ARIA/heregulin, and its putative receptors, ErbB2 and ErbB3, in developing mammalian muscle. *Dev Biol*, 172, 158-169.

Musaro, A., McCullagh, K.J., Naya, F.J., Olson, E.N. and Rosenthal, N. (1999) IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. *Nature*, 400, 581-585.

Napoli, C., Lemieux, C. and Jorgensen, R. (1990) Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell*, 2, 279-289.

Ngo, H., Tschudi, C., Gull, K. and Ullu, E. (1998) Double-stranded RNA induces mRNA degradation in Trypanosoma brucei. *Proc Natl Acad Sci U S A*, 95, 14687-14692.

Noakes, P.G., Phillips, W.D., Hanley, T.A., Sanes, J.R. and Merlie, J.P. (1993) 43K protein and acetylcholine receptors colocalize during the initial stages of neuromuscular synapse formation in vivo. *Dev Biol*, 155, 275-280.

Nykanen, A., Haley, B. and Zamore, P.D. (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell*, 107, 309-321.

Ogata, T. (1988) Structure of motor endplates in the different fiber types of vertebrate skeletal muscles. *Arch Histol Cytol*, 51, 385-424.

Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J. and Conklin, D.S. (2002a) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev*, 16, 948-958.

Paddison, P.J., Caudy, A.A. and Hannon, G.J. (2002b) Stable suppression of gene expression by RNAi in mammalian cells. *Proc Natl Acad Sci U S A*, 99, 1443-1448.

Paddison, P.J. and Hannon, G.J. (2002) RNA interference: the new somatic cell genetics? *Cancer Cell*, 2, 17-23.

Pain, V.M. and Garlick, P.J. (1974) Effect of streptozotocin diabetes and insulin treatment on the rate of protein synthesis in tissues of the rat in vivo. *J Biol Chem*, 249, 4510-4514.

Pal-Bhadra, M., Bhadra, U. and Birchler, J.A. (1997) Cosuppression in Drosophila: gene silencing of Alcohol dehydrogenase by white-Adh transgenes is Polycomb dependent. *Cell*, 90, 479-490.

Pal-Bhadra, M., Bhadra, U. and Birchler, J.A. (2002) RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in Drosophila. *Mol Cell*, 9, 315-327.

Pallafacchina, G., Calabria, E., Serrano, A.L., Kalhovde, J.M. and Schiaffino, S. (2002) A protein kinase B-dependent and rapamycin-sensitive pathway controls skeletal muscle growth but not fiber type specification. *Proc Natl Acad Sci U S A*, 99, 9213-9218.

Pardo, J.V., Siliciano, J.D. and Craig, S.W. (1983) A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements mark sites of attachment between myofibrils and sarcolemma. *Proc Natl Acad Sci U S A*, 80, 1008-1012.

Parrish, S., Fleenor, J., Xu, S., Mello, C. and Fire, A. (2000) Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. *Mol Cell*, 6, 1077-1087.

Patton, B.L., Miner, J.H., Chiu, A.Y. and Sanes, J.R. (1997) Distribution and function of laminins in the neuromuscular system of developing, adult, and mutant mice. *J Cell Biol*, 139, 1507-1521.

Paul, C.P., Good, P.D., Winer, I. and Engelke, D.R. (2002) Effective expression of small interfering RNA in human cells. *Nat Biotechnol*, 20, 505-508.

Pekarik, V., Bourikas, D., Miglino, N., Joset, P., Preiswerk, S. and Stoeckli, E.T. (2003) Screening for gene function in chicken embryo using RNAi and electroporation. *Nat Biotechnol*, 21, 93-96.

Peters, M.F., Sadoulet-Puccio, H.M., Grady, M.R., Kramarcy, N.R., Kunkel, L.M., Sanes, J.R., Sealock, R. and Froehner, S.C. (1998) Differential membrane localization and

intermolecular associations of alpha-dystrobrevin isoforms in skeletal muscle. *J Cell Biol*, 142, 1269-1278.

Piette, J., Huchet, M., Houzelstein, D. and Changeux, J.P. (1993) Compartmentalized expression of the alpha- and gamma-subunits of the acetylcholine receptor in recently fused myofibers. *Dev Biol*, 157, 205-213.

Plasterk, R.H. (2002) RNA silencing: the genome's immune system. *Science*, 296, 1263-1265.

Potter, C.J., Pedraza, L.G. and Xu, T. (2002) Akt regulates growth by directly phosphorylating Tsc2. *Nat Cell Biol*, 4, 658-665.

Price, S.R., Bailey, J.L., Wang, X., Jurkovitz, C., England, B.K., Ding, X., Phillips, L.S. and Mitch, W.E. (1996) Muscle wasting in insulinopenic rats results from activation of the ATP-dependent, ubiquitin-proteasome proteolytic pathway by a mechanism including gene transcription. *J Clin Invest*, 98, 1703-1708.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R. and Ruvkun, G. (2000) The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature*, 403, 901-906.

Reist, N.E., Werle, M.J. and McMahan, U.J. (1992) Agrin released by motor neurons induces the aggregation of acetylcholine receptors at neuromuscular junctions. *Neuron*, 8, 865-868.

Reynolds, T.H.t., Bodine, S.C. and Lawrence, J.C., Jr. (2002) Control of Ser2448 phosphorylation in the mammalian target of rapamycin by insulin and skeletal muscle load. *J Biol Chem*, 277, 17657-17662.

Rimer, M., Cohen, I., Lomo, T., Burden, S.J. and McMahan, U.J. (1998) Neuregulins and erbB receptors at neuromuscular junctions and at agrin- induced postsynaptic-like apparatus in skeletal muscle. *Mol Cell Neurosci*, 12, 1-15.

Rimer, M., Mathiesen, I., Lomo, T. and McMahan, U.J. (1997) g-AChR/e-AChR switch at agrin-induced postsynaptic-like apparatus in skeletal muscle. *Mol. Cell. Neurosci.*, 9, 254-263.

Romano, N. and Macino, G. (1992) Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. *Mol Microbiol*, 6, 3343-3353.

Rommel, C., Bodine, S.C., Clarke, B.A., Rossman, R., Nunez, L., Stitt, T.N., Yancopoulos, G.D. and Glass, D.J. (2001) Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol*, 3, 1009-1013.

Roy, R.R., Talmadge, R.J., Fox, K., Lee, M., Ishihara, A. and Edgerton, V.R. (1997) Modulation of MHC isoforms in functionally overloaded and exercised rat plantaris fibers. *J Appl Physiol*, 83, 280-290.

Rubinson, D.A., Dillon, C.P., Kwiatkowski, A.V., Sievers, C., Yang, L., Kopinja, J., Rooney, D.L., Ihrig, M.M., McManus, M.T., Gertler, F.B., Scott, M.L. and Van Parijs, L. (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet*, 33, 401-406.

Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P. and Snyder, S.H. (1994) RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin- dependent fashion and is homologous to yeast TORs. *Cell*, 78, 35-43.

Sabers, C.J., Martin, M.M., Brunn, G.J., Williams, J.M., Dumont, F.J., Wiederrecht, G. and Abraham, R.T. (1995) Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. *J Biol Chem*, 270, 815-822.

Salpeter, M.M. and Loring, R.H. (1985) Nicotinic acetylcholine receptors in vertebrate muscle: properties, distribution and neural control. *Prog Neurobiol*, 25, 297-325.

Sanchez Alvarado, A. and Newmark, P.A. (1999) Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc Natl Acad Sci U S A*, 96, 5049-5054.

Sanes, J.R. and Lichtman, J.W. (1999) Development of the vertebrate neuromuscular junction. *Annu Rev Neurosci*, 22, 389-442.

Sanes, J.R. and Lichtman, J.W. (2001) Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat Rev Neurosci*, 2, 791-805.

Saucedo, L.J., Gao, X., Chiarelli, D.A., Li, L., Pan, D. and Edgar, B.A. (2003) Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nat Cell Biol*, 5, 566-571.

Saunders, R.N., Metcalfe, M.S. and Nicholson, M.L. (2001) Rapamycin in transplantation: a review of the evidence. *Kidney Int*, 59, 3-16.

Saxena, S., Jonsson, Z.O. and Dutta, A. (2003) Small RNAs with imperfect match to endogenous mRNA repress translation: implications for off-target activity of siRNA in mammalian cells. *J Biol Chem*.

Sealock, R., Wray, B.E. and Froehner, S.C. (1984) Ultrastructural localization of the Mr 43,000 protein and the acetylcholine receptor in Torpedo postsynaptic membranes using monoclonal antibodies. *J Cell Biol*, 98, 2239-2244.

Semizarov, D., Frost, L., Sarthy, A., Kroeger, P., Halbert, D.N. and Fesik, S.W. (2003) Specificity of short interfering RNA determined through gene expression signatures. *Proc Natl Acad Sci U S A*, 100, 6347-6352.

Semsarian, C., Wu, M.J., Ju, Y.K., Marciniec, T., Yeoh, T., Allen, D.G., Harvey, R.P. and Graham, R.M. (1999) Skeletal muscle hypertrophy is mediated by a Ca2+-dependent calcineurin signalling pathway. *Nature*, 400, 576-581.

Shah, O.J., Anthony, J.C., Kimball, S.R. and Jefferson, L.S. (2000) 4E-BP1 and S6K1: translational integration sites for nutritional and hormonal information in muscle. *Am J Physiol Endocrinol Metab*, 279, E715-729.

Shioi, T., McMullen, J.R., Kang, P.M., Douglas, P.S., Obata, T., Franke, T.F., Cantley, L.C. and Izumo, S. (2002) Akt/protein kinase B promotes organ growth in transgenic mice. *Mol Cell Biol*, 22, 2799-2809.

Shuey, D.J., McCallus, D.E. and Giordano, T. (2002) RNAi: gene-silencing in therapeutic intervention. *Drug Discov Today*, 7, 1040-1046.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H. and Fire, A. (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell*, 107, 465-476.

Sledz, C.A., Holko, M., de Veer, M.J., Silverman, R.H. and Williams, B.R. (2003) Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol*, 5, 834-839.

Song, E., Lee, S.K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P. and Lieberman, J. (2003) RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat Med*, 9, 347-351.

Sparagana, S.P. and Roach, E.S. (2000) Tuberous sclerosis complex. *Curr Opin Neurol*, 13, 115-119.

Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H. and Schreiber, R.D. (1998) How cells respond to interferons. *Annu Rev Biochem*, 67, 227-264.

Stein, P., Svoboda, P. and Schultz, R.M. (2003) Transgenic RNAi in mouse oocytes: a simple and fast approach to study gene function. *Dev Biol*, 256, 187-193.

Sternberg, E.A., Spizz, G., Perry, W.M., Vizard, D., Weil, T. and Olson, E.N. (1988) Identification of upstream and intragenic regulatory elements that confer cell-type-restricted and differentiation-specific expression on the muscle creatine kinase gene. *Mol Cell Biol*, 8, 2896-2909.

Stocker, H. and Hafen, E. (2000) Genetic control of cell size. *Curr Opin Genet Dev*, 10, 529-535.

Stojdl, D.F., Lichty, B., Knowles, S., Marius, R., Atkins, H., Sonenberg, N. and Bell, J.C. (2000) Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat Med*, 6, 821-825.

Sui, G., Soohoo, C., Affar el, B., Gay, F., Shi, Y. and Forrester, W.C. (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci U S A*, 99, 5515-5520.

Sullenger, B.A. and Gilboa, E. (2002) Emerging clinical applications of RNA. *Nature*, 418, 252-258.

Svoboda, P., Stein, P., Hayashi, H. and Schultz, R.M. (2000) Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. *Development*, 127, 4147-4156.

Svoboda, P., Stein, P. and Schultz, R.M. (2001) RNAi in mouse oocytes and preimplantation embryos: effectiveness of hairpin dsRNA. *Biochem Biophys Res Commun*, 287, 1099-1104.

Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A. and Mello, C.C. (1999) The rde-1 gene, RNA interference, and transposon silencing in C. elegans. *Cell*, 99, 123-132.

Tee, A.R., Fingar, D.C., Manning, B.D., Kwiatkowski, D.J., Cantley, L.C. and Blenis, J. (2002) Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. *Proc Natl Acad Sci U S A*, 99, 13571-13576.

Thomason, D.B., Herrick, R.E., Surdyka, D. and Baldwin, K.M. (1987) Time course of soleus muscle myosin expression during hindlimb suspension and recovery. *J Appl Physiol*, 63, 130-137.

Tijsterman, M., Ketting, R.F. and Plasterk, R.H. (2002) The genetics of RNA silencing. *Annu Rev Genet*, 36, 489-519.

Tiscornia, G., Singer, O., Ikawa, M. and Verma, I.M. (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc Natl Acad Sci U S A*, 100, 1844-1848.

Tsay, H.J. and Schmidt, J. (1989) Skeletal muscle denervation activates acetylcholine receptor genes. *J Cell Biol*, 108, 1523-1526.

Tsen, G., Halfter, W., Kroger, S. and Cole, G.J. (1995) Agrin is a heparan sulfate proteoglycan. *J Biol Chem*, 270, 3392-3399.

Usdin, T.B. and Fischbach, G.D. (1986) Purification and characterization of a polypeptide from chick brain that promotes the accumulation of acetylcholine receptors in chick myotubes. *J Cell Biol*, 103, 493-507.

Valenzuela, D.M., Stitt, T.N., DiStefano, P.S., Rojas, E., Mattsson, K., Compton, D.L., Nunez, L., Park, J.S., Stark, J.L., Gies, D.R., Thomas, S., Le, B.M.M., Fernald, A.A., Copeland, N.G., Jenkins, N.A., Burden, S.J., Glass, D.J. and Yancopoulos, G.D. (1995) Receptor tyrosine kinase specific for the skeletal muscle lineage:

Expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron*, 15, 573-584.

van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N. and Stuitje, A.R. (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell*, 2, 291-299.

Vickers, T.A., Koo, S., Bennett, C.F., Crooke, S.T., Dean, N.M. and Baker, B.F. (2003) Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis. *J Biol Chem*, 278, 7108-7118.

Vogt, P.K. (2001) PI 3-kinase, mTOR, protein synthesis and cancer. *Trends Mol Med*, 7, 482-484.

Wianny, F. and Zernicka-Goetz, M. (2000) Specific interference with gene function by double-stranded RNA in early mouse development. *Nat Cell Biol*, 2, 70-75.

Wightman, B., Ha, I. and Ruvkun, G. (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell*, 75, 855-862.

Winston, W.M., Molodowitch, C. and Hunter, C.P. (2002) Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. *Science*, 295, 2456-2459.

Witzemann, V., Barg, B., Nishikawa, Y., Sakmann, B. and Numa, S. (1987) Differential regulation of muscle acetylcholine receptor gamma- and epsilon-subunit mRNAs. *FEBS Lett*, 223, 104-112.

Witzemann, V., Brenner, H.R. and Sakmann, B. (1991) Neural factors regulate AChR subunit mRNAs at rat neuromuscular synapses. *J Cell Biol*, 114, 125-141.

Wood, S.J. and Slater, C.R. (1998) beta-Spectrin is colocalized with both voltage-gated sodium channels and ankyrinG at the adult rat neuromuscular junction. *J Cell Biol*, 140, 675-684.

Xia, H., Mao, Q., Paulson, H.L. and Davidson, B.L. (2002) siRNA-mediated gene silencing in vitro and in vivo. *Nat Biotechnol*, 20, 1006-1010.

Yaffe, D. and Saxel, O. (1977) Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature*, 270, 725-727.

Yang, D., Buchholz, F., Huang, Z., Goga, A., Chen, C.Y., Brodsky, F.M. and Bishop, J.M. (2002) Short RNA duplexes produced by hydrolysis with Escherichia coli RNase III mediate effective RNA interference in mammalian cells. *Proc Natl Acad Sci U S A*, 99, 9942-9947.

Yang, D., Lu, H. and Erickson, J.W. (2000) Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in Drosophila embryos. *Curr Biol*, 10, 1191-1200.

Yi, C.E., Bekker, J.M., Miller, G., Hill, K.L. and Crosbie, R.H. (2003) Specific and potent RNA interference in terminally differentiated myotubes. *J Biol Chem*, 278, 934-939.

Yu, J.Y., DeRuiter, S.L. and Turner, D.L. (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci U S A*, 99, 6047-6052.

Zamore, P.D. (2002) Ancient pathways programmed by small RNAs. *Science*, 296, 1265-1269.

Zamore, P.D., Tuschl, T., Sharp, P.A. and Bartel, D.P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*, 101, 25-33.

Zhang, Y., Gao, X., Saucedo, L.J., Ru, B., Edgar, B.A. and Pan, D. (2003) Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat Cell Biol*, 5, 578-581.

Zhou, Y., Ching, Y.P., Kok, K.H., Kung, H.F. and Jin, D.Y. (2002) Post-transcriptional suppression of gene expression in Xenopus embryos by small interfering RNA. *Nucleic Acids Res*, 30, 1664-1669.

Zhu, X.J., Lai, C., Thomas, S. and Burden, S.J. (1995) Neuregulin receptors, erbB3 and erbB4, are localized at neuromuscular synapses. *Embo J*, 14, 5842-5848.

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