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Adverse effects induced by short hairpin RNA expression in porcine fetal fibroblasts

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ABSTRACT

RNA interference is a recent, gene silencing technique that could be extremely valuable in studying gene function, treating diseases, and developing novel animal models for human diseases. Here, we investigated the feasibility of applying shRNA-mediated RNA interference in fetal fibroblasts for silencing of the myostatin gene and investigate adverse effects of RNAi. We report that up to 97% silencing of myostatin mRNA was achieved using shRNA constructs in transiently and stably transfected fetal fibroblasts ($p < 0.05$). At the same time we also demonstrate that high level of shRNA expression resulted in 10- to 1000-fold induction of interferon responsive genes (OAS1, IFN-β) ($p < 0.05$). In addition we also report novel adverse effect of shRNA expression in stably transfected cells—interference with microRNA processing/transport which led to 500-fold increase in the level of miR21 precursors ($p < 0.05$). Reduction of these side effects will be essential to obtain long term stable RNAi silencing.

RNA interference (RNAi) become a popular techniques to achieve sequence-specific knockdown of target mRNA as it displays 100- to 1000-fold higher efficiency than antisense oligonucleotides or ribozymes [1,2]. Two approaches are generally used to induce RNA interference in mammalian cells. One relies on transfection with synthetic small interfering RNA (siRNA) and while very effective will only induce transient silencing. Stable induction of RNAi usually utilizes short hairpin RNA (shRNA) expressed from plasmid or viral vectors. Such shRNA mimic structure of endogenous microRNA (miRNA) and utilize their processing and transport pathways [3,4]. The miRNAs are endogenous RNAs that play important regulatory roles by targeting mRNAs for cleavage or translational repression. These short RNA species are derived from primary miRNA (pri-miRNAs) transcripts that are single-stranded precursors up to 2 kb in length. These precursors are identified and processed in the nucleus by the complex of RNase III enzyme Drosha, DGCR8 and RNASEN to long (~70 nt) endogenous hairpin precursors (pre-miRNAs) [5,6]. These 70-nt-long hairpin precursors are exported out of the nucleus by Ran-GTP and a receptor Exportin-5. Mature 21–25 nt miRNAs are produced in cytoplasm by Dicer nuclease cleavage of precursors to produce mature miRNA with antisense strand being incorporated into the RISC complex and mediating gene silencing. Ability to obtain potent and stable RNAi silencing is critical for number of application especially for gene knockdown in transgenic animals. Yet the side effects of stable long term silencing are still poorly understood as majority of RNAi studies investigated short term transient gene silencing using synthetic siRNAs. Also many experiments use established cell lines which might not faithfully represent *in vivo* conditions especially possible adverse effects of RNAi [7].

Here we examined RNAi silencing of myostatin using transient and stably transfected primary fetal fibroblasts. Myostatin (GDF-8) functions as a secreted negative regulator of skeletal muscle growth and mutations inactivating this gene in cattle and mice led to increased muscle mass [8]. We achieved highly efficient silencing of myostatin in both transient and stably transfected cells. At the same time we also demonstrate that high level of shRNA expression resulted in induction of interferon (IFN) responsive genes (OAS1, IFN-β) despite introducing G:U mismatches into shRNA which were claimed to abolish IFN response [9,10]. In addition we also detected novel adverse effect of shRNA expression leading to interference with processing of endogenous miR21 miRNA.

Materials and methods

Cell culture. Porcine fibroblast cells were isolated from 55-day old fetuses as described and were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) H21 (Gibco) supplemented with 15% fetal bovine serum (FBS) (Gibco) at 37 °C and 5% carbon dioxide (CO_2) [11]. Twenty-four hours before transfection, 2.5×10^5 cells were seeded in each well of a 24-well plate (Costar) and cultured in growth medium without antibiotics to achieve greater than 90% confluence on the day of transfection. For transfection, 0.6 μg of each shRNA expression construct was used per well of cells. Lipofectamine 2000 Reagent (Invitrogen) was used as the

Abbreviations: RNAi, RNA interference; shRNA, short hairpin RNA; sh, shRNA expression construct; IFN, interferon; miRNA, microRNA.

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transfection reagent in a ratio of 1 μg of DNA:1.5 μl lipofectamine according to the manufacturer's instructions. Transfection efficiency was determined using green fluorescent protein expressing construct pEGFP-N1 (BD Biosciences). As controls, non-transfected and scrambled shRNA transfected cells were used. The lipofectamine/DNA complexes were removed after 6 h and fresh medium was added to the cells. To produce stably transfected cells, after transfection with linearized shRNA expression construct, 200 μg/ml Geneticin (G-418) was added at 48 h to the medium (DMEM + 15% FBS) to select for clones containing the insert. The cells were left in selective medium for 10 days after which they were trypsinized and re-cultured in selective medium for propagation.

Construction of shRNA plasmids. The mRNA sequence of porcine myostatin (AF188638) was placed in the Dharmacon si*DESIGN* Center (http://www.dharmacon.com/sidesign/) to design siRNAs (Suppl. Table 1). The five shRNAs that ranked the highest were selected for testing. The siRNAs were converted to shRNAs using a single loop sequence derived from miRNA (miR-26b), and some C nucleotides in the sense strand were changed to T's to increase stability of the vectors in bacteria, simplify sequencing and decrease IFN response. The shRNA-annealed oligonucleotides were cloned into the p*Silencer*™ 3.1-H1 neo (Ambion). All constructs were sequenced to ensure the correct design.

Real-Time RT-PCR analysis. Total RNA was isolated 48 h after transfection using Trizol (Invitrogen) according to the manufacturer's instructions. The quality of RNA was checked by Agilent 2100 Bioanalyzer. cDNA was prepared using oligo (dT) primers (Sigma) and Superscript II Reverse transcriptase (Invitrogen). Primers were designed for myostatin (AF188638), OAS1 (AY550259) and IFN-β (AY687281) across introns to prevent amplification of genomic DNA using Primer 3 (Suppl. Table 2). Primers for ssc-mir-21 (MI0002459) were designed using sequence retrieved from miRBase (http://microrna.sanger.ac.uk/) [12]. The concentration of prepared cDNAs was determined by using Picogreen dsDNA Quantitation (Molecular Probes). Real-Time RT-PCR (ABI Prism 7000) was performed using Platinum SYBR Green (Invitrogen) following the manufacturer's protocol. PCR conditions consisted of 45 cycles at 95 °C for 3 min, 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The standard curve method was used to determine the relative amounts of RNA transcripts for the genes of interest. Two housekeeping genes (β-actin and HPRT) were tested and HPRT was selected as the most stably expressed control gene for normalization. The specificity of amplification was verified by checking for correct size of amplification products on agarose gel, sequencing, and evaluation of melting curve during Real-Time RT-PCR. A RT-PCR containing all components except reverse transcriptase was also prepared as a negative control.

Statistical analysis. The Student's *t*-test was performed to identify significant changes. All experiments were repeated in triplicate. Pearson product-moment correlation coefficient (r) was calculated using the Excel Analysis ToolPak. The level of significance chosen to determine whether treatments were significantly different to controls is $p < 0.05$.

Results

Transient silencing of myostatin

The ability of porcine fetal fibroblasts to produce RNAi was tested in transiently transfected cells. Out of the five anti-myostatin shRNA constructs, three resulted in 37–92% reductions ($p < 0.05$) in the level of myostatin mRNA compared to the non-transfected cells (Fig. 1). Unexpectedly, transfection of the scrambled control and sh1 constructs lead to the 2.5- to 5-fold increase in the level of myostatin mRNA ($p < 0.05$).

The ability of RNAi to silence myostatin expression was also tested in stably transfected cells. For this experiment, the most efficient anti-myostatin constructs sh2 and sh4 were chosen with scrambled shRNA serving as a control. Results show that RNAi silencing of myostatin by same shRNA constructs was 4- to 7-fold less efficient in stably transfected cells as compare to transiently transfected cells (Fig. 1). Still myostatin mRNA level was reduced by 50% by sh2 and by 32% by sh4 shRNA constructs compared to the non-transfected cells ($p < 0.05$). Stably transfected scrambled shRNA construct again led to the 2.5-fold increase in the level of myostatin mRNA ($p < 0.05$).

Interferon response in cells transiently and stably transfected with shRNA constructs

Induction of the IFN response by anti-myostatin shRNA constructs was monitored by developing Real-Time RT-PCR for two classic IFN stimulated genes (OAS1, IFN-β) that are commonly used to detect induction of IFN response. As a positive controls for IFN induction poly (IC), a synthetic dsRNA that mimics a viral-infected state in cells [13], and cloning vector pBluescript as control for unmethylated CpG motifs were used [14]. Our results show that every shRNA construct, including the scrambled control, induced 200- to 1000-fold induction of OAS1 (Fig. 2) ($p < 0.05$) and 10- to 50-fold induction of IFN-β genes (Fig. 3) ($p < 0.05$) in transiently transfected fetal fibroblasts when compared to the non-transfected control. The induction of OAS1 and IFN-β genes by shRNA constructs was similar in magnitude to induction caused by poly (IC) and pBluescript positive controls. Similarly, 40- to 150-fold induction of OAS1 ($p < 0.05$) and 2- to 10-fold induction of IFN-β genes ($p < 0.05$) was detected in fetal fibroblasts stably transfected with shRNA constructs (Figs. 2 and 3). It seems that induction of IFN response was caused by all shRNA expressing constructs in both transiently and stably transfected fetal fibroblasts. Only weak correlation was found between level of myostatin and OAS1 ($r = 0.31$) and IFN-β ($r = -0.08$) mRNA levels.

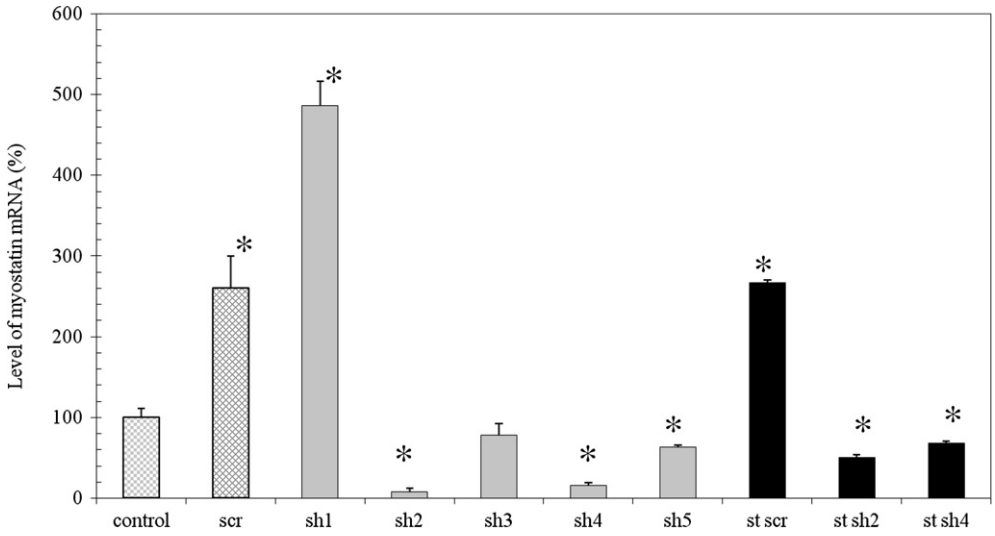

Fig. 1. Silencing of myostatin using shRNA constructs. The amount of myostatin mRNA was determined using Real-Time RT-PCR and normalized to HPRT mRNA. All experiments were done in triplicates. Control, non-transfected cells; scr, scrambled shRNA control; sh1-5, anti-myostatin shRNA construct; st, cells stably transfected with shRNA construct. *Statistically significant difference with non-transfected control.

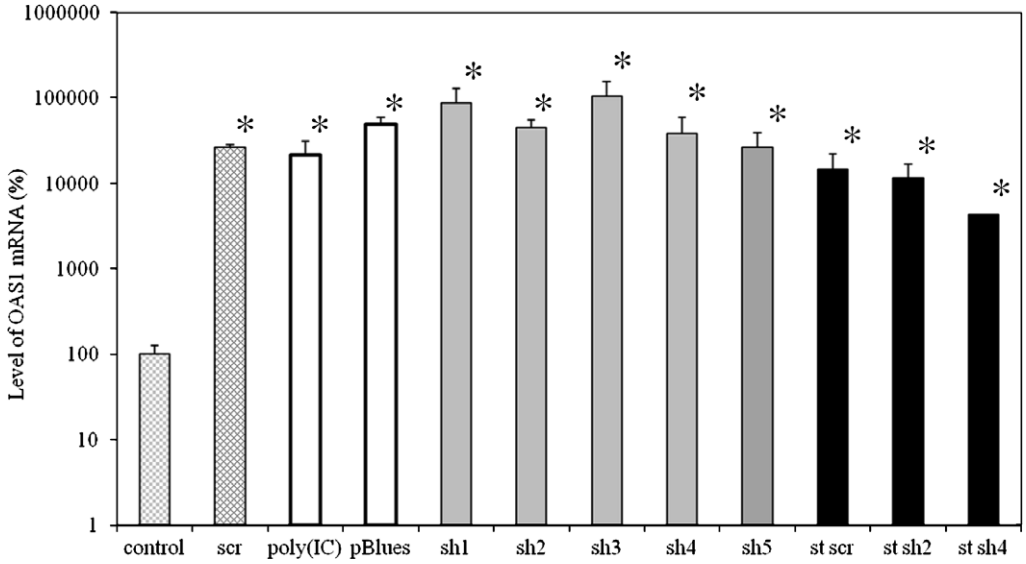

Fig. 2. Induction of interferon responsive genes (OAS1) with shRNA constructs. The amount of OAS1 mRNA was determined using Real-Time RT-PCR and normalized to HPRT mRNA. Transfection with each construct was done in triplicates. Control, non-transfected cells; scr, scrambled shRNA control; sh1-5, anti-myostatin shRNA construct; st, cells stably transfected with shRNA construct; pBlues, pBluescript. *Statistically significant difference with non-transfected control.

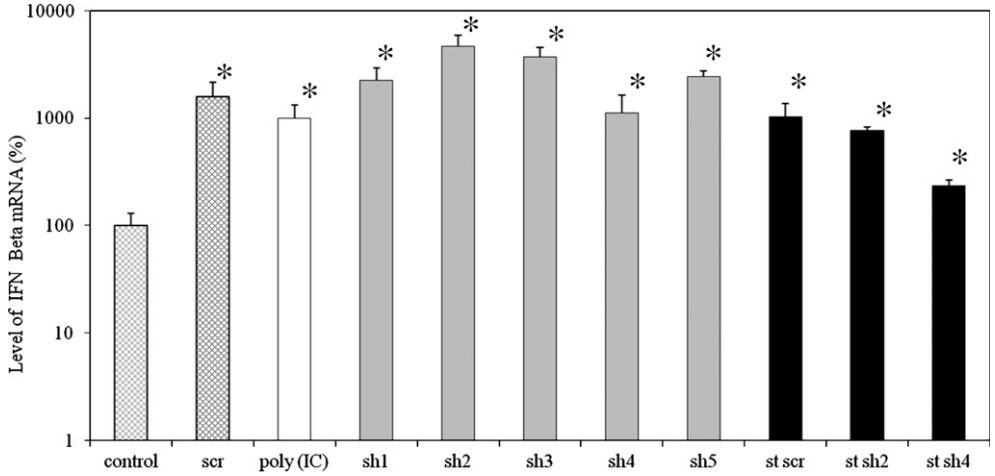

Fig. 3. Induction of interferon responsive genes (IFN-β) with shRNA constructs. The amount of IFN-β mRNA was determined using Real-Time RT-PCR and normalized to HPRT mRNA. Transfection with each construct was done in triplicates. Control, non-transfected cells; scr, scrambled shRNA control; sh1-5, anti-myostatin shRNA construct; st, cells stably transfected with shRNA construct. *Statistically significant difference with non-transfected control.

Interference with endogenous miRNA pathway

MiRNAs are recently discovered endogenous non-coding RNAs. As shRNAs use same processing/transport pathway, the interference with endogenous miRNA is of a great concern for RNAi experiments. We tested several miRNAs and developed Real-Time RT-PCR assay to monitor level of hairpin precursors for highly expressed miR21. Transient transfection of shRNA constructs had no significant effect on processing/transport of miR21 (Fig. 4). In contrast, in fetal fibroblasts stably transfected with shRNA expressing constructs there was 300- to 500-fold increase in the level of miR21 precursors ($p < 0.05$). Interference with miR21 processing/transport pathway was caused by all shRNA constructs including the scrambled control. No correlation was found between myostatin and miR21 mRNA levels ($r = -0.02$).

Discussion

In our experiments we detected two adverse effects of shRNA expressing constructs in fetal fibroblasts: induction of IFN response and interference with miRNA processing. Significant induction of two classic IFN stimulated genes (OAS1, IFN-β) was observed in both transiently and stably transfected cells. Induction was sequence independent and was caused by a factor common to all constructs including the scrambled control. In our experiment two possible sequence independent triggers of IFN response were identified including presence of unmethylated CpG DNA motifs and expression of dsRNA [15,16]. It has been known that introduction of DNA with unmethylated CpG motifs such as bacterial DNA, synthetic oligonucleotides or plasmids led to induction of strong immune response *in vitro* and *in vivo* [17]. It was also demonstrated that the transfection of empty shRNA vector or pBluescript led to induction of IFN response in another RNAi experiment [14]. We demonstrated that fetal fibroblasts did respond to challenge with pBluescript by inducing IFN response. While presence of CpG motifs might explain induction of IFN response in transiently transfected cells, it seems unlikely to explain induction of IFN response in stably transfected cells. When a plasmid vector is stably integrated into the genome, the CpG motifs are *de novo* methylated which should make vector indistinguishable from mammalian

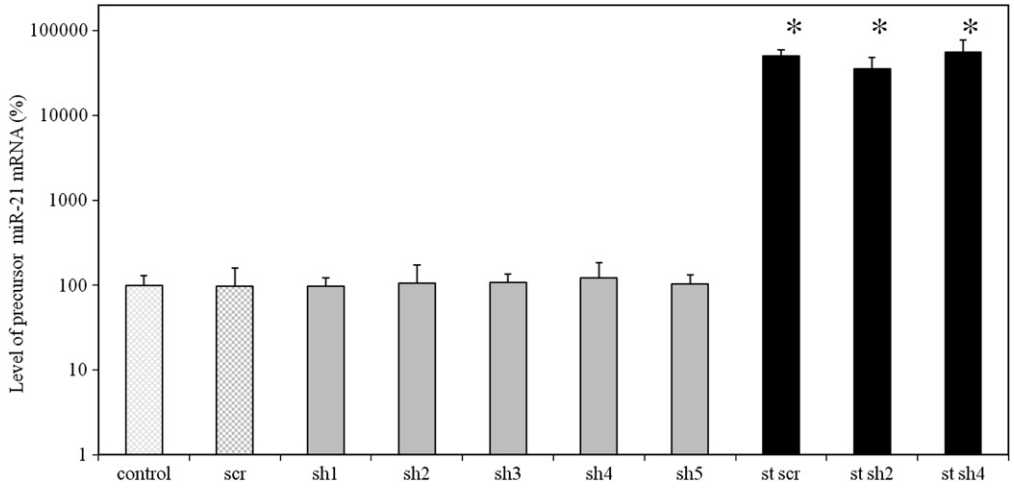

Fig. 4. Interference with endogenous miR-21 microRNA pathway by shRNA constructs. The amount of precursor miR-21 mRNA was determined using Real-Time RT-PCR and normalized to HPRT mRNA. Transfection with each construct was done in triplicates. Control, non-transfected cells; scr, scrambled shRNA control; sh1-5, anti-myostatin shRNA construct; st, cells stably transfected with shRNA construct. *Statistically significant difference with non-transfected control.

DNA [18]. We also demonstrated that challenge of fetal fibroblasts with synthetic dsRNA poly(IC) induced IFN response. It has been long known that exposure of mammalian cells to long dsRNA, regardless of its sequence, triggers a global repression of protein synthesis, degradation of mRNA and IFN secretion [19]. It is usually assumed that introduction of short (<30 nt) dsRNAs with 2-base 3′ overhangs resembling Dicer processing should not activate IFN pathway [20]. Also expression of shRNA from vector in the nucleus should resemble endogenous miRNA and was reported not to activate IFN response [21]. In contrast others reported that even short shRNA can induce IFN response [14,22,23]. For example, 500-fold induction of OAS1 gene by both U6 and H1 expressed shRNA in primary lung fibroblasts was reported [14]. Similarly, introduction of H1 and U6 promoter-based shRNA constructs by pronuclear microinjection led to induction of the OAS1 gene and early embryo lethality [23]. It was also reported that protein sensors of dsRNA including PKR, OAS, RIG-1, TLR7, and TLR8 might be activated by sequences shorter than 19 bp [16]. To prevent such induction of IFN response by shRNA we disrupted duplex dsRNA structure by introducing mutations in the sense strand to create G:U wobble base-pairing between sense and antisense strands. It has been recently reported that such mismatches reduce possibility of IFN response in long hairpin RNA (>30 nt) by decreasing the length of perfect dsRNA duplex thus evading recognition by dsRNA sensors [9,10]. In contrast in our experiment despite introducing 2–3 mutations within the sense strand leading to very short regions of dsRNA (<10 nt) significant induction of IFN response was detected for all constructs. Also there was no significant differences in induction of OAS1 and IFN-β genes between scrambled control which had no G:U mismatches and other shRNA constructs which had 2–4 G:U mismatches between sense and antisense strand of shRNA. Primary fetal fibroblasts used in our experiments are more likely to reflect *in vivo* situation. Established cell lines are often characterized by a host of genetic abnormalities that provide them with growth/survival advantage over their normal counterparts including defects in the IFN response pathway [7]. Similarly, others have shown that challenge with long hairpin RNA with G:U mismatches still led to activation of nuclear factor-κB (NF-κB) and IFN-β (IFN-β) [24,25]. Recently, introduction of 1 nt symmetrical bulge was suggested as a way to overcome IFN induction by expressed shRNA [26]. Such bulges are often found in endogenous miRNA that presumably were selected to avoid non-specific induction of IFN response.

It was previously suggested that high level of shRNA expression might lead to accumulation of unprocessed or aberrantly processed transcripts triggering IFN response [14]. The most likely target of such interference will be endogenous miRNA as both utilized same pathway for processing and export. We monitored level of hairpin precursor (pre-miRNAs) for miR21 that was shown to be highly expressed in several tissues and would be more likely to be affected by shRNA interference [27]. While no significant interference was found in transiently transfected fetal fibroblasts, there was up to 500-fold significant increase in the level of miR21 precursors in stably transfected cells. As effect was seen only in stably transfected cells it seems unlikely that accumulation of unprocessed transcripts was responsible for IFN response in both transiently and stably transfected fetal fibroblasts. We only found one other report which detected interference from shRNA expression on endogenous miRNA pathway. Recently it was shown that high level of shRNA expression for 36 out of 49 construct against six different targets in mouse liver interfered with endogenous miRNA pathway resulting in liver toxicity and in some cases in animal death [28]. Interference from expressed shRNA affected highly abundant miR-122 and occurred due to saturation of Exportin-5 used by both shRNA and miRNA for export out of the nucleus. Direct correlation was found between level of shRNA expression and toxicity, and low level of shRNA expression using weaker H1 and 7SK promoters were tolerated. Other groups also reported concentration dependent cytotoxic effect in cultured cells expressing shRNA although the cause of cytotoxicity were not uncovered [29,30].

We did achieve efficient silencing of myostatin with up to 92% significant reductions in the level of myostatin mRNA in transiently transfected cells and up to 50% in stably transfected cells. Previously it was shown that introduction of synthetic siRNA targeting myostatin in chicken embryonic myoblasts reduced myostatin transcript level up to 75% [31]. Similarly, transient electroporation of anti-myostatin shRNA expressing plasmid into the rat muscle reduced myostatin mRNA by 27% [32]. RNAi silencing of myostatin is likely unrelated to IFN induction or interference with miRNA as no correlation was found between RNAi silencing and the level of OAS1 or miR21 mRNA. Also scrambled control which affected both IFN response and level of miR21 precursor did not decreased myostatin mRNA. Similarly, while all shRNA constructs induced similar level of IFN response and miRNA interference, there was large variation in myostatin silencing efficiencies among constructs. For example, significant 10-fold difference in

silencing efficiency was found when the target region was shifted only by a single nucleotide (sh2/sh3). These results are consistent with previous studies that show that even single nucleotide shift in the target site might lead to large differences in silencing efficiency [33]. Transfection of scrambled and sh1 constructs actually resulted in significant 3- to 5-fold up regulation of myostatin expression. The up regulation of myostatin expression is likely caused by induction of IFN response in cells. Promoter region of the myostatin gene is known to contain several NF-κB and glucocorticoid transcriptional response elements, and it has been suggested that inflammatory cytokines may activate myostatin transcription [34]. Increase in myostatin expression was found in the dexamethasone treated C2C12 myotubes, in pigs with viral and bacterial pneumonia and in HIV-infected men [35–37]. If up regulation of myostatin expression happened for all shRNA constructs then the efficiency of myostatin silencing was even greater. For example, decrease in myostatin mRNA by sh2 construct is around 34-fold during transient transfection and 5-fold in stably transfected cells when compared to scrambled control.

In conclusion, while transient transfection of anti-myostatin siRNA might be a useful method for short term treatment; the increase in muscle mass in the transgenic animals will require long term stable expression of anti-myostatin shRNA in the muscle. In this case side effects of RNAi become increasingly important and all constructs should be tested not only for high RNAi efficiency but also for induction of IFN response and interference with endogenous miRNA in stably transfected primary cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.03.041.

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