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DNA constructs designed to produce short hairpin, interfering RNAs in transgenic mice sometimes show early lethality and an interferon response

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Abstract. Arylamine N-acetyltransferase (*NAT*) genes were targeted for inhibition using short hairpin RNA (shRNA) using two different RNA polymerase III promoters. Constructs were developed for *NAT1* and *NAT2*, the endogenous mouse genes, and for human *NAT1*. There were fetal and neonatal deaths with these constructs, perhaps due in part to an interferon response as reflected in increases in oligoadenylate synthetase I mRNA levels. Seven out of 8 founders with the U6 promoter generated offspring but only 2 gave positive offspring. Out of 15 founders for H1 promoted constructs, only 4 had positive offspring. When transgenic lines were successfully established, the expression of the targeted genes was variable between animals and was not generally inhibitory.

Key words: embryo death, interfering RNA, interferon response, silencing RNA, short hairpin RNA, transgenics.

Introduction

There has been great interest in the use of interfering RNA (RNAi) to inhibit gene expression. Fire et al. (1998) discovered that long double-stranded RNA can induce gene silencing in *Caenorhabditis elegans* while Hamilton and Baulcombe (1999) found that short double-stranded RNAs caused gene silencing in plants. Tushl et al. (1999) demonstrated that the short RNA forms which are intermediates of the double-stranded RNA-induced silencing process are suppressive and coined the term "short interfering", (si)RNA. It was soon apparent that the genome uses inhibitory microRNAs (reviewed in Denli and Hannon 2003). These endogenous RNAs arise from fold-back structures and are cut by Dicer. However, they do not use the RNA induced silencing complex (RISC) and, in animals, do not require perfect complementarity to their target.

Successful methods for creating animal models of genetic disease by the use of RNAi have been greatly sought. Recently, one group reported failure using short hairpin RNA (shRNA) constructs to inhibit gene expression in transgenic mice while such constructs introduced into embryonic stem cells were both inhibitory and successfully transmitted through the germ line (Carmell et al. 2003).

RNA silencing or inhibition, seems to be a universal property of eukaryotes (Hannon 2002; Plasterk 2002) and the enzymes involved, e.g. Dicer, are essential for development (Bernstein et al. 2003). The long double-stranded RNA used to induce RNAi in many species provokes a strong cytotoxic response in mammalian cells (Hunter et al. 1975). However, introduction of long double-stranded RNAs function as RNAi in the preimplantation embryo (Wianny and Zernicka-Goetz 2000; Svoboda et al. 2000; Feodoriw et al. 2004),

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embryonal cells (Paddison et al. 2002) and may be an explanation of the successful use of antisense RNA in preimplantation embryos (Bevilacqua et al. 1988, 1989a, 1989b). The introduction of short, double-stranded RNA complexes by transfection has been found to be a very efficient way to "knock down" gene expression in cultured mammalian cells (Elbashir et al. 2001, 2002) and, when introduced by injection, in embryos (Calegari et al. 2002). Very recently, a number of laboratories have shown that plasmids directing the synthesis of shRNAs promoted by polymerase III promoters would effectively result in gene suppression by RNAi in cultured cells (Brummelkamp et al. 2002; Miyagishi and Tiara 2002; Lee et al. 2002; Paul et al. 2002; Paddison et al. 2002; Sui et al. 2002). In direct comparisons, RNAi was much more effective than antisense oligodeoxynucleotides (ODNs) targeted to the same gene (Miyagashi et al. 2003) and showed a longer duration of effect (Bertrand et al. 2002). Lack of specificity, as previously shown with ODNs (Woolf 1992) has been found (Oates et al. 2000; Jackson et al. 2003).

Attempts to create mice with genes silenced by RNAi have also been made. Both tail-vein injection of short inhibitory double-stranded RNAs and/or plasmids resulted in short term inhibition of gene expression in mouse livers (Lewis et al. 2002; McCaffrey et al. 2002). However, this massive volume dilution (a volume equivalent to 5–10 % of the mouse's weight) is not generally applicable and the RNAi effect was short lived. Thus, methods of generating transgenic mice with long term expression of shRNAs have been sought. Carmell et al. (2003) did not find distinct or reproducible phenotypes that were expected for hypomorphic alleles (of 8 genes with visible phenotypes which were each targeted by 3 constructs). There was successful germ line transmission with lower levels of gene suppression with plasmids, which were selected in embryonic stem cells (Carmell et al. 2003), our constructs frequently do not show germline transmission.

The shRNA constructs we used targeted arylamine N-acetyltransferases (NAT). NATs catalyze the acetylation of the extracyclic amino groups of aromatic amine and hydrazines. The successful inhibition of the endogenous *Nat1* and *2* would not be expected to be harmful since the knockout of *Nat2* (Cornish et al. 2003) and both *Nat1* and *2* (Sugamori et al. 2003) did not show a phenotype. However, there was poor transmission to offspring with little inhibition by the shRNA constructs.

Material and methods

The siRNA sequences targeting mouse and human N-acetyltransferase genes were synthesized by MGW Biotech Inc. (Table 1) and terminated in 5 thymidines to provide the P0l III termination signal.

H1 promoter

The H1-RNA promoter was PCR amplified using the following primers: 5'CCATGGAAT TCGAACGCTGACGTC-3' and 5'GCAAGCT TAGATCTGTGGTCTCATACAGAACTTATA AGATTCCC-3' (Brummelkamp et al. 2002). The PCR product was digested with *EcoR* I and *Hind* III enzymes and cloned into pBKSII. The SiRNA sequences were designed with the *Bg1* II and *Hind* III site in the two ends and cloned into the *Bg1* II-*Hind* III sites of pSUPER. A purified 335 pb fragment including the H1 promoter and duplex siRNA were cut from pH1-siNat1, pH1siNat2, and pH1-siNAT1 plasmids with *Xba* I and *Sal* I and purified for injection.

Table 1. Design of shRNA for transgenic injection

Target gene	Promoter	Position from ATG	Length base pair	Sequence for dsRNA*
Nat1	H1	78	21	AGTTCTTCAGCACCAGATGCG
Nat1	U6	78	21	AGTTCTTCAGCACCAGATGCG
Nat2	H1	123	19	ACATCCATTGTGGGGAATC
NAT1	H1	138	21	GCCATGGACTTAGGCTTAGAG
NAT1	U6	138	21	GCCATGGACTTAGGCTTAGAG

*all with loop sequence TTCAAGAGA and 5 terminal Ts.

U6 promoter

The mU6pro vector contains the mouse U6 RNA promoter (Paul et al. 2002). DNA oligos were designed (Table 1) with the *BamH* I and *Xba* I sites in both ends and subcloned into the *BamH* I and *Xba* I sites of U6 RNA promoter. Digests of pU6-siNat1 and pU6-siNAT1 with *Hind* III and *Pvu* II resulted in a 800 bp fragment including the U6 promoter and duplex siRNA which were purified from gel for injection.

Injection

DNA was injected into C57Bl/6 × DBA/2J zygotes (Genetically Modified Mouse Service, University of Arizona). Founder animals and their offspring were identified as described below.

Typing

Mice were tail-tipped, DNA was prepared and typing for the presence of a transgene was performed using the primers in Table 2. The PCR reactions were performed in a 25 μL volume with 40 cycles with a 95°C denaturation for 30 s, 58°C annealing for 45 s and 72°C extension for 1 minute.

RT-PCR for *Nat1, Nat2* mRNA quantitation

Tissue Samples

Liver, lung, and kidney were taken from positive mice and were immediately frozen in liquid nitrogen. Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Genomic DNA potentially present in RNA samples was removed by incubating the RNA with RNAse free DNase I (Promega). RNA quantification was performed by spectrophotometry at 260 nm, and integrity of the RNA was verified by electrophoresis of 1 μg of total RNA on a 1.2% agarose gel stained with ethidium bromide.

Synthesis of cDNA and real time Reverse Transcription PCR

Two μg total RNA was reverse-transcribed into first strand cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Roche). In order to optimize the real time PCR conditions we used a 10X SYBR Green I master mixture of 100 mM Tris-HC1, pH8.5, 500 mM KC1, 25 mM $MgCl_2$, and 1.5% X-100 (Karsai et al). Real time PCR was performed using a thermal cycler system (Cepheid, USA).

Table 2. Primers for typing transgenic mice

Constructs	Primer	Product
H1-Nat1	5'-GAATTCGAACGCTGACGTCA-3' 5'-TGAACGCATCTGGTGCTFAA-3'	249 bp
H1-Nat2	5'-GAATTCGAACFCTGACGTCA-3' 5'-GAAGGATTCCCCACAATGGA-3'	246 bp
U6-Nat1	5'-CCCACTAGTATCCGACGCCCGGATCTCTA-3' 5'-TGAACGCATCTGGTFCTFAA-3'	353 bp
H1-NAT1	5'-GAATTCGAACFCTFACFTCA-3' 5'-GAACTCTAAGCCTAAGTCC-3'	249 bp
U6-NAT1	5'-CCCACTAGTATCCFACFCCFCCATCTCTA-3' 5'-GAACTCTAAGCCTAAGTCC-3'	353 bp

Table 3. Primers for quantitating target mRNA and oligoadenylate synthetase 1

	Primer	Product	Concentration in PCR	Anneal temperature C°
Nat1	5-TGT,TAA,CTC,AGA,CCT,CCT,TG-3 5-TAC,AAA,CAC,AGA,TGC,TGG,CG-3	130 bp	11.7 pmol	58
Nat2	5-GAG,AGC,AGT,ATG,TTC,CAA,ACC-3 5-AGA,CGC,TGG,TGA,TGT,CTG,AA-3	148 bp	15 pmol	60
Oas1	5'TGCTGCCAGCCTATGATTTA-3' 5'-CGACAGTTCAGGAAGTACTT-3'	156 bp	16.7 pmol	56
GAPDH*	5-TTC,ACC,ACC,ATG,GAG,AAG,GC-3 5-GGC,ATG,GAC,TGT,GGT,CAT,GA-3	236 bp	16.7 pmol	60

*The GAPDG primer is located in different exons that only mRNA is measured.

The reactions were performed in a 25 μL volume with concentrations and annealing temperatures optimized for each specific primer (Table 3). A typical protocol included a 300 s denaturation step followed by 45 cycles with 95°C denaturation for 30 s, annealing for 45 s and 72°C extension for 1 minute. To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis and subsequent agarose gel electrophoresis. All experiments were performed at least three times with separate RNA preparations.

For a standard curve, 2–4 fold serial dilutions were made starting from the original concentration. The parameters were: Nat1: $Y = -3.5315X + 23.227$, $R^2 + 0.999$ (2 fold dilution); Nat2: $Y = -2.315X + 23.552$, $R^2 = 0.993$ (4 fold dilution); Oas1: $Y = -3.2821X + 28.907$, $R^2 = 0.987$ (2 fold dilution); and GAPDH: $Y = -3.87X + 16.016$, $R^2 = 0.991$ (4 fold dilution).

Results

U6 promoter

Mouse *Nat1* and human *NAT1* were targeted in separate gene injections with shRNA driven by the U6 promoter. Human *NAT1* was chosen as an exogenous target since there might be toxicity related to endogenous targets; *NAT1* was expressed in other transgenic mice which we planned to cross with these transgenic mice. Multiple founders for both constructs were obtained but there were relatively few positive offspring to study. Five out of six founders generated offspring. Two of these gave 10 positive versus 31 negative ($\chi^2 \leq 0.002$) among surviving pups while the remaining 3 had no positive offspring among 39 surviving. There were higher proportions of transgenics among dead fetuses removed before they were eaten: 3/4 for 1 transmitting founder and 2/5 for another; 1/1 for 1 non-transmitting founder. There was no consistent pattern of inhibition in liver, kidney or lung (Table 4). Neither of two U6-*NAT1* founders transmitted (in crosses to CMV-promoted, *NAT1* transgenics; 2 offspring for 1 founder; 22 for the other) so the degree of inhibition of the exogenous targeted transgene could not be studied.

Table 4. U6-promoted, shRNA effects on endogenous *Nat1* mRNA levels

	% inhibition*		
mouse # founders	lung	kidney	liver
283	89.7	84.2	13.6
285	91.6	–95.8	71.9
offspring			
40 (from 283)	19.2	–258.3	–26.1
124 (from 284)	–19.5	–73.9	–21.4
127 (from 284)	60.1	22.5	19.7

* means, std. dev. not presented since the triplicates were from single mice

H1 promoter

The human polymerase III H1-RNA gene promoter has also been successfully used to drive expression of shRNAs to inhibit gene expression (Brummelkamp et al. 2002). We separately targeted mouse *Nat1* and *Nat2* and human *NAT1* with shRNA driven by this promoter. The *NAT1* target also provided the potential to test a nonendogenous gene. While only 2 founders

Table 5. Transmission of H1-Nat1 RNAi transgenes

Founder #	Sex	Bred to	Alive		Dead		Fetuses	
			positive	negative	positive	negative	positive	negative
118	female	B6		15	3	8	2	7
119	female	B6		10	2		2	10
168	male	ICR ICR	4	7			6*	11*
169	male	B6 ICR B6		3				13 10
170	male	B6 ICR					5*	7 13*
171	male	ICR B6	8	8			2*	6*
173	female	B6		26				

* fetus < 13d

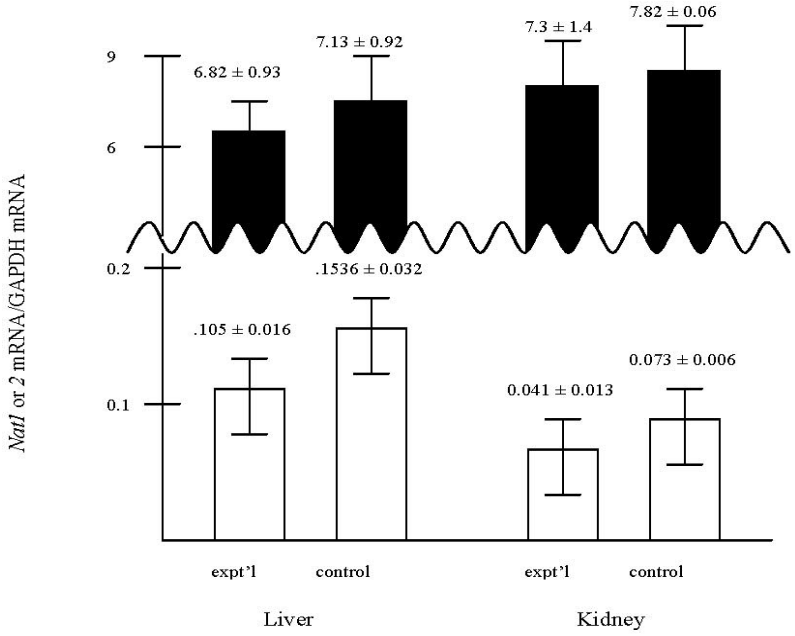

Figure 1. Real time RT-PCR quantitation of target *Nat1* or *2* mRNA levels in H1-transgene positive mice compared to controls (mean ± std error) in liver and kidney. *Nat1*□, *Nat 2*■

transmitted the transgene to liveborn offspring, 5 transmitted to dead newborns or fetuses (where the mother was sacrificed for these studies) with a trend to a higher percentage of positive offspring in younger fetuses (Table 5). When *Nat1* was the target, inhibition of target endogenous mRNA was inconsistent (Table 6). Three founders with constructs targeting human *NAT1* did not have positive offspring among liveborns (50), dead newborns (6), or fetuses (10 from just 1 founder). Thus, early death was not explained by toxicity related to an endogenous target since it was also found with an exogenous target. Two out of 5 founders transgenic for the H1-*Nat2* construct had positive offspring but at possibly lower frequencies than expected: 12 positive versus 22 negative (χ^2, p = 0.09). There was no significant inhibition of endogenous *Nat2* mRNA (lung: 70%, 36%; kidney: 61%, 257%; liver: 186%, 53% in 2 founders).

Table 6. H1-promoted shRNA effects on endogenous Nat1 mRNA levels in 5 founders % inhibition*

Mouse #	Lung	Kidney	Liver
119	93.1	86.7	75.8
118	24.7	–70.8	–8.6
48	98.6	–20.4	91.9
168	5.8	–316.7	–38.8
171	73.3	–139.6	–30.1

* means, std. dev. not presented since the triplicates were from single mice

Transgenic lines containing H1-driven shRNAs targeted to *Nat1* and *Nat2* were eventually established. There was little or no inhibition of expression of the endogenous genes (Figure 1) which could now be studied in multiple mice.

2'5' oligoadenylate synthetase (OAS) induction

Several recent papers have indicated that, contrary to earlier studies, RNAi can stimulate the interferon response in mammalian cells (Bridge et al. 2003; Sledz et al. 2003). Inasmuch as this response could be toxic to the early embryo and result in the pattern of more positive transgenics in younger versus older fetuses that we observed, OAS mRNA levels were measured. Transgenic fetuses from 3 different founders had variably elevated levels (Table 7).

Table 7. Oligoadenylate synthase 1 mRNA levels in transgenic fetuses from various founders by real time RT-PCR

	mRNA/GAPDH mRNA
Non-transgenic fetuses	(3) 0.114 ± 0.067*
Transgene positive from 119	(2) 1.75 ± 0.12^{+}
Transgene positive from 168	(4) 0.787 ± 0.54
Transgene positive from 171	(2) 0.157 ± 0.067

* (n) mean ± std dev
$^{+}$ p ≤ 0.001 against non-transgenics

Discussion

N-acetyltransferases were chosen as targets since their knockouts are viable (Cornish et al. 2003; Sugamori et al. 2003). Two genes, *NAT1* and *NAT2,* have been isolated and characterized from humans and several animal species (see review Hein et al. 2002). Human NAT1 preferentially catalyzes the acetylation of substrates like p-aminobenzoic acid (PABA) while isoniazid (INH) is a substrate for human NAT2. Little is known about the physiological significance of these enzymes. Human NAT1 catalyzes the acetylation of a breakdown product of folic acid, p-aminobenzoylglutamate, leading to the suggestion that NAT1 may play an as yet undefined role in folic acid metabolism (Minchin 1995; Ward et al. 1995; Payton et al. 1999; Upton et al. 2000; Smelt et al. 2000). All the NAT genes are located on chromosome 8 in mice and humans (Mattano et al. 1988; Blum et al. 1991; Hickman et al. 1994; Fakis et al. 2000).

While the percentage of liveborn mice that were transgene positive after pronuclear injection with shRNA constructs ranged from 5.9% for U6NAT1 to 13.9% for H1Nat1, in the general range for other constructs, the transmission to subsequent generations was deficient. This was unusual. Generally, only about 15% of transgenic founders are mosaics which do not transmit to subsequent generations while up to 30% are germline mosaics (Wilkie et al. 1986). There were low transmission ratios to offspring that survived to weaning (15%) with higher ratios among dead newborns (38%), and early fetuses (30%), but not late fetuses (8%) (Table 6). This suggests that the shRNA constructs were toxic to the developing embryos. The fact that the same lack of transmission occurred with a non-endogenous target makes it unlikely that the toxicity was related to reaction with the endogenous target, *Nat1* or *2*. Although mice deficient in *Nat2* or both *Nat1* and *Nat2* have no phenotype (Cornish et al. 2003; Sugamori et al. 2003), the silencing complex attached to the target gene could potentially be toxic.

One possibility is that induction of an interferon response, expected for double-stranded RNAs of 30 pb or longer, but not for the shRNAs, was responsible for this fetal lethality. Induction of the mRNA of *Oas1*, an indicator for this response, ranged from 1.4 to 15.3 fold, comparable to the about 3 fold increases found using microarrays (Sledz et al. 2003) but lower than the 100 fold responses found by Bridge et al. (2003). Thus, an interferon response to highly expressing shRNA constructs may contribute to apparent early fetal lethality of these constructs. This interferon response could be related to the amount of shRNA generated by the transgene and it is very possible that the transgenic founders which transmitted and allowed the establishment of lines had lower levels of shRNA than those which did not.

The constructs in this study were designed to function as siRNAs, not miRNAs. Recent advances in understanding of the mechanisms of RNA silencing have led to better designs for RNAi (Silva et al. 2003). Schwarz et al. (2003) showed that the antisense strand of a double-stranded RNA was more efficiently incorporated into the RNA silencing complex and was associated with less stable hydrogen-bonding at the 5' end. Comparative analyses of natural silencing RNAs led to a similar conclusion (Khvorova et al. 2003). Effective double-stranded silencing RNAs also were thermodynamically less stable internally from positions ± 10 to ± 15. The targeted sequences for *Nat1* and *Nat2* (Table 1) fit these specifications quite well. However, the target sequence for *NAT1* (a human transgene present in other lines), does not fit these specifications since it was G-C rich in those internal positions. Other aspects of shRNA (chemistry included) have recently been explored (Harborth et al. 2003).

It is interesting to compare the current results and those of Carmell et al. (2003) who also had highly variable degrees of inhibition of target genes with transgenics expressing shRNA but did not comment on a deficiency in transmission of the transgenes. It is possible that the difference is the target gene, an enzyme involved in phase II xenobiotic metabolism with no visible phenotype in knockout mice, in contrast to Carmell et al. (2003) who targeted genes encoding visible phenotypes. Important differences may also be the promoter used. Carmell et al. (2003) did not list the promoters for their constructs and they are not given in the supplementary information to that paper. However, the H1 promoter was used for all 3 shRNA constructs expressed in embryonic stem cells and may have been used in the transgenics. Differences in promoters may be important since products of different polymerase III promoters localize to different sub-regions of cells (Ilves et al. 1996).

More recently, lentiviral vectors have been shown to effectively deliver polymerase III promoted, shRNA constructs for knockdown

transgenesis (Rubinson et al. 2003; Tiscornia et al. 2003). For CD8 silencing, copy numbers ranged from 2–6 and did not correlate with percent decrease in expression (Rubinson et al. 2003). Although numbers of offspring are not presented, it appears that there is a discrepancy between the percentage of positive liveborns (decreased) and embryos in the work of Tiscornia et al. (2003). Thus, the lentiviral approach to shRNA transgenics may suffer from some of the same limitations that we have found with "standard" pronuclear injection transgenics.

The mechanism of action of the RNAi produced from the shRNA constructs is also not known. Hall et al. (2002) showed that a shRNA driven from the U6 promoter but with strand reversal, such that an accessible anti-sense 3 terminus was not available, still markedly suppressed the target gene expression. Thus, these shRNAs might not require primer extension amplification. Perhaps gene silencing involving heterochromatin modification which has been found to occur with RNAi in fission yeast (Volpe et al. 2002; Hall et al. 2002), could be involved. It is also of interest that the shRNA constructs which efficiently suppress gene expression in mammalian cells do not contain introns and polyadenylation signals. Thus, they may have to function in the nucleus or are efficiently transported to the cytoplasm despite lacking these export signals. Constructs with introns work better than ones without in plants (Smith et al. 2000) but, perhaps, for other reasons.

The interest in using RNAi in transgenic mice is partly driven by the potency of RNAi compared to antisense oligodeoxy-nucleotides. Two studies have found shRNAs to be much more potent than similarly targeted antisense oligodeoxynucleotides (Bertrand et al. 2002; Miyagashi et al. 2003). A polymerase II promoter has been shown to be effective with shRNA in ES cells suggesting that these cells may have an increased sensitivity to RNAi (Grabarek et al. 2003). Transgenic incorporation of polymerase III driven shRNA by electroporation of ES cells has led to variable degrees of inhibition in embryos (Kunath et al. 2003) and in the adults resulting from chimeric mice resulting from blastocyst injection of the ES cells (Carmell et al. 2003).

Conclusions

In conclusion, a large number of founders were generated from embryo injection of constructs using Pol III promoters to generate shRNA. Poor transmission to the next generation suggests toxicity of the constructs which may be explained by an interferon response. The transgenic mouse lines which were eventually established showed little or no inhibition of expression of the target genes.

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