Gene silencing in chick embryos with a vector-based small interfering RNA system

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In this paper, the use of vector-based RNA interference (RNAi) to specifically interfere with gene expression in chick embryos is reported. In ovo electroporation was carried out to transfer a small interfering RNA (siRNA) expression vector into chick embryos. En2 was chosen for the target gene because the family gene, En1, is expressed in a similar pattern. Four sets of 19-mer sequences were designed with the En2 open reading frame region connected to a sequence of short hairpin RNA (shRNA), which exerts siRNA effects after being transcribed, and inserted into pSilencer U6-1.0 vector. En2 and En1 expression were suppressed by the siRNA whose sequence completely matched En2 and En1. Suppression occurred when the siRNA sequence differed by up to two nucleotides from the target sequence. The sequence that differed by four nucleotides from the target gene did not show siRNA effects. One set that completely matched the En2 target did not show siRNA effects, which may be due to location of the siRNA in the target gene. Thus, multiple sets of shRNA must be prepared if we are to consider. This system will greatly contribute to the analysis of function of genes of interest, because the target gene can be silenced in a locally and temporally desired manner.

Key words: chick, engrailed, in ovo electroporation, RNAi, shRNA, siRNA.

Introduction

In the field of experimental embryology, chick embryos have been used as a model animal because of accessibility to the embryos. Since in ovo gene transfer by electroporation was developed (Mura-matsu et al. 1997; Ogino & Yasuda 1998; Funahashi et al. 1999), we can analyze the function of a gene of interest conventionally. But in chick embryos, it is still difficult to knock down function of certain genes, because it is not possible to make mutant animals by gene targeting. In some studies, antagonistic constructs have been used (Araki & Nakamura 1999; Matsunaga et al. 2000), but this method is limited to certain kinds of molecule. Morpholino antisense oligonucleotides have recently been used in chick embryos, and have been shown to be efficient for gene silencing (Kos et al. 2001; Kos et al. 2003; Sugiyama & Nakamura 2003). We can transfer fluorescein-conjugated morpholino antisense oligonucleotides by electroporation, but because these molecules interfere with translation, we need antibody against the molecule to evaluate the results precisely.

Powerful gene silencing by double-stranded RNA, known as RNA interference (RNAi), was developed in Caenorhabditis elegans and Drosophila (Fire et al. 1998; Kennerdell & Carthew 1998). Double-stranded RNA molecules of 21 bp, termed small interfering RNA (siRNA), also have gene-silencing activity in mammalian cells (Caplen et al. 2001; Elbashir et al. 2001a; Harborth et al. 2001a; Harborth et al. 2001b). Recently, it was shown that short hairpin forming 45–50-mer RNA (shRNA) that is complementary to the gene of interest has RNAi effects (Svoboda et al. 2001; Brummelkamp et al. 2002; Paddison et al. 2002; Paul et al. 2002; Sui et al. 2002; Yu et al. 2002). An siRNA expression vector for mammalian cells has been developed, and gene silencing by transfecting cultured cells with the expression vector has been reported (Svoboda et al. 2001; Brummelkamp et al. 2002; Lee et al. 2002; Miyagishi & Taira 2002; Paddison et al. 2002; Paul et al. 2002; Sui et al. 2002; Yu et al. 2002).

We wondered if vector-based siRNA could be realized by in ovo electroporation in chick embryos. Because siRNA is reported to exert its effects by degrading mRNA in an RNase III-dependent manner, the effect could be conveniently detected by in situ hybridization. To confirm the sequence specificity and the site dependency of siRNA for the target gene, we tested the En2 gene, because the engrailed family En1 is expressed in a similar expression pattern to...
En2 in the midbrain–hindbrain (Gardner et al. 1988; Patel et al. 1989). Thus, En1 would be a good control.

Materials and Methods

Design of shRNA sequences

We designed shRNA to interfere with En2 expression, referring to technical information of Ambion (Austin, TX, USA) and NipponBioService (Asaka, Saitama, Japan). Four sets of 19-mer oligonucleotides, immediately downstream of an AA dinucleotide, were selected from the chick En2 open reading frame (ORF) sequence (Fig. 1a) and were named En2-150, En2-582, En2-648 and En2-846, according to the number from the first base of the start codon (ATG). En2-150, in the engrailed homology region (EH) 1 domain, was completely homologous to the corresponding part of the En1 sequence (Logan et al. 1992). En2-648, in the homeodomain, differed by two nucleotides from the corresponding part of the En1 sequence; En2-582, in the EH3 domain, differed by six nucleotides from the corresponding part of the En1 sequence; and En2-846 differed by eight nucleotides from the corresponding part of the En1 sequence. An oligonucleotide in which four nucleotides were substituted from En2-648 was designed, and designated as En2-648s4 (Fig. 1b). We confirmed that selected oligonucleotide sets did not have homology to any other genes by a BLAST search, so that they would not interfere with other genes.

The oligonucleotides were synthesized and column-purified at NipponBioService. The 19-mer sense siRNA sequence and antisense siRNA sequence were linked with a nine nucleotide spacer (TTCAAGAGA) as a loop. Six T bases and 6 A bases were added as a termination signal to the 3’ end of the forward oligonucleotides and 5’ end of the reverse oligonucleotides, respectively. Then four nucleotides corresponding to the EcoRI (AATT) and ApaI (GGCC) restriction sites were added to the 5’ and 3’ end of the reverse oligonucleotides, respectively (Fig. 1c).

Selection of a clone

Forward and reverse oligonucleotides were incubated in annealing buffer (100 mM K-acetate, 30 mM HEPES-KOH (pH 7.4) and 2 mM Mg-acetate) for 3 min at 90°C, followed by incubation for 1 h at 37°C. The annealed DNA for siRNA was ligated with linearized pSilencer1.0-U6 siRNA expression vector (Ambion) at ApaI and EcoRI sites. After transfection, many clones were picked and sequenced from both sides. Many clones contained no insert or mutated insert, and some inserts were hard to sequence because they formed very complex structures. To improve sequencing, we added dimethylsulfoxide (DMSO) up to 5% in

![Fig. 1. Design of vector-based En2 short hairpin RNA (shRNA).](image-url)
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the reaction mixture. We used a clone that does not contain mutation.

In ovo electroporation

Fertile chick eggs obtained from a local farm were incubated at 38°C. They were staged according to Hamburger and Hamilton (1951). In ovo electroporation was carried out to transfect the plasmid to stage 10 chick embryos as previously reported (Funahashi et al. 1999; Nakamura et al. 2000). Green fluorescence protein (GFP) expression vector (pEGFP-N1; Clontech, Palo Alto, CA, USA) was co-electroporated with siRNA constructs to check efficiency. Plasmid solution was 1–2 μg/μL. As described previously (Funahashi et al. 1999; Nakamura et al. 2000), transfection occurs on the hemilateral side of the neural tube, and the other side serves as a control.

In situ hybridization and immunohistochemistry

Specimens were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). In embryos at a later stage than 14, the epidermal and mesenchymal tissues were removed from embryos, and the neural tube was exposed. Whole-mount in situ hybridization was carried out as previously described by Bally-Cuif et al. (1992), except that the hybridization and wash were carried out at 65°C rather than at 70°C. Digoxigenin (DIG)-labeled antisense RNA probe was used. Alkaline phosphatase (ALP)-conjugated anti-DIG (Roche, Mannheim, Germany) was used for detection, with the color substrates 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). After whole-mount in situ hybridization, embryos were immunostained with anti-En2 monoclonal antibody 4D9 (Patel et al. 1989) as a primary antibody, and horse radish peroxidase (HRP)-conjugated antimouse antibody (Jackson, West Grove, PA, USA) as a secondary antibody (Funahashi et al. 1999). NBT/BCIP color was removed to detect only the HRP immunostaining by incubating in N,N-dimethylformamide (DMF) at 55°C for 3 h.

En2 antisense probe was a 687 bp fragment (–130–457). En1 antisense probe was a 590 bp fragment (–38–552), as described previously (Itasaki & Nakamura 1992).

Results

Gene silencing by electroporation with shRNA expression plasmids

To explore the conventional RNA interference system in chick embryos, we attempted to apply electroporation to transfected plasmids that encode shRNA. We selected En2 for silencing, because En1 could be used as a control. Four sets of 19-mer oligonucleotides that are in the En2 ORF were designed: En2-150, which completely matches the corresponding En1 sequence. Control side (A,D,G,J). Experimental side (B,E,H,K). mes, mesencephalon; met, metencephalon. Bars, 100 μm.
by two and six nucleotides, respectively, from the corresponding sequence of En1; and En2-846, which differs by eight nucleotides from the corresponding sequence of En1 (Fig. 1a,b).

Expression vector of GFP was co-electroporated to assess the transfection site of the shRNA. shRNA was transcribed by RNA polymerase III, and GFP was transcribed by RNA polymerase II. Because both plasmids assure ubiquitous expression, GFP-expressing cells were regarded as shRNA-transfected cells.

Electroporation with En2-150 expression vector interfered with expression of both En2 and En1 (n = 11 and n = 9, respectively; Fig. 2A–F). It is difficult to compare the degree of interference between En2 and En1, because the expression pattern of En1 (Fig. 2D) and En2 (Fig. 2A) are a little different from each other. However, it appeared that En2-150 interfered similarly with En2 and En1.

Electroporation with En2-582 interfered with En2 expression (n = 3) in a somewhat different manner from that by En2-150 (Fig. 2B,H). Repression by En2-582 seemed weaker than by En2-150, which may be due to the fact that En2-582 contains four consecutive C bases (the manufacturer of oligonucleotides recommends to avoid more than three consecutive C bases). Alternatively, it may be due to the fact that siRNA may be less accessible to the target gene because of the complex secondary structure in this region of the mRNA (Leirdal & Sioud 2002; Miyagishi & Taira 2002). En2-582 differed by six nucleotides from the corresponding En1 sequence, and did not affect En1 expression (n = 3; Fig. 2J–L).

Electroporation with En2-846 did not interfere with En2 expression (n = 6), which indicates that the matching site of En2-846, near the C-terminal end of the En2 ORF, may be located in the complex secondary structure of the mRNA. Therefore, En2-846 could not access the target mRNA. En1 expression was not affected by En2-846 either (n = 6, data not shown).

**Specificity of gene silencing by shRNA**

The specificity of the shRNA sequence was tested by En2-648 and En2-648s4. En2-648 differs by two nucleotides from the corresponding sequence of En1. Four nucleotides were substituted in En2-648s4. Consequently, En2-648s4 differs by four nucleotides from En2 and six nucleotides from En1 (Fig. 1b).

En2-648 silenced En2 expression as did En2-150, as was assessed by in situ hybridization (Figs 2A–C, 3A–C). En1 expression was also affected, but the degree of interference of En1 was less than that of En2 (Fig. 3D–F). The results indicate that shRNA could interfere with a sequence that differed by two nucleotides from the target gene.

En2-648s4, in which four nucleotides were substituted from the En2 sequence, did not interfere with En2 expression (n = 3; Fig. 3G–I). En2-648s4, which differed by six nucleotides from the target sequence, did not repress En1 expression (n = 3; Fig. 3J–L). In summary, shRNA could affect sequences that differed by two nucleotides from the target sequence. Sequences that differed by more than four nucleotides could not be affected.

**Fig. 3.** Sequence specificity of the RNA interference (RNAi) effects in the midbrain–hindbrain region 24 h after electroporation. Whole-mount in situ hybridization for En2 (A,B,G,H) and En1 (D,E,J,K). Green fluorescent protein (GFP) fluorescence shows the transfected sites (C,F,I,L). Photographs on the same row are from the same embryo. En2-648 suppressed En2 as clearly as En2-150 (B), and suppressed En1 weakly (E). This result indicates that short hairpin RNA (shRNA) could interfere with the sequence that differed by two nucleotides from the target sequence (Fig. 1B). En2-648s4, a four nucleotide substitution from En2 and six nucleotide substitution from En1, did not affect En2 and En1 expression (H,K). Control side (A,D,G,J). Experimental side (B,E,H,K). mes, mesencephalon; met, metencephalon. Bars, 100 µm.
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Time-course of gene silencing

Effects of shRNA were detectable 6 h after electroporation by in situ hybridization (n = 6; Fig. 4A–B). At 6 h after electroporation, reduction of RNA signal was slight (Fig. 4B), and immunostaining on the same embryo showed that reduction in the protein was at an undetectable level (Fig. 4C).

By 12 h after electroporation, effects of shRNA became clear. In situ hybridization and immunostaining on the same embryo revealed that En2 expression was silenced at the site where strong GFP fluorescence was observed (n = 6; Fig. 4D–F).

We followed the effects of En2-150 and En2-648 until 48 h after electroporation. Silencing could be detected until the stage we examined (n = 4, data not shown). Because En2 expression becomes very weak after that stage, we did not follow its expression further.

Discussion

The present study has shown that in ovo transfection with shRNA expression vector effectively repressed target gene expression. We designed four sets of oligonucleotides that make hairpin loops and contain 19-mer forward and reverse sequences in the En2 ORF. The oligonucleotides were inserted into pSilencer 1.0-U6, and transfected by electroporation into the midbrain–hindbrain region. Two siRNA, En2-150 and En2-648, effectively repressed En2 expression, as assessed by in situ hybridization. This may indicate that shRNA caused mRNA degradation, because the probe for in situ hybridization is the N-terminal region, and does not cover the En2-648 region. In addition, repression of translation product of En2 by shRNA was assessed by immunohistochemistry with anti-En2 antibody. Another siRNA, En2-582, also repressed En2 expression, but its effect seemed weaker than that of En2-150 and En2-648. This may be due to the fact that En2-582 contained four consecutive C bases (not recommended by the manufacturer for siRNA), or due to less accessibility of siRNA because of the secondary structure of mRNA. The other siRNA, En2-846, did not affect En2 expression. En2-846 matches the sequence very near to the C-terminal region of the En2 ORF, and this site may be difficult for the siRNA to access because of the complex secondary structure of the target mRNA. Therefore, multiple sets of shRNA need to be prepared to silence the gene of interest (Jarvis & Ford 2001; Holen et al. 2002; Lee et al. 2002; Sørensen et al. 2003).

Some researchers have reported that even a one nucleotide mismatch of shRNA failed to suppress target gene expression (Elbashir et al. 2001a; Elbashir et al. 2001b; Brummelkamp et al. 2002; Yu et al. 2002; Zhang et al. 2003). Elbashir et al. (2001b) reported that substitution of only one nucleotide near the center of the siRNA abolished its effects, but that a two to four nucleotide substitution near the 3′ end of the siRNA did not affect its effects significantly. In the present study, shRNA that mismatched by two nucleotides suppressed gene expression. shRNA that differed by more than four nucleotides from the target gene did not exert RNAi effects.

It has been shown that silencing of the target gene can be detected by 4 h after application of synthesized siRNA to cultured cells (Byrom et al. 2002). In the present study, we transfected expression vector of En2 shRNA to live chick embryos by electro-
poration. A slight decrease in En2 mRNA could be detected by 6 h after electroporation. The results suggest that shRNA may have been transcribed rapidly to silence the target gene. Reduction in translation product was very subtle at 6 h after electroporation, but was clearly detected by 12 h after electroporation.

Realization of gene silencing in chick embryos has been long awaited. To date, morpholino antisense oligonucleotides have been used as a tool for gene silencing in chick embryos (Kos et al. 2001; Kos et al. 2003; Sugiyama & Nakamura 2003), but with this system, expensive antibodies against the gene products are needed to check if the genes have really been silenced or not. An shRNA system with an expression vector is very convenient to prepare and to apply to the embryo. This method will assure locally and temporally restricted gene silencing, and will greatly contribute to functional analysis of genes of interest.

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References


