Small interfering RNA and gene silencing in transgenic mice and rats

Hidetoshi Hasuwa\textsuperscript{a}, Kazuhiro Kaseda\textsuperscript{b}, Thorbjorn Einarsdottir\textsuperscript{a}, Masaru Okabe\textsuperscript{a,\*}

\textsuperscript{a}Genome Information Research Center, Osaka University, Yamadaoka 3-1, Suita, Osaka 565-0871, Japan
\textsuperscript{b}Research Institute for Microbial Diseases, Osaka University, Yamadaoka 3-1, Suita, Osaka 565-0871, Japan

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Abstract After short duplexes of synthetic 21–23 nt RNAs (siRNA) were reported to be effective in silencing specific genes, a vector-based approach for siRNAs was demonstrated in mammalian cultured cell lines. However, the effect of RNA interference (RNAi) on various differentiated cells in live animals remains unknown. In this report, we demonstrate that transcriptionally supplied siRNA can silence ubiquitously expressed enhanced green fluorescent protein in every part of the mouse and rat body. These results suggest that transgenic RNAi could function as an alternative method of gene silencing by applying homologous recombination to embryonic stem (ES) cells, and should be successful even in species where ES cell lines remain unestablished.

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1. Introduction

Double-stranded RNA (dsRNA)-based gene silencing or RNA interference (RNAi) is an ancient and evolutionarily conserved mechanism for sequence-specific post-transcriptional gene silencing among species from various kingdoms (reviewed in [1,2]). The system probably is the most well-studied in \textit{Caenorhabditis elegans} where long dsRNAs, ingested as part of the food source, are processed into short (19–25 nucleotides) small interfering RNAs (siRNA) by a ribonuclease-III–protein complex. The shorter siRNAs then prompt the specific degradation of homologous cellular RNA. Until recently, the use of RNAi to silence mammalian genes was not applicable because the introduction of dsRNAs longer than 30 nt elicits a viral response that is sequence-non-specific [3]. However, the introduction of short duplexes of synthetic 21–23 nt RNAs (siRNA) into mammalian cells have a gene-specific silencing function [4]. The limitation of this method is that the transfected synthetic siRNA works for only a few days in mammalian cells. To circumvent this problem, a vector-based approach for the synthesis of siRNAs which were driven by RNA polymerase III (pol III) promoter was demonstrated to silence various genes in mammalian cultured cell lines [5–11]. Recently, RNAi from an exogenously administrated plasmid was shown to silence a concomitantly administered transgene expression in adult mouse livers [12]. However, the effect was observed only in a transient expression system and the effect was shown only in the liver. In this report, we establish a transgene-based RNAi system using a transgene containing an enhanced green fluorescent protein (EGFP) siRNA driven by a pol III promoter, suggesting that it may be an alternative method for gene disruption. This system was also successful in silencing EGFP expressed in the rat, indicating that transgenic RNAi could analyze the function of genes in animals where homologous recombination is not possible due to the lack of embryonic stem (ES) cell lines.

2. Materials and methods

2.1. Plasmid construction

The Hi promoter [13] was PCR amplified from genomic DNA of the human 293 cell line, using the following primers: 5’-CCGCTCGAGAAGCTTCTCTGAGGATCAGTTCAAGAGACTGGGTGCTCAGGTAGTGTAAGATTCCC-3’ and 5’-GAGCACCCAGTTCAAGAGACTGGGTGCTCAGGTAGTGTAAGATTCCC-3’. The PCR product was digested with EcoRV and XhoI, and cloned into the XhoI site of pH1 (pH1/siRNAEGFP). The HcRed1 gene was amplified from the human 293 cell line, using the following primers: 5’-CCGCTCGAGAAGCTTCTCTGAGGATCAGTTCAAGAGACTGGGTGCTCAGGTAGTGTAAGATTCCC-3’ and 5’-GAGCACCCAGTTCAAGAGACTGGGTGCTCAGGTAGTGTAAGATTCCC-3’. The PCR product was digested with EcoRV and XhoI, and cloned into the XhoI site of pH1 (pH1/XhoI/SiRNAEGFP).

CAG/HcRed1 was constructed from the pCX-EGFP [14] and pHcRed1-N1 (Clontech) plasmids. NovI and XhoI sites were introduced between the CAG promoter and the rabbit \(\beta\)-globin polyA region of pCX-EGFP (pCXNX). The HcRed1 gene was amplified by PCR using primers including NovI or XhoI sites at their 5’ ends. Both the PCR product and pCXNX were digested with NovI and XhoI and ligated (pRed). To produce the siRNA transgenic animals with red fluorescence as an RNAi transgene marker, pH1/siRNAEGFP was digested with BamHI and HindIII, and inserted into the corresponding restriction sites of pRed (pGtoR).

2.2. Animals and DNA injection

The ‘green’ mouse line, C57BL/6 Tg[act-EGFP]OobC14-Y01-FM131, and the ‘green’ rat line, Rat SD TgN(act-EGFP)Osb4, have been established as described previously [14,15]. Fertilized green mouse eggs were prepared by fertilizing wild-type (B6D2F1) eggs in vitro with sperm from homozygous ‘green mice’. Fertilized rat eggs were obtained by mating wild-type (SD) females with heterozygous ‘green rat’ males. The pGtoR and pRed plasmids, which were digested with SalI and BamHI and purified from an agarose gel, were injected into the pronuclear stage of fertilized eggs. The injected embryos were cultured in KSOM until the blastocyst stage or immediately implanted into pseudopregnant animals to generate transgenic animals.

2.3. Microscopic analysis

DNA-injected embryos and newborn animals were observed on a...
fluorescence inverted microscope IX70 (Olympus) and fluorescence stereoscopic microscope SZX12 (Olympus). The green and red fluoroses were photographed under 510 nm (green, EGFP) and 610 nm (red, HcRed1) band pass filters equipped with a CoolSNAP fx and cex color digital camera (Roper).

2.4. Genotype analysis
Genotypes of all mice and fetuses were determined by PCR analysis on tail or fetal DNA. PCR was carried out using the EGFP (GAATTCGCCACATGTGAGC, GAATTCTTACTTGTACAGCTCGTCC) and human H1 promoter primers (GAACGCTGACGTCATAACC, TGGTCTACAGAACCTTAATAAGATCC).

2.5. Immunoblot analysis
Total cellular proteins were extracted with 30 mM 3-(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate/phosphate-buffered saline with 1 mM phenylmethylsulfonyl fluoride, separated by SDS-PAGE on 12.5% polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes. EGFP, CD9, and Shc were visualized using an ECL detection system (Amersham Bioscience) after incubation with rat anti-EGFP monoclonal antibody (kindly provided by Dr. Fujita of Mitsubishi-Kasei Institute of Life Sciences), anti-CD9 antibody KMC8.8 (Pharmingen) or anti-Shc antibody c-20 (Santa Cruz Biotechnology). Either a horseradish peroxidase-linked goat anti-rat or rabbit IgG (Chemicon) was used for the secondary antibody. The visualized signals were quantified by the NIH image program.

3. Results and discussion
Although long dsRNAs are thought to be detrimental to mammalian cells [3], this may not be applicable in early embryos. In this regard, when mouse eggs are injected with dsRNA corresponding to GFP, RNA-induced gene silencing was reported [16,17]. However, the silencing effect continued for only a few days. The injection of synthesized siRNA (21-mer) of EGFP into ‘green’ one cell eggs is also effective in silencing the expression of the transgene, but again only for a limited time period (data not shown). Fluorescence began to re-appear after the blastocyst stage. This was probably due to a dilution of injected siRNA by a rapid increase of embryonic body mass. To circumvent this limitation, we investigated the possibility of a transgene-based gene silencing system to maintain the RNAi effect in animals for an extended period. ‘Green’ mice [14] and rats [15], i.e. mice and rats that express EGFP in all tissues and cells, were used as a model system. The silencing EGFP transgenic construct consisted of the pol III human H1 promoter [13] with a hairpin precursor sequence for siRNA (Fig. 1a). The transgene also contained HcRed1 driven by a CAG promoter, which was designed to monitor the genomic integration of the transgene by turning the cells ‘red’ while the dsRNA silences the ‘green’. This ‘green to red’ transgenic construct (pGtoR) was injected into the pronuclei of ‘green’ mouse eggs prepared by fertilizing wild-type B6D2F1 eggs in vitro with sperm from homozygous ‘green’ mice. Initially, silencing of EGFP was examined in mouse preimplantation blastocysts. The embryos were cultured for 3 days in vitro in kSOM medium [18] following transgene injection. The blastocysts appeared ‘red’ due to the expression of the HcRed1 portion of the transgenic construct, indicating injection was successful. Significantly, EGFP fluorescence was greatly reduced. This EGFP silencing was not due to the expression of HcRed1 because when only the pRed (CAG/HcRed1) construct was injected, the intensity of EGFP fluorescence in the blastocysts was not reduced (Fig. 1b).

Following injection of the transgenes, the mouse eggs were

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**Fig. 1.** Transgenic-based gene silencing in mouse embryos. a: A pGtoR (pH1/siRNAEGFP-CAG/HcRed1) transgene was constructed. Two oligonucleotides containing sense and antisense 21 nt sequences from the EGFP coding region (blue colored sequence), a 9 nt spacer sequence which provided a loop structure, and a transcription termination signal of five thymidines (red colored sequence) were annealed and inserted downstream of the H1 promoter (pH1/siRNAEGFP). b: Green eggs, obtained from wild-type females mated with homozygous ‘green’ males, were injected with pGtoR, cultured in kSOM for 3 days, and observed for HcRed (middle column) and EGFP (right column) expression by fluorescence microscopy. ‘Green’ blastocysts with no additional transgene (top row), ‘green’ blastocysts with the pRed transgene (middle row) and ‘silenced’ blastocysts with the pGtoR transgene (bottom row). c: Day 10.5 embryos were recovered and their fluorescence observed (from the left, ‘green’ embryo with pRed GM# #1, with pGtoR GM# #1, 2 and 3, respectively). d: Immunoblot analysis of EGFP in day 10.5 embryos. The lysates from E10.5 embryo of one pRed integrated transgenic (GM# #1) and three pGtoR integrated embryos (GM# #1, 2 and 3 as shown above) were analyzed by immunoblotting with anti-EGFP (top) and anti-CD9 (bottom) antibody. e: The intensity of the bands in panel d was quantified and normalized to the control EGFP amount.
transplanted to the oviducts of pseudopregnant females. Transgenic founder embryos were recovered at day 10.5 and examined for EGFP silencing. In these embryos, the green fluorescence was diminished in all organs including the heart which showed the strongest expression of EGFP at this stage of development (Fig. 1c). In contrast, when synthesized siRNA was injected into one cell stage embryos, the EGFP silencing effect disappeared by day 10.5 and all the embryos showed comparable green fluorescence to normal green embryos (data not shown), indicating that the transgenic approach extended the silencing effect. Proteins from one pRed transgenic embryo GM<sub>R</sub> #1 (as a control) and three pGtoR transgenic embryos GM<sub>GR</sub> #1 to 3 that showed ‘red’ fluorescence and were positive for human H1 promoter sequence in genomic PCR screening were prepared by homogenizing whole embryos and the amount of EGFP was analyzed by immunoblotting (Fig. 1d). Following quantification, EGFP expression of the three founder mice was reduced to 18, 4 and 24% of the control level, respectively (Fig. 1e). In contrast, the amount of CD9 was not affected by the integration of the transgene (Fig. 1d).

Newborn founder mice which had incorporated the transgenic construct were identified by their ‘red’ fluorescence appearance and genomic PCR analysis. ‘Red mice’ that contained the pRed transgene alone showed no decrease in EGFP fluorescence when compared to the original ‘green’ mice. However, mice containing the pGtoR transgene (e.g. GM<sub>GR</sub> #4) showed a significant decrease in their ‘green’ fluorescence (Fig. 2a). To observe the silencing of EGFP in various tissues, brain, heart, liver, pancreas, kidney and skin were collected from the newborn mice with a genetic background of ‘green’ (GM #2), ‘green with pRed’ (GM<sub>R</sub> #3) and ‘green with pGtoR’ (GM<sub>GR</sub> #5). The silencing effect was nearly complete in both the heart and pancreas, two organs that express EGFP intensively (Fig. 2b). Furthermore, all the tis-

![Fig. 2. Transgenic-based gene silencing in newborn mice. Mice were obtained by injecting ‘green eggs’ with pRed or pGtoR that contains the siRNA<sub>EGFP</sub> sequence. a: A newborn ‘green’ mouse without an additional transgene (GM #1), a ‘green’ mouse containing the pRed transgene (GM<sub>R</sub> #2), and a ‘silenced green’ mouse with the pGtoR transgene (GM<sub>GR</sub> #4). b: Various organs were removed from newborn founder mice (GM #2, GM<sub>R</sub> #3, GM<sub>GR</sub> #5) containing the genetic backgrounds indicated above and photographed under normal lighting (left), 510 nm (right) and 610 nm (center) band pass filter. The exposure time for each panel was adjusted to obtain optimal images. The absolute fluorescence in muscle and pancreas is brighter than in other tissues.

![Fig. 3. Transgenic-based EGFP silencing in adult mice and the F1 generation. a: Fluorescent view of abdominal muscle obtained from 8 week old ‘green’ mouse (GM #3) and from ‘green with pGtoR’ transgenic mouse (GM<sub>GR</sub> #4 in Fig. 2a). b: Immunoblot of proteins extracted from abdominal muscle and skin. Eight week old double transgenic mice with a ‘green’ genetic background and with or without pGtoR integration were compared for EGFP silencing. A B6D2F1 mouse was used to analyze a wild-type mouse. c: Fluorescent view of day 12.5 embryos obtained from B6D2F1 females mated with the GM<sub>GR</sub> #4 transgenic male from b. Genetic backgrounds of embryos #1 to #4 are as indicated in the table shown in d. d: The embryos shown in c were homogenized and analyzed by immunoblotting. The + and – in the table indicate the result of genotyping the EGFP and H1 promoter sequences by PCR.


EGFP expression in abdominal muscle and skin in the 8 week RNAi silencing effect was transmitted to the next generation. The success of transgenic silencing will bring several important advances in the field of gene function analysis in mammals. First, since the silencing effect is inheritable, the system can provide a new type of gene ‘knockout’ or ‘knockdown’ method. While homologous recombination using ES cell lines is a well-established method to study the function of a particular gene, the method is time- and labor-intensive. In contrast, the RNAi approach is relatively rapid. Since a RNAi corresponding to a particular gene functions in ‘trans’, the phenotypic effect of gene silencing experiments can be examined in hemizygous mice. Second, the results in this paper demonstrate that silencing could be effective in any type of organ and any kind of cell from the early embryo until the adult. Third, silencing in rats indicates that silencing could become a powerful tool to produce genetically modified animals in those species where ES cell lines are not established and homologous recombination has not been demonstrated. Finally, in the future it should be possible to express a particular RNAi in selected organs or at a specific developmental time by using methods such as the Cre/loxP recombination system and/or the tetracycline switch promoter [19,20].

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References


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