—Mini Review—

RNA Interference as a Tool for Producing Knockdown Mice

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Abstract: *Analysis at the individual level using genetically-engineered mice has allowed conclusions to be reached regarding the actual function of a target gene. In recent years, the "knockdown" method using RNA interference (RNAi) has been established as a powerful tool for analyzing gene function. In this review, we focus on RNAi knockdown technology for producing genetically-engineered mice and describe the value of this approach from the perspective of both basic research and therapeutic potential. First, we introduce the basic mechanism of RNAi and development of knockdown animals from worms to mice. Next, we describe strategies to produce knockdown mice using DNA-based expression vectors introduced into zygotes or embryonic stem cells. Finally, we refer to the trends of research for clinical application. By way of illustration, we show the production of knockdown mice for treatment of neurodegenerative disease and mention the prospect of therapeutic potential of RNAi technology.*

Key words: *RNAi, dsRNA, siRNA, Knockdown, ES cells*

Introduction

The decoding of the human genome has been a turning point in the study of life sciences. Analysis at the individual level using genetically-engineered mice has allowed conclusions to be reached regarding the actual function of a target gene on the basis of phenotypic analysis. Genetic information from mice has become more and more important, because decoding of the mouse genome has made it possible to apply such information to humans, and data from genetically-engineered mice provide findings for the target gene that cannot be obtained from *in vitro* experiments.

To date, genetically-engineered mice are classified into "transgenic mice" and "gene-targeted mice", such as knock-out mice or knock-in mice. The former are produced by microinjection of a DNA fragment into the pronucleus of fertilized eggs [1], and the latter are based on gene targeting that destroys or alters the object gene by exchange with an exogenous targeting vector sequence, using homologous recombination in embryonic stem cells [2]. In recent years, the "knockdown" method using RNA interference (RNAi) has been established in lower animals [3–5] and has also been applied to mammalian cultured cells [6–10] as a powerful tool for analyzing gene function. More recently, production of "knockdown mice" by RNAi has become possible [11, 12].

In this review, we focus on RNAi knockdown for producing genetically-engineered mice and describe the value of this approach from the perspective of both basic research and therapeutic potential.

The development of Knockdown Animals by RNAi from Worms to Mice

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing mechanism, which is triggered by double-stranded RNA (dsRNA) and consequently induces degradation of mRNAs with the same sequence of the sense strand of dsRNA (Fig. 1) [13–25]. In 1998, Fire *et al.* first showed this phenomenon in the nematode worm *Caenorhabditis elegans*. When dsRNA was injected into the worm the corresponding gene products disappeared from both the somatic cells of the organism and its F1 progeny [3]. This discovery has led to a new technology allowing rapid reverse genetic analysis, and similar effects were

Received: September 7, 2005
Accepted: September 20, 2005
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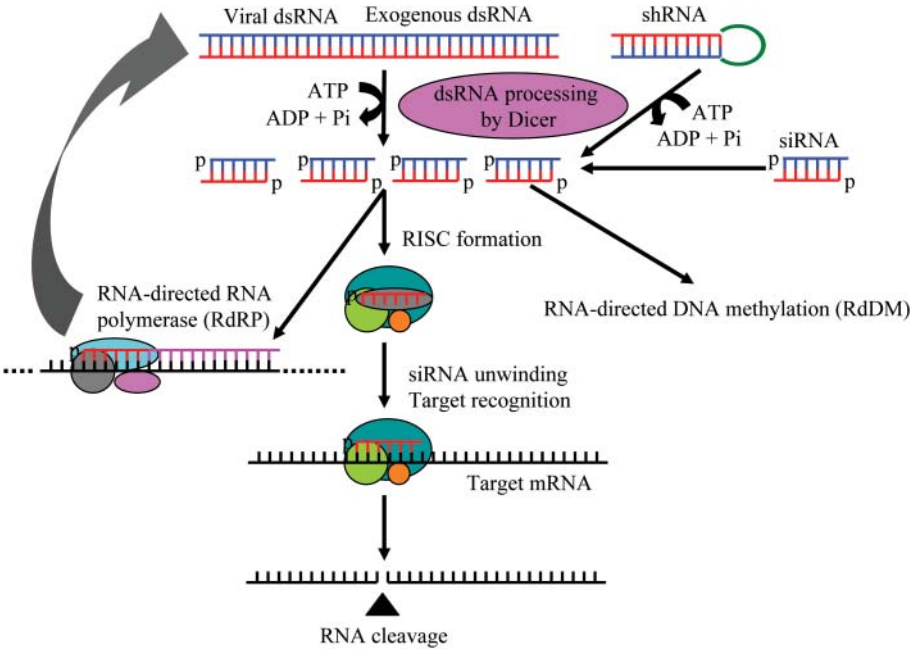

Fig. 1. A model for the mechanism of RNAi. dsRNAs or shRNAs are cleaved by RNase III-type enzymes such as Dicer into 21–25 nt siRNAs. These siRNAs or the introduced siRNAs are then incorporated into a protein complex termed RNA-induced silencing complex (RISC). The unwinding siRNA guides the RISC to homologous region of the target mRNA, ensuring its degradation. The unwinding siRNAs can also serve as primers on the target RNA in association with RNA-dependent RNA polymerases (RdRPs) to subsequently generate secondary siRNAs. Furthermore, besides this post-transcriptional gene silencing (PTGS), siRNAs mediate RNA-directed DNA methylation (RdDM) for silencing the target gene.

immediately demonstrated in fungi such as *Neurospora crassa* [26], and other animals such as *Tripanosoma brucei* [27], *Drosophila melanogaster* [28–31] Zebrafish [32–34] and *Xenopus laevis* [35].

Similar phenomena, referred to as post-transcriptional gene silencing (PTGS) in plants and quelling in fungi, have actually been known for many years [36–40]. Since the RNAi effect was subsequently shown in a wide range of eukaryotes, it was anticipated that a similar effect would occur in mammals. However, gene silencing by RNAi in cultured mammalian cells has been hampered by an antiviral response via dsRNA-triggered pathways that mediate non-specific suppression of gene expression (Fig. 2) [41–46]. dsRNA induces type I interferon (IFN) synthesis, which activates two classes of IFN-induced enzymes: PKR, a dsRNA-dependent protein kinase, which phosphorylates eIF-2α and induces inhibition of translation [42, 43, 45, 46]; and 2',5'-oligoadenylate synthetase, whose products activate the ribonuclease RNase L [43–45]. These pathways induce cell death via apoptosis. Surprisingly, dsRNA can induce sequence-specific gene silencing only in mammalian embryos, because of their lack of such IFN responses against dsRNA. Indeed, microinjection of dsRNA into mouse zygotes results in the RNAi phenomenon for both exogenous transgenes such as green fluorescent protein (*GFP*) [47] and endogenous genes such as c-mos, with phenotypes equivalent to the gene-deficient mice [47, 48]. Tuschl *et al.* first demonstrated that RNA duplexes of 21–23 nt (short interfering RNA: siRNA), which were designed to mimic the naturally processed products from long dsRNA produced by the RNase III enzyme, Dicer, could evade the antiviral response via IFN-induced pathways and induce sequence-specific silencing *in vitro* in Drosophila embryo extracts [49–51]. These results were successfully extended to cultured mammalian somatic cells [6, 52] making the use of siRNA possible as a sequence-specific "gene knockdown" approach, and it has rapidly become popular as a convenient tool for analyzing gene function. However, siRNA can only transiently suppress the expression of target genes,

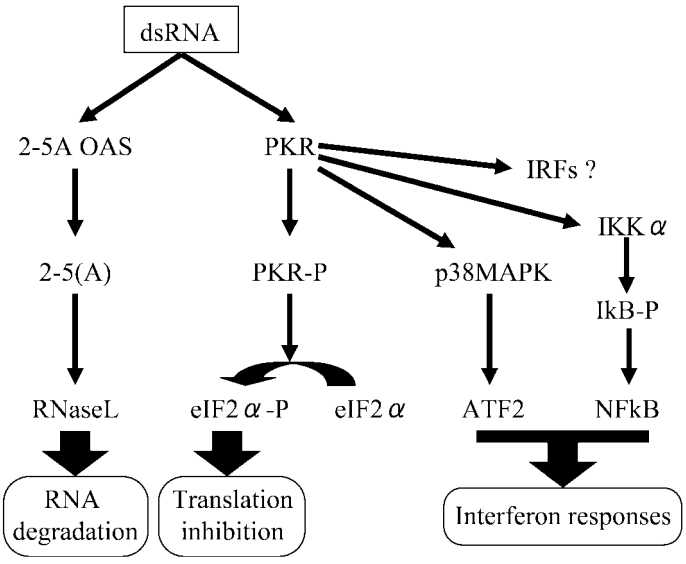

Fig. 2. Model for the diverse actions triggered by dsRNA in mammalian cells. dsRNA activates latent 2'-5'-oligoadenylate synthetase (2–5 OAS) and this enzyme activates RNase L via 2'-5'-oligoadenylates (2–5(A)). Activated RNase L non-specifically degrades single-stranded RNAs. dsRNA also activates protein kinase R (PKR). Activated PKR inhibits protein synthesis by phosphorylation of transcriptional initiation factor eIF2α. Moreover, PKR can function as a signal-transducing kinase interacting with IkB kinase (IKK) and p38 MAPK to mediate the antiviral actions of type I interferons.

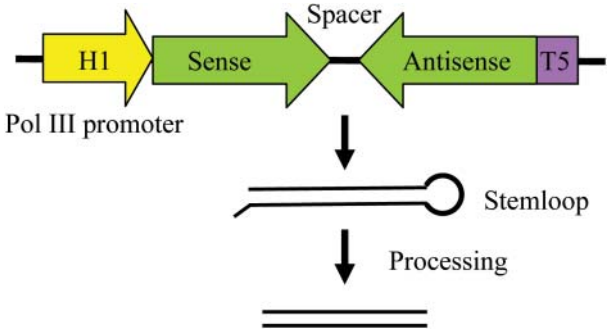

Fig. 3. Strategy for generating siRNA from DNA template *in vivo*. The 19–23 nt long of the sense and antisense regions that target the gene of interest are separated by a spacer of 6–8 nt and are followed by the transcriptional termination signal of five thymidines (T5). The shRNA is formed by folding back into a loop structure and is consequently processed into siRNA.

which seriously restricts its application in the analysis of mammalian organisms. To circumvent this limitation, on the basis of an *in vivo* RNAi expression strategy [53], a mammalian expression vector that synthesizes siRNA-like transcripts has been designed [54, 55]. The transcripts are synthesized under the control of RNA polymerase III (Pol III)-dependent promoters, such as H1 or U6, that effectively express small RNAs lacking poly adenosine tails in a fold-back stem-loop structure, named the short hairpin RNA (shRNA), which are subsequently processed into active siRNAs (Fig. 3). This DNA vector-based approach gave rise to stable suppression of target gene expression. Subsequently, the production of knockdown mice using shRNA expression vectors has been examined.

Generation of Knockdown Mice using a Conventional Transgenic Method: Microinjection of a shRNA-expression Vector into Fertilized Eggs

Hasuwa *et al.* produced transgenic mice by introducing a shRNA-expression vector specific for enhanced green fluorescent protein (*EGFP*) [56]. These mice were mated with *EGFP*-transgenic mice, "green mice", and the double transgenic individuals showed dramatic silencing of *EGFP* throughout their lifetime. This experiment first proved that stable gene

silencing by RNAi was possible in mammalian organisms. However, the RNAi effect disappeared in some F1 progeny derived from founders that showed effective gene silencing, owing to fade-away of shRNA expression, and this observation identified a serious problem that needs to be resolved to allow RNAi technology to be used in mice [57]. Hence, future development of a method for heritable and stable gene silencing in organisms is required to establish stable knockdown mouse strains as a powerful tool for reverse genetics.

To date, suppression of endogenous genes in mammalian organisms by microinjection of a shRNA-expression vector into zygotes has been unsuccessful [11], and we were also unable to produce knockdown mice by this method, as discussed below. Carmell *et al.* constructed shRNA expression vectors directed against a variety of targets with well-known phenotypes, such as tyrosinase (albino), myosin VIIa (shaker), *Bmp-5* (crinkled ears), homogentisate 1,2-dioxygenase (urine turns black upon exposure to air), *Hoxa10* (limb defects), *Hairless* (hair loss) and melanocortin 1 receptor (yellow), and injected these vectors into fertilized mouse eggs. Despite the presence of transgenes in some animals, virtually none of the animals showed a distinct or reproducible phenotype associated with the particular target gene that was expected based on the phenotype of a null mutant or knockout mouse.

Recently, Chang *et al.* produced a knockdown mice using RNAi directed against an endogenous gene, *ABCA1*, which is a member of the ABC transporter protein family, using inducible regulation of RNAi by a combination of Cre-*loxP*- and tetracycline systems in a time- or tissue-dependent manner [58]. In their study, knockdown mice were produced by a conventional transgenic method. This is one of the rare cases reported to date in which a shRNA expression vector has been used in the successful silencing of an endogenous gene, and the reason for the success in this particular case is still unclear. In addition to such conventional methods, viral vectors can also be used to produce transgenic mice [59, 60]. Lentiviral vectors capable of introducing exogenous DNA into zygotes by mixing with zona-free eggs have been developed [60]. Using this strategy, it has been demonstrated that lentiviral vectors expressing siRNA against *GFP*, as illustrated in Fig. 4a, can reduce fluorescence in *GFP*-transgenic embryos and in mice after transduction of eggs [61]. Notably, it has been observed that the lentiviral vector avoids transcriptional shut-off from integrated viral DNA, which might account for the appearance of siRNA expression and the resulting down-regulation of a specific gene, not only in embryos but also in organisms after birth. This methodology has been applied to conditional gene silencing, as described below [58, 62–64].

Generation of Knockdown Mice by Introduction of a siRNA-expression Vector into Embryonic Stem Cells

Theoretically, embryonic stem cells (ES cells) should be suitable for production of knockdown mice. Similar to gene targeting, a shRNA expression vector is introduced into ES cells. Stable integrants that effectively silence the target gene are injected into host blastocysts, and the resulting chimeric mice are mated with wild type mice to produce F1 progeny with transgenes, which show gene silencing of the target gene. Since Carmell *et al.* failed to make knockdown mice by the microinjection method using mouse zygotes, as described above [11], they examined this strategy as an alternative. A shRNA expression vector directed against a novel gene, *Neil1*, was introduced into ES cells, and *Neil1* knockdown mice were successfully produced using stable integrants with strong silencing of the target gene. *Neil1* is a member of a newly discovered family of mammalian DNA *N*-glycosylases, related to the Fpg/Nei family of proteins from *Escherichia coli*, and has been proposed to have a role in DNA repair. Germline transmission of the shRNA expression transgene from chimeras to F1 progeny was achieved and a consequent reduction of the level of Neil1 protein was confirmed. It should be noted that suppression of the target gene expression in F1 mice was similar to that in the ES cells used. These results indicate that the use of ES cells provides a significant advantage in that the knockdown level of the target gene can be practically evaluated in ES cells in culture prior to the production of knockdown mice, which is quite difficult using the microinjection method.

Cells with a shRNA transgene exhibit dominantly gene silencing, and because of this the tetraploid aggregation method [65], in which the embryos are completely derived from ES cells, may make it possible to assess embryonic phenotypes directly. In tetraploid embryos aggregated with ES cells, the host tetraploid cells contribute to the extra-embryonic tissues, including the trophoblast tissue of the placenta and the extra-embryonic endoderm component of the yolk sac, but they are rigorously excluded from the embryo proper,

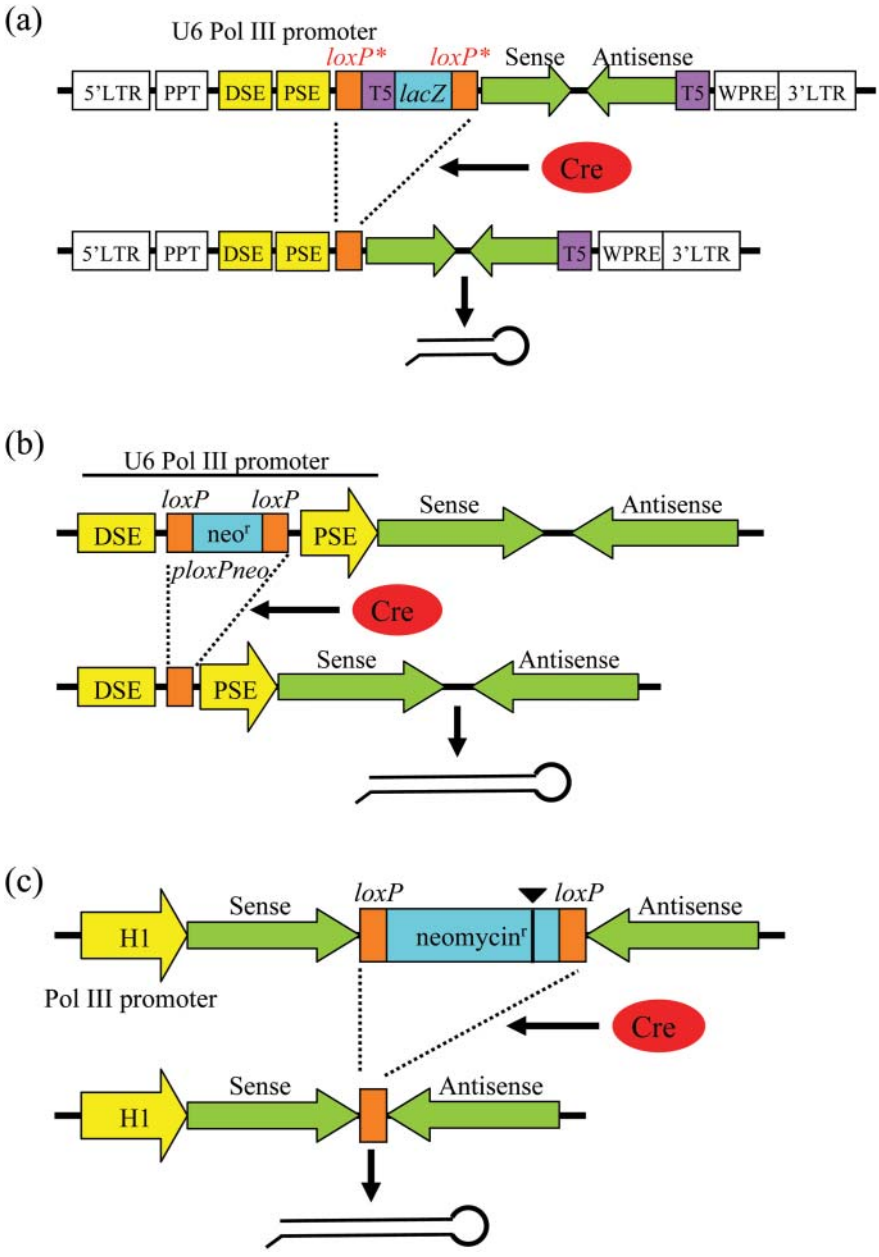

Fig. 4. Diagram of the siRNA expression vector using Cre-*loxP* system. (a) Lentiviral vector for expressing siRNA is inserted with a DNA stuffer sequence (T5/*lacZ*) flanked by two modified *loxP** sites. The *loxP** sequence contains two mutations, which generate the intercalated *loxP* sequence acting as a U6 TATA box after the loop-out of the stuffer sequence by Cre recombinase [63]. (b) A neomycin cassette flanked by two *loxP* sites (*ploxPneo*) separates a Proximal Site Enhancer (PSE) with a Distal Site Enhancer (DSE) of a U6 promoter. Cre recombinase eliminates *ploxP*, allowing the siRNA expression [67, 68]. (c) A neomycin cassette with a Pol III stop codon of 5 thymidines (T5) (arrowhead) is flanked by two *loxP* sites. This cassette is inserted downstream of the sense strand of the inverted repeat sequence. In the presence of Cre recombinase, the neomycin cassette is eliminated and the resulting intercalated *loxP* sequence acts as a loop to fold back the antisense strand [69].

which results in the embryo being composed entirely of ES cells. Using this strategy, it has been demonstrated that knockdown ES cell-derived embryos reproduce a genetic null phenotype [12]. In at experiment, ES cells were transfected with a shRNA expression vector directed against an endogenous gene, p120-Ras GTPase-activating protein (*RasGAP*), followed by injection into enhanced green fluorescent protein transgenic tetraploid embryos. As the tetraploid cells were derived from a transgenic line that ubiquitously expressed *EGFP*, contamination of host tetraploid cells in the embryo proper was detectible by their fluorescence. Indeed, *RasGAP* shRNA ES cell-derived embryos exhibited phenotypes that were strikingly similar to the null phenotype. Again, the reduction in expression of the target protein in ES cell lines was reflected by the degree of the phenotype in the embryos.

In contrast to homologous recombination, introduction of a shRNA expression vector into ES cells has similar problems to the microinjection of a vector construct into zygotes; that is, the copy number and integration site of the transgene are uncontrollable. Regarding these limitations, controllable integration of a single copy of a shRNA transgene into a defined locus *rosa26* has been demonstrated [66]. In addition to this site-specific insertion, the Cre-*loxP* system was combined at the rosa26 locus to induce tissue-specific shRNA expression. Furthermore, injection of ES cells with a shRNA transgene into tetraploid blastocysts has been shown to produce shRNA transgenic ES cell-derived mice at a frequency of 3% [66].

Generation of Conditional Knockdown Mice

Since the phenotypic effect of the shRNA transgene emerges in hemizygous mice, it is difficult to establish shRNA transgenic mouse strains and to analyze the function of the target gene in adult tissues when silencing of the target gene causes embryonic or juvenile lethality. To circumvent this limitation, effective conditional knockdown systems under the spatial and temporal control of RNAi have been developed. Recently, two knockdown systems, a combined RNAi and Cre-*loxP* system [59, 62–64, 66–69] and a pDECAP vector system [70], have been proposed to have the potential which generate transgenic mice with controllable gene silencing. These systems are expected to advance markedly the functional characterization of genes involved in development and disease.

The Cre-*loxP* system has been widely utilized for conditional gene knockout [71]. This system uses the Cre recombinase encoded by the bacteriophage P1 to catalyze recombination between consensus DNA sequences, called *loxP* recognition sites. To develop inducible regulation of RNAi, Coumoul *et al.* modified the Pol III U6 promoter to make a spatio-temporal switch controlled by the Cre-*loxP* system, as illustrated in Fig. 4b. The insertion of a *loxP*-flanked neomycin (*ploxPneo*) cassette between the regulatory elements of the U6 promoter impairs its ability to express a downstream shRNA targeting *Fgfr2* [67]. This inducible shRNA expression vector was injected into mouse zygotes and transgenic mice were obtained [68]. These mice did not express shRNA, owing to an interruption by a *ploxPneo* cassette, but showed germline transmission of the transgene. Mice carrying the *Fgfr2*-shRNA transgene were crossed with two kinds of *Cre* transgenic mice, *EIIa-Cre* expressed in the germline and *Ap2-Cre* expressed in the progress zone of the limb, to obtain transgenic mice with an *Fgfr2*-shRNA transgene and Cre recombinase. In these double transgenic (bigenic) mice, the *ploxPneo* cassette was looped-out by Cre recombinase in a Pol II-dependent manner, and the U6 promoter retrieved its activity. The resulting mice showed a dramatic reduction of Fgfr2 expression in a tissue-specific manner, and consequently embryonic lethality was induced in *Fgfr2* shRNA/*EIIa* mice and the malformation of digits of both the forelimbs and hindlimbs occurred in *Fgfr2* shRNA/*Ap2* mice. As demonstrated by Coumoul *et al.*, even a gene whose mutation or suppression induces embryonic lethality can be silenced *in vivo* using this method, such that its function can be analyzed. Fritsch *et al.* developed a Cre-*loxP* controlled shRNA expression vector in a different way as illustrated in Fig. 4c. In this vector, the neomycin cassette with the Pol III stop codon was used as terminator to interrupt complete transcription of an inverted repeat at the spacer (loop) sequence. Nowadays, a wide variety of transgenic mouse strains expressing Cre recombinase from tissue-specific promoters have been developed to achieve spatial and temporal control of gene targeting. An adenoviral vector encoding Cre recombinase can also transiently express this enzyme in transfected cells, and hence may also be useful for conditional gene silencing [64].

Another example of an approach designed to gain both spatial and temporal control over Cre recombinase transcription is provided by the use of a tetracycline (*tet*)-responsive system [58]. The tet system is based

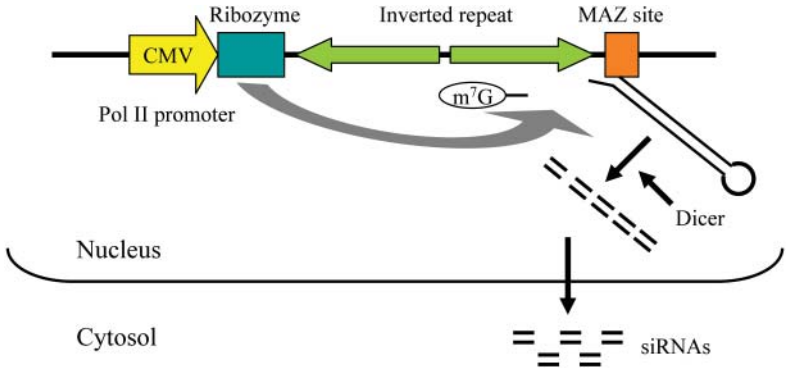

Fig. 5. Structure of pDECAP vector [70]. The pDECAP vector contains the Pol II promoter (e.g. CMV promoter), a ribozyme cassette and MAZ site. DNA template of an inverted repeat separated by a spacer is inserted between the ribozyme cassette and MAZ site. Transcription from the Pol II promoter is paused and the resulting long dsRNA is anchored at the MAZ site, which is cut off the m^7G cap structure by the ribozyme and is processed into siRNAs by RNase (Dicer) localized in the nucleus. The siRNAs then move to the cytosol.

on binding of the tet repressor to the tet operator (*tetO*). Tetracycline binds to the repressor, inducing a conformational change that causes the repressor to dissociate from *tetO*. If the tet repressor is fused to the transcriptional activation domain of the herpes simplex virus VP16 protein, a conditional transcription activator is obtained that can be shut off or, in another variation of the system, turned on by the addition of doxycycline, which is much less toxic to vertebrate cells than to *E.coli*. This inducible gene knockdown system, named the SIRIUS-Cre system, combines three elements: siRNA for specific gene silencing, Cre-*loxP* for tissue-specific expression and tetracycline for inducible expression. Transgenic mice were generated by mating two independent transgenic mouse strains which contained *ABCA1* siRNA expression vector with a *loxP*-flanked *EGFP* cassette and a tissue-specific Cre recombinase under tetracycline control. The bigenic mice have been shown to avoid embryonic lethality and to provide a successful mimic of Tangier disease, which is caused by mutation of the *ABCA1* gene. The combination of the RNAi and Cre-*loxP* approaches may facilitate the deciphering of the true functions of genes *in vivo*. However, a significant disadvantage of this system is that the Cre recombinase does not act in 100% of cells, and its induction results in a genetically heterogeneous population of target cells. Therefore, when a small number of Cre non-induced cells act complementarily in the target organs, the phenotypes of the transgenic mice may be complex. Hence, adoption of this system requires some care to confirm the correctness of the observed phenotype.

The pDECAP system may also have the potential to generate tissue-specific gene knockdown animals (Fig. 5) [70]. This novel vector expresses hairpin-type long dsRNA under the control of a Pol II promoter, instead of a Pol III promoter. To avoid the interferon response against long dsRNA, the vector is engineered to transcribe dsRNA that lacks both the 5'-cap structure and the 3'-poly adenylate tail needed for export to the cytosol. The transcripts form dsRNA structure, are processed into small siRNAs in the nucleus, and are then transferred to the cytosol, where they direct the degradation of the target mRNA without eliciting the interferon response. Using this system, a pDECAP vector expressing long dsRNA of the endogenous *Ski* gene from a CMV promoter was injected into mouse zygotes. pDECAP-*Ski* transgenic mouse embryos had recapitulated phenotypes that were remarkably similar to those of *Ski*-deficient embryos, with defects in neural tube closure and eye formation. Although Shinagawa and Ishii used a CMV promoter to control the pDECAP vector, it may be possible to express siRNAs derived from long dsRNAs using tissue-specific promoters.

Since oocytes and early embryos lack interferon-induced pathways, long dsRNAs can silence specific genes at these developmental stages [72]. Hence, microinjection of a long dsRNA directed against the *Mos* gene from the oocyte-specific ZP3 promoter has been used to generate transgenic mice. These founder

animals appeared healthy, but while males were fertile, females were sterile in accordance with the genetic null phenotype. Germline transmission of the transgene was confirmed from the founder males to F1 progeny. In the transgenic F1 females, the amount of *Mos* mRNA was reduced specifically, resulting in suppression of MAP kinase and H1 kinase activities in MII eggs; these eggs underwent spontaneous parthenogenetic activation. These observations demonstrate the recapitulations of the phenotype of the *Mos* null mutant.

Proof of Principle of siRNA-mediated Gene Silencing for the Correction of Genetic Diseases, using Knockdown Mice

RNAi has been employed as an innovative and powerful tool to investigate gene function *in vitro* and *in vivo*. Furthermore, this technology has fascinating potential applications as a therapeutic approach [73–77]. To date, to suppress viral replication, various vaccines and drugs targeting envelope proteins or enzymes derived from the virus have been investigated. The advent of RNAi has raised the possibility of developing a novel approach for targeting the viral genome. Recently, specific siRNAs targeted against replication of viruses such as the human immunodeficiency virus (HIV) [78, 79], poliovirus [80], SARS virus [81] and hepatitis B [82, 83] and C [84–86] viruses have been developed. For example, Yokota and colleagues have shown efficient inhibition of hepatitis C virus replication by both synthetic and vector-derived siRNAs using an HCV replicon system in the human hepatoma cell line Huh7 [84].

Furthermore, suppression of mutant proteins that cause disease provides a new strategic approach for the treatment of inherited diseases. Dominantly inherited diseases are caused by a mutant allele of a gene causing loss of function or gain of toxic function. In cases of loss of function caused by the mutant allele in which the normal allele has an insufficient compensatory effect, disease is caused by loss of function, whereas if the mutant gene induces a novel altered function or disturbs the metabolic conditions, pathological symptoms are caused by a gain of toxic function. Using the RNAi strategy, effective clinical treatment for intractable neurodegenerative diseases such as Alzheimer's disease and Parkinson's syndrome may be possible. Amyotrophic lateral sclerosis (ALS) is another fatal neurodegenerative disease characterized by degeneration of motor neurons in the central nervous system, and 20% of such cases are caused by gain of toxic function due to a mutated Cu/Zn superoxide dismutase (*SOD1*) enzyme. Therefore, inhibition of mutant *SOD1* expression may provide a direct approach to treatment for this type of familial ALS, in which onset and progression of the disease are prevented. In culture cells, siRNA can effectively suppress the expression of mutant proteins in various neurodegenerative diseases, including ALS [87]. Furthermore, virus-mediated siRNA delivered by direct injection of viral vectors to the spinal cord or muscles has been shown to delay the onset or progression of ALS in an ALS mouse model [88,89]. However, the most difficult problem in *in vivo* therapy with siRNA is that a sophisticated method of siRNA delivery throughout the central nervous system has yet to be developed. To address this issue, we generated *SOD1* knockdown mice in which siRNA is ubiquitously expressed in the whole body, including the brain, and then crossed these mice with $SOD1^{G93A}$ transgenic mice, a widely used animal model of ALS, to investigate the true efficacy of siRNA *in vivo*, especially in the central nervous system. In treating autosomal dominant inheritance, the siRNA sequence should in principle be selected to target only the mutant transcripts, but in the case of the *SOD1* gene, *SOD1*-deficient mice show no severe pathological phenotypes, except for fragility of nerve cells after an axonal injury, hearing loss and female sterility [90]. We therefore first designed a siRNA sequence for ubiquitous suppression of both the normal and mutant SOD1 proteins.

Microinjection of a shRNA expression vector into mouse zygotes

We first tried to generate *SOD1* knockdown mice using a conventional transgenic method, as used in other studies [11, 56]. The shRNA expression vector was constructed by inserting the stem-loop type *SOD1* siRNA cassette immediately downstream of the human U6, H1 promoter or tRNA promoter in pUC19 with a pgk1/ neomycin cassette. When these vectors were introduced into the pronucleus of the mouse fertilized eggs, no suppression of SOD1 protein was observed in either case, despite integration of the shRNA transgene into the genome (Fig. 6, unpublished data). Consequently, as also found by Carmell *et al*. [11], we were unable to generate siRNA transgenic mice by the microinjection method.

Generation of SOD1 knockdown mice from ES cells using a shRNA expression vector

As an alternative approach, we also examined the ES

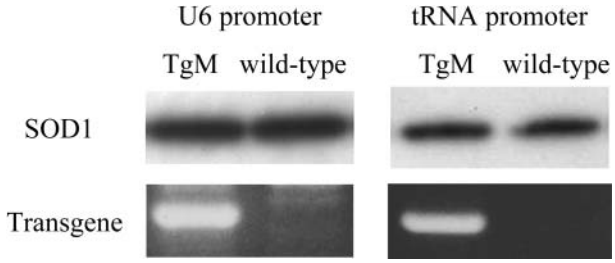

Fig. 6. Microinjection of the *SOD1* siRNA expression vectors into the mouse zygotes. Human U6 promoter and tRNA promoter were used as the Pol III promoter. Western blot analysis showed no down-regulation of the endogenous SOD1 protein (upper) despite the integration of transgene into the mouse genome being detected by PCR analysis.

cell method for production of shRNA transgenic mice that express siRNA. A *SOD1* shRNA expression vector was introduced into 129/Sv ES cells by electroporation, followed by drug selection with G418. As a result, some stable integrants showed more than an 80% reduction level of endogenous SOD1 protein by western blot analysis (Fig. 7a). The ES clones were injected into C57BL/6 blastocysts, and the resulting chimeric male mice were crossed with wild type C57BL/6 female mice. Germline transmission of the shRNA transgene was achieved in F1 progeny. The presence of the transgene in genomic DNA from the tails of these progeny was proved by PCR analysis, and a remarkable reduction of the level of *SOD1* transcripts was detected by northern blot analysis, which resulted in about 80% suppression of the SOD1 protein level on western blot analysis (Fig. 7b).

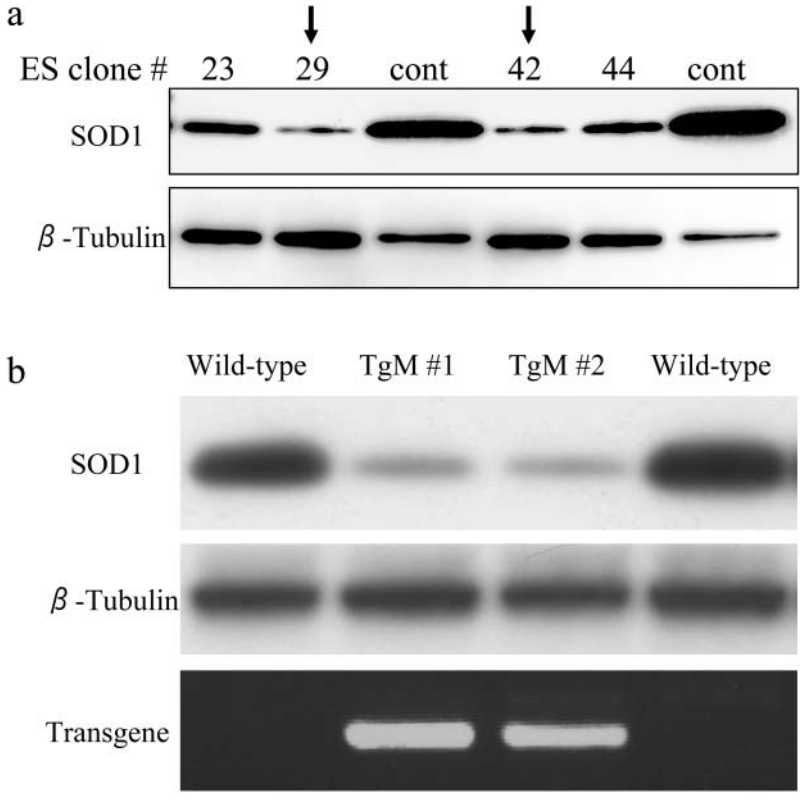

Fig. 7. Production of *SOD1* knockdown mice derived from ES cells. (a) Western blot analysis of whole cell lysate from individual ES cell clones for *SOD1* and tubulin. Suppression of the *SOD1* expression was successfully observed in many ES cell clones, some of which showed drastically enhanced down-regulation of the endogenous *SOD1* (Arrows). (b) Analysis of *SOD1* shRNA transgenic mice generated from the "knockdown" ES cells. Transgenic mice confirmed the integration of the transgene by PCR analysis in the genome from the tails (lower) showed the inhibition of *SOD1* expression by western blot analysis (upper) similar to that in the ES cells of their origin.

Similar to *SOD1*-deficient mice, the *SOD1* knockdown mice did not show any obvious phenotype, showing neither growth retardation nor motor signs, except for infertility in female mice.

siRNA directed against the SOD1 gene suppresses the onset of ALS in SOD1^{G93A} transgenic mice

Two different groups have recently demonstrated that injection of lentiviral vectors which express siRNA specifically targeting the human *SOD1* gene has a therapeutic effect in an ALS animal model [88, 89]. In these reports, human *SOD1* siRNA expression lentiviral vectors were delivered by intraspinal injection [88] or intramuscular injection [89]. Both treatments showed a retardation of the onset of ALS, an extension of survival and an improvement of motor performance in the mice. These results clearly demonstrate that siRNA has the potential to reduce the gain of toxic function caused by mutant proteins, which will encourage the exploration of gene therapy using RNAi technology.

We have also investigated the onset and progression of ALS in *SOD1* shRNA/*SOD1^{G93A}* bigenic mice generated by crossing *SOD1* knockdown mice with *SOD1^{G93A}* transgenic mice. If the expression level of both mutant human *SOD1^{G93A}* and mouse endogenous *SOD1* are simultaneously reduced in the bigenic mice, the *SOD1* shRNA/*SOD1^{G93A}* bigenic mice are expected to appear normal and to grow up healthy without any pathological features of ALS. Our approach of expressing siRNA constitutively in the whole body makes it possible to investigate the true physiological response in all the cell types that express the mutant gene with toxic function. Therefore, knockdown mice will facilitate investigation of the mechanism of the onset of genetic disorders, and will allow harnessing of RNAi technology for the development of new therapeutic approaches.

Conclusions and Future Prospects

The major advantage of production of RNAi knockdown mice is that the basic structure of a DNA-based knockdown vector and its introduction into cells are relatively simple. These features make it possible to regulate the suppression of splicing variants or multiple genes simultaneously [91], which requires quite time-consuming and laborious work using knock-out or knock-in methods based on homologous recombination. On the other hand, RNAi technology still has some disadvantages. In knockdown mice, non-specific and off-target effects of siRNAs may complicate the phenotype, and the efficacy of suppression of gene expression varies with both the target genes and organs. Guidelines for the RNAi approach are being developed to avoid these problems, including software algorithms to design the most effective and specific siRNA sequence against a given target gene [92–94].

Finally, although the application of RNAi to animals has yet to be investigated thoroughly, knockdown mice can facilitate the understanding of the true function of numerous genes *in vivo*. This fascinating technology represents an essential tool that will provide answers to many questions and enhance progress in the life sciences. Furthermore, genetic manipulation using RNAi is likely to develop as a novel technology with applications in non-human primates and domestic animals, in addition to its value as a therapeutic approach.

Acknowledgements

This work was supported in part by the Wakayama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence of the JST (T.M.), and the Ministry of Education, Culture, Science, Sports and Technology, Japan (T.Y.), from the Ministry of Health, Labour and Welfare, Japan (T.Y.).

Supplementary Note

Our study on ALS using *SOD1* knockdown mice, entitled “Transgenic siRNA halts ALS in a mouse model” by Saito, Y., Yokota, T., Mitani, T. *et al.*, has been accepted in J. Biol. Chem., and published on line on October 12, 2005. In this study, we demonstrated that *SOD1* knockdown mice expressing modified siRNA with multiple mismatch alterations could successfully suppress both human *SOD1^{G93A}* mutant protein and endogenous mouse SOD1 protein throughout lifetime and at least over four generations. As expectedly, in the *SOD1* shRNA/*SOD1^{G93A}* bigenic mice, siRNA prevented the development of disease in the central nervous system. Our findings clearly prove the principle that siRNA-mediated gene silencing can halt the development of certain autosomal dominant diseases.

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