DEVELOPMENTAL DYNAMICS 231:592–600, 2004

TECHNIQUES

In Vivo Comparative Study of RNAi Methodologies by In Ovo Electroporation in the Chick Embryo

M. Rao, J.H. Baraban, F. Rajaii, and S. Sockanathan*

The combination of emergent RNA interference (RNAi) technology with in ovo electroporation in the chick embryo has the potential to provide a powerful and rapid means for functional analyses of novel genes in vivo. In this study, we show that electroporation of short 21-bp RNA duplexes (siRNAs) is a quick and simple method for silencing exogenous and endogenous gene expression in vivo. Quantitative comparisons with two other RNAi protocols that use long double-stranded RNA duplexes and endonuclease-digested duplexes (esiRNAs) demonstrate that siRNAs are significantly more effective at reducing gene expression. Furthermore, we also find that much higher amounts of siRNA are required for silencing of endogenous gene expression relative to plasmid-borne reporter constructs. In short, these results demonstrate that siRNAs are the most effective type of double-stranded RNA duplex for silencing gene expression and suggest that there might be important differences between silencing endogenous and exogenous genes. Finally, we review the parameters for each of these RNA-based methods of RNAi and the controls required to analyze RNAi data in the context of the developing vertebrate embryo. *Developmental Dynamics 231: 592–600, 2004.* © 2004 Wiley-Liss, Inc.

Key words: RNAi; chick; embryo; spinal cord

Received 16 January 2004; Revised 12 May 2004; Accepted 14 June 2004

INTRODUCTION

Recent advances in genomic technology and high-throughput screens have resulted in the identification of an increasing number of novel genes implicated in a myriad of biological processes. The major challenge now is to define the functions of these genes to assemble the available information into coherent networks. Two approaches commonly used to assess gene function in vivo are gain- and loss-of-function analyses. However, technical constraints often limit certain methodologies to subsets of animal model systems. For example, in *Caenorhabditis elegans* the generation of transgenic animals for gain-of-function experiments in addition to the application of RNA interference for loss-of-function studies has allowed significant progress in the understanding of gene function (reviewed by Grishok and Mello, 2002). In vertebrates, however, while gain-of-function strategies such as in ovo electroporation in the chick (Nakamura and Funahashi, 2001) and transgenesis in the mouse have become routine in assessing gene function, loss-of-function approaches have remained problematic.

To date, the zebrafish model system has proved the most amenable to high-throughput loss-of-function screens due to the availability of morpholinos and antisense DNA oligos. However, both of these antisense technologies are often plagued by nonspecific side effects as well as variable penetrance and phenotype (reviewed by Heasman, 2002; Kos et al., 2003). While morpholinos are less toxic and more nuclease-resistant than standard DNA oligos, their use demands reliable sequence information for a specific 30-bp stretch of nucleotides spanning the start codon. Furthermore, both of these approaches target translation, meaning that antibodies specific to the protein of interest would be nec-

Department of Neuroscience, Johns Hopkins School of Medicine, Baltimore, Maryland
Grant sponsor: National Institute of Neurological Disease and Stroke (National Institutes of Health); Grant number: RO1NS046336; Grant sponsor: the Whitehall Foundation; Grant sponsor: Muscular Dystrophy Association.
J.H. Baraban's present address is Yale University, P.O. Box 200579, New Haven, CT 06520.
*Correspondence to: S. Sockanathan, Department of Neuroscience, Johns Hopkins School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205. E-mail: ssockan1@jhmi.edu

DOI 10.1002/dvdy.20161
Published online 16 September 2004 in Wiley InterScience (www.interscience.wiley.com).

essary to confirm loss of the gene product. Thus, there is a clear need for a rapid and reliable means of evaluating loss of gene function in the vertebrate embryo, using methodologies that require minimal sequence information or immunohistochemical reagents.

One of the principal vertebrate model systems used in developmental biology is the chick embryo. The main advantages of the chick system are first, the generation of large numbers of embryos is relatively economical; second, embryos at all stages of development are accessible for experimental manipulation, and third, in ovo electroporation has provided a simple and effective means of introducing nucleic acid molecules into chick embryos in vivo (reviewed by Itasaki et al., 1999; Swartz et al., 2001; Krull, 2004). These properties make it an ideal model system for rapid and high-throughput analysis of gene function in the developing vertebrate embryo. One potential technology that could be combined with chick in ovo electroporation to enable loss-of-function experiments is RNA interference (RNAi).

RNAi is the biological phenomenon whereby double-stranded RNA (dsRNA) duplexes target homologous mRNAs in the cell for degradation (reviewed by McManus and Sharp, 2002). Advantages of this method include that very little sequence information is required for effective "knockdown" to occur, loss of target gene expression can be judged at the level of the mRNA, and exogenously introduced dsRNA duplexes appear to be relatively stable in vitro and in vivo (Elbashir et al., 2002). Several variations of this technique have been used to varying degrees of success in vertebrate embryos. In ovo electroporation of long (400 bp–2 kb) dsRNA duplexes in the chick spinal cord resulted in the transient loss of gene function (Pekarik et al., 2003), whereas similar results were obtained in mouse embryos electroporated with long dsRNA duplexes that had been digested into 15- to 40-bp dsRNA fragments with an endonuclease (esiRNAs; Calegari et al., 2002). A third method of gene silencing using small, chemically synthesized 21-bp dsRNA (siRNA) has been shown to be effective in mammalian cells (Elbashir et al., 2001); however, there have been few reports of the use of these RNA oligonucleotides in vivo. Finally, in yet one more variation of RNAi technology, cells can be transfected with DNA plasmids encoding small hairpin RNA (shRNA; Sui et al., 2002). These shRNAs are transcribed by RNA polymerase III and proceed to target their cognate mRNAs for destruction. Vector-based RNAi has also been demonstrated to have some efficacy in the chick model system (Katahira and Nakamura, 2003; Chestnutt and Niswander, 2004).

Here, we have focused on determining which of the emerging RNA-based RNAi methodologies in combination with in ovo electroporation, has the best potential to be a simple and effective means of carrying out in vivo loss-of-function analyses in the chick embryo. We have conducted a quantitative comparison of the efficacy of silencing the exogenous reporter gene DsRed, as well as that of an endogenous gene, Raldh2, using three different methods of RNAi (siRNA, esiRNA, and long dsRNA duplexes). Based on the data, we conclude that siRNAs are highly effective at silencing gene expression in vivo. Second, relative to esiRNAs and long dsRNA duplexes, siRNAs are the most efficient means of producing gene-specific RNA interference in the chick. Third, significantly higher concentrations of siRNA are required to silence endogenous gene expression as opposed to expression of exogenous reporter genes. Fourth, electroporation of any of these three dsRNA substrates into the developing neural tube is relatively nontoxic. In summary, in ovo electroporation of chemically synthesized siRNAs is a fast, simple, and powerful method for conducting temporally and spatially specific loss-of-function analyses in vivo.

RESULTS

Short 21-bp RNA Duplexes Efficiently Silence Reporter Gene Expression

Electroporation of long RNA duplexes (450 bp–2.2 kb) and endonuclease-digested RNA duplexes has been shown previously to reduce target gene expression in chick and mouse embryos, respectively (Calegari et al., 2002; Pekarik et al., 2003). However, there had been no reports that siRNA is similarly effective in vivo. To determine whether short duplexes would silence gene expression in the chick, we obtained commercially synthesized 21-bp siRNA duplexes corresponding to two different target sequences in the open reading frame (ORF) of the DsRed reporter gene, located 125 bp and 362 bp from the start codon. The spinal cords of control embryos were coinjected with a plasmid expressing enhanced green fluorescent protein (eGFP) to control for electroporation efficiency and a plasmid expressing the fluorescent reporter gene DsRed (Fig. 1A,B,G). A parallel set of embryos was injected with both of these plasmids as well as equal amounts of each of the two DsRed siRNAs (Fig. 1C–F). Embryos were allowed to develop for 2 days before scoring the number of eGFP and DsRed-expressing cells. Control embryos had many cells expressing eGFP and DsRed; however, coelectroporation of 5 ng/μl of siRNA corresponding to DsRed was sufficient to reduce DsRed expression by 40% without affecting the number of eGFP-expressing cells (Fig. 1A–D,H). Titration of siRNA concentration revealed that 200 ng/μl was sufficient to consistently achieve specific knockdown of DsRed expression by 85–90% (Fig. 1E,F,H). Gene silencing was found to saturate at this level as further increases in siRNA concentration up to 1.75 μg/μl did not lead to further reductions in DsRed expression (Fig. 1H). Coelectroporation of siRNAs of unrelated sequences to the eGFP and DsRed reporters did not affect DsRed expression (data not shown).

siRNAs Are the Most Effective Form of dsRNA for Reducing Reporter Expression

We next wanted to determine which of the three RNA-based methods described to date, namely siRNA, long dsRNA duplexes, and esiRNA (Fig. 2E), would be the most effec-

tive in abrogating target gene expression in the chick using in ovo electroporation. Embryos were coelectroporated as described above with plasmids expressing DsRed and eGFP, as well as 200 ng/μl of a 706-bp RNA duplex corresponding to the DsRed ORF (dsRNA), or a 200 ng/μl mixture of shorter RNAs resulting from RNAse III digestion of the same long duplex (esiRNA). In all cases where RNA duplexes were electroporated, expression of eGFP was seen to be similar to that of control embryos electroporated with plasmid alone (compare Figs. 1A, 2A, and 2C); however, specific loss of DsRed expression was observed to varying degrees. Electroporation of the long dsRNA duplexes led to 53% knock-down of DsRed expression (Fig. 2B,F), whereas electroporation of esiRNAs resulted in a 29% loss of DsRed expression (Fig. 2D,F). When compared with the 90% efficiency of knockdown obtained with the same concentration of siRNAs (Figs. 1F, 2F), neither long dsRNA duplexes nor esiRNAs were as effective at producing RNAi in chick.

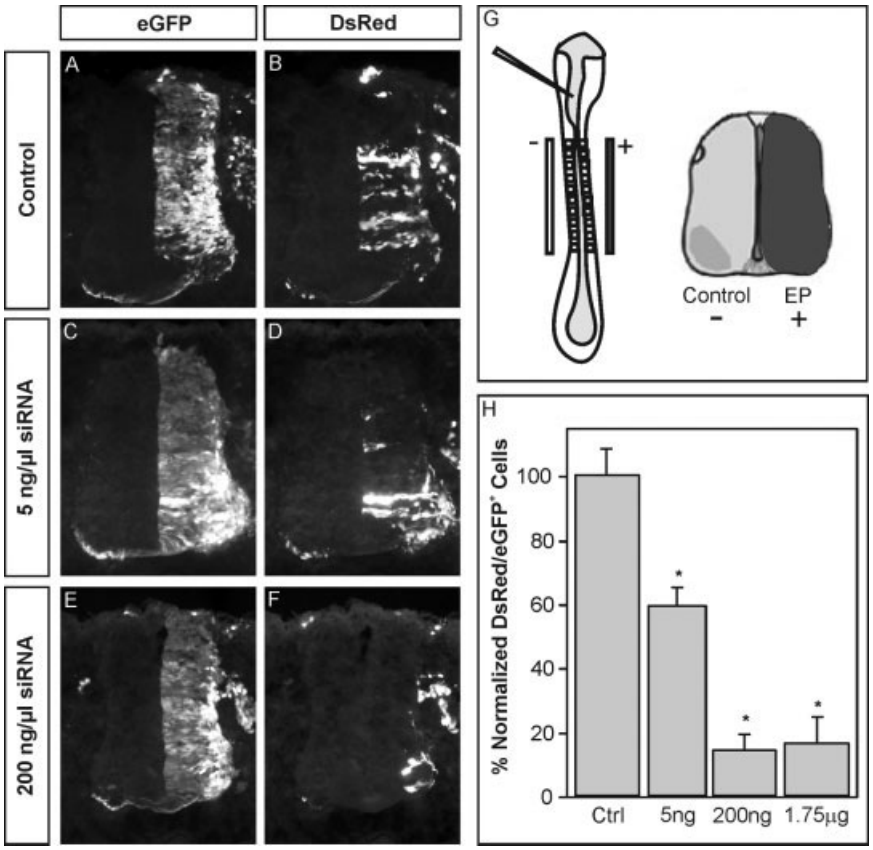

Fig. 1. Titration of electroporated short 21-bp RNA duplexes (siRNAs) results in effective gene silencing. In all images, the right side of the embryo is electroporated. All images shown are of Hamburger–Hamilton stage 21–23 embryos. **A,B:** Fluorescence photomicrographs of a spinal cord section from a representative control embryo coelectroporated with reporter plasmids expressing enhanced green fluorescent protein (eGFP) and DsRed. **C,D:** Images of a representative embryo coelectroporated with the same amounts of eGFP and DsRed as previously, together with 5 ng/μl siRNA against DsRed. **E,F:** Images of a representative embryo coelectroporated with eGFP and DsRed plasmids, as well as 200 ng/μl siRNA against DsRed. **G:** Schematic showing electroporation (EP) of constructs and RNA duplexes into the chick spinal cord. **H:** Histogram showing the percentage of $DsRed^+$ cells to $eGFP^+$ cells in control and siRNA coelectroporations. Data were obtained from three to six embryos for each condition ($*P < 0.001$).

siRNAs Can Silence Endogenous Gene Expression

While electroporation of various dsRNA substrates has proven to be powerful at reducing reporter gene expression, the more critical question for developing an in vivo loss-of-function assay is whether they can similarly silence endogenous gene expression. To address this issue, we decided to test the efficacy of these three RNAi methodologies at limiting the expression of the retinoid-synthesizing enzyme Raldh2 (Zhao et al., 1996). Raldh2 is first expressed very early in chick development and then later expressed within the limb-level population of spinal motor neurons, which constitute the lateral motor column (Sockanathan and Jessell, 1998). Embryos were coelectroporated as before with a reporter plasmid expressing eGFP or nuclear LacZ (nLacZ), as well as either 200 ng/μl of a 700-bp RNA duplex corresponding to a portion of the Raldh2 ORF, or a 200 ng/μl mixture of esiRNAs resulting from RNAse III digestion of this duplex. Embryos were harvested at 72 hr postelectroporation, sectioned, and subjected to in situ hybridization for Raldh2 mRNA to assess the efficiency of Raldh2 knockdown. The numbers of $Raldh2^+$ cells in the control and electroporated halves of the spinal cord were compared. Surprisingly, neither dsRNA nor esiRNA was able to reduce endogenous Raldh2 mRNA or protein expression to any significant degree at a concentration previously shown to reduce reporter expression (Fig. 3A–C,E–G,P). We also obtained siRNAs corresponding to two different sequences in the ORF of Raldh2, located 125 bp and 1,042 bp from the start codon. When a 200 ng/μl equimolar mixture of these duplexes was coelectroporated into chick spinal cords along with a reporter plasmid, the number of Raldh2-expressing cells was found to decrease by 30% on the electroporated side (Fig. 3I–K,P). Electroporation with almost ninefold more siRNA (1.75 μg/μl) was able to boost the efficiency of Raldh2 silencing to 50% (Fig. 3L–N,P). At this higher concentration of siRNA, there was a significant amount of variability in gene silencing from one embryo to another with some embryos exhibiting close to 100% knockdown of Raldh2 expression; however, more typical examples are shown here with a 50% reduction. Immunohistochemical staining using an RALDH2-specific polyclonal antibody showed analogous levels of protein loss under all of these conditions (Fig. 3C,G,K,N). In

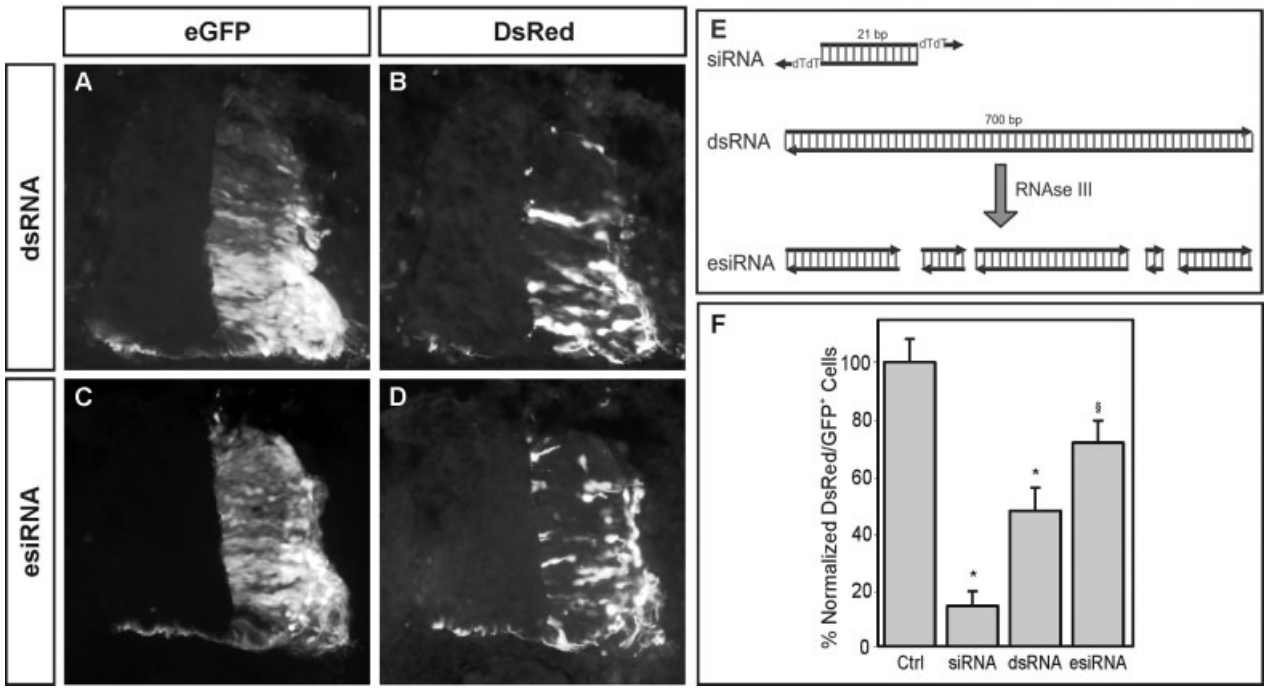

Fig. 2. Comparison of DsRed silencing by short 21-bp RNA duplexes (siRNAs), double-stranded RNA duplexes (dsRNAs), and endonuclease-digested short RNA duplexes (esiRNAs). **A,B:** Images of a representative embryo coelectroporated with enhanced green fluorescent protein (eGFP) and DsRed plasmids, as well as 200 ng/μl of dsRNA targeting DsRed. **C,D:** Images of a representative embryos coelectroporated with eGFP and DsRed plasmids, along with 200 ng/μl of esiRNA targeting DsRed. **E:** Schematic showing siRNA, dsRNA, and generation of esiRNA. **F:** Histogram showing loss of DsRed-expressing cells when embryos were independently coelectroporated with DsRed and eGFP reporter plasmids (control, Ctrl), or both plasmids with 200 ng/μl each of siRNAs, dsRNAs, or esiRNAs against DsRed target sequences. Data were obtained from three to six embryos for each condition (*$P < 0.001$; §$P < 0.02$).

these experiments, siRNAs are significantly more capable of silencing endogenous gene expression than long dsRNA duplexes or esiRNAs. However, there appears to be a sizable disparity between the siRNA concentrations required for targeting endogenous genes versus exogenous genes.

siRNA-Mediated Gene Silencing Is Nontoxic and Target-Specific

One reservation with regard to the use of RNAi for analysis of gene function is the observed potential for dsRNA substrates to induce an interferon response and subsequent cell death in mammalian cells (Sledz et al., 2003). To assess the toxicity of long dsRNA duplexes, esiRNAs, and siRNAs in the developing neural tube, embryos were electroporated with each of the above dsRNA substrates targeting Raldh2 and assayed for cell death by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining (Fig. 3D,H,O). There was no detectable cell death in either the control or electroporated halves of the spinal cord under any of these conditions, suggesting that in ovo electroporation of dsRNA of any type is not toxic to the developing chick neural tube. Thus, the relative levels of gene silencing observed by RNAi in these experiments reflect loss of gene product rather than loss of cell number.

A critical criterion for the interpretation of RNAi-based loss of function analyses is the specificity of target gene silencing. While all of the siRNA target sequences chosen in this study were screened by BLAST searches to minimize cross-reactivity, we wanted to further confirm the specificity of silencing for the targeted endogenous gene Raldh2. First, we evaluated expression of a nontargeted gene, NeuroM, in embryos electroporated with 1.75 μg/μl of siRNAs corresponding to Raldh2. NeuroM is a transcription factor transiently expressed by immature neurons in the ventricular zone just after they withdraw from the cell cycle (Roztocil et al., 1997). If siRNA electroporation leads to a global or nonspecific decline in gene expression, then we would expect to observe fewer NeuroM+ cells on the electroporated side of the neural tube. However, we observed no difference in the levels of NeuroM expression between control and electroporated sides, confirming that Raldh2 mRNA and protein loss were not due to global transcriptional silencing (Fig. 3T). Next, to determine whether Raldh2 silencing was indeed dependent on siRNA sequence or could be induced by any siRNAs, embryos were electroporated with 1.75 μg/μl of siRNAs targeting the fluorescent reporter DsRed. While loss of DsRed fluorescence confirmed that the RNAi mechanism had been engaged (data not shown), silencing proved to be target-specific because there was no change in levels of Raldh2 mRNA or protein (Fig. 3R,S). Taken together, these experiments demon-

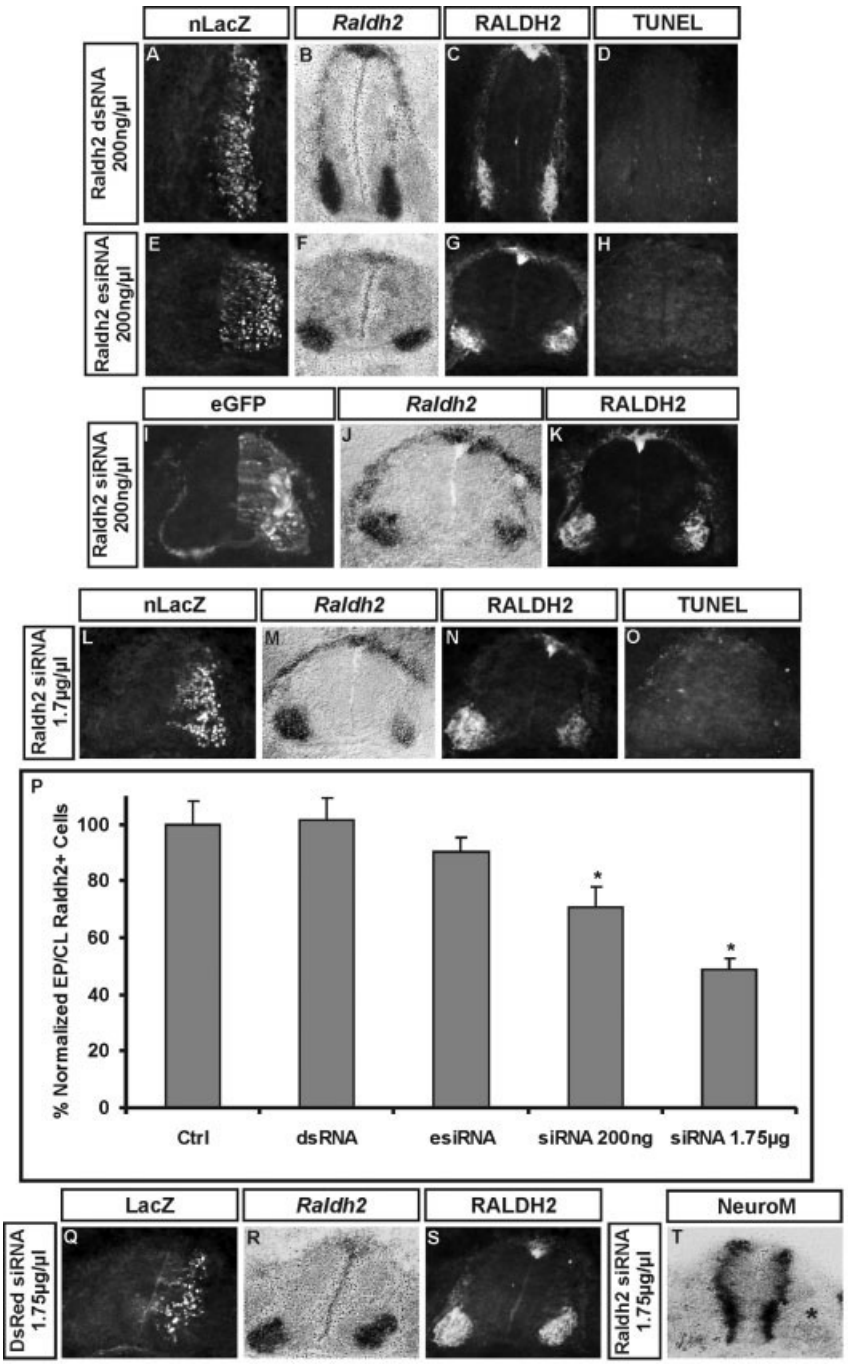

Fig. 3. Comparison of Raldh2 silencing by double-stranded RNA duplexes (dsRNAs), endonuclease-digested short RNA duplexes (esiRNAs), and short 21-bp RNA duplexes (siRNAs). **A–D:** Images from a representative embryo coelectroporated with a nuclear LacZ (nLacZ) plasmid and 200 ng/µl of dsRNA targeting Raldh2. **E–H:** Images from a representative embryo coelectroporated with an nLacZ plasmid and 200 ng/µl of esiRNA targeting Raldh2. **I–K:** Images from a representative embryo coelectroporated with an enhanced green fluorescent protein (eGFP) plasmid and 200 ng/µl of siRNA targeting Raldh2. **L–O:** Images from a representative embryo coelectroporated with an nLacZ plasmid and 1.75 µg/µl of siRNA targeting Raldh2. **Q–S:** Images from a representative embryo coelectroporated with nLacZ and DsRed plasmids as well as 1.75 µg/µl of siRNA targeting DsRed. A,E,I,L,Q: eGFP fluorescence or nLacZ immunostaining identifies the electroporated side of the spinal cord. B,F,J,M,R: In situ hybridization of Hamburger–Hamilton stage 25–27 chick embryonic spinal cord sections showing Raldh2 mRNA expression in motor neurons. C,G,K,N,S: Immunostaining for Raldh2 shows Raldh2 protein expression in motor neurons. D,H,O: Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) cell death detection assay. **P:** Quantitation of Raldh2 expressing cells on electroporated (EP) and contralateral (CL) sides of the embryo using 200 ng/µl of dsRNA, 200 ng/µl esiRNA, 200 ng/µl siRNA, and 1.75 µg/µl siRNAs against Raldh2. Data were obtained from three to five embryos for each condition (*$P < 0.001$). **T:** In situ hybridization of an embryo electroporated with 1.75 µg/µl of siRNA targeting Raldh2 shows no loss of NeuroM expression on the electroporated side of the spinal cord (asterisk).

strate that in ovo electroporation of siRNAs is a simple, nontoxic means of achieving target-specific gene silencing in the developing vertebrate neural tube.

DISCUSSION

The developing chick embryo provides an excellent system for the study of many aspects of developmental biology. Recent advances in gene transfer using in ovo electroporation have raised the possibility that the chick model system may be used for functional high-throughput screens based on loss-of-function approaches in vivo. To determine whether RNAi may constitute such an approach in the developing chick embryo, we conducted a systematic and quantitative trial of the available RNA-based RNAi methodologies on an exogenous as well as an endogenous gene. Our results demonstrate that microinjection and electroporation of siRNAs into the developing chick spinal cord can specifically silence target gene expression. Moreover, siRNAs are significantly more effective at gene silencing than long dsRNA duplexes or esiRNAs.

RNA interference using siRNAs offers several advantages over other existing oligonucleotide-based loss-of-function approaches including

morpholinos (Heasman, 2002) and standard antisense DNA methods (Kos et al., 2003). We have not observed any toxicity upon siRNA administration in ovo and their use requires minimal sequence information as siRNA targets can be derived from almost any portion of the coding region of a gene, as opposed to a single defined region spanning the start codon. Moreover, preliminary results suggest that efficient knockdown of gene expression can also be obtained from siRNA sequences designed against untranslated regions of the transcript (M. Rao and S. Sockanathan, unpublished observations). Furthermore, whereas antisense DNA technologies function by limiting the translation of specific mRNAs into protein, RNA interference targets the mRNAs themselves for destruction (reviewed by Scherer and Rossi, 2003). This distinction allows one to monitor loss of a gene product by in situ hybridization rather than by immunohistochemical methods, which require the generation of antibodies against proteins of interest.

While RNAi represents a powerful tool for studying vertebrate development in vivo, there are several critical issues that need to be addressed before drawing conclusions about gene function based on loss-of-function phenotypes. The foremost issue is the specificity of target gene silencing. Several groups have reported that introducing exogenous dsRNA into cells can trigger global transcriptional silencing or deregulation of the expression of genes other than the desired target (Persengiev et al., 2004). Alternatively, siRNAs could also silence genes with significant homology to their target sequences (Jackson et al., 2003). In this study, we minimized the possibility of cross-reactive silencing by using BLAST to screen all of our siRNA targets against expressed sequence tag (EST) databases. To verify the actual specificity of the silencing in vivo, we confirmed that expression of untargeted genes was unaffected and that silencing of the target gene was dependent on siRNA sequence (i.e., a siRNA of unrelated sequence was not able to reduce target gene expression). When RNAi is used to analyze gene function, the most compelling demonstration of specificity would be to show rescue of a particular loss-of-function phenotype upon target gene expression. Although this strategy was not addressed in this study, siRNA sequences targeting the untranslated regions of a gene's transcript can be coelectroporated along with an expression plasmid encoding the ORF of the gene. Silencing of the endogenous gene with simultaneous rescue of the loss-of-function phenotype by gene expression from the plasmid, thus, can be obtained.

Another phenomenon that can bolster the argument for specificity is the dose-response of RNA interference. Our results show that the level of knockdown observed upon electroporation of siRNAs targeting the reporter gene DsRed was dependent upon the concentration of duplexes injected. As little as 5 ng/μl of siRNA was found to result in a 40% loss of DsRed expression, whereas increasing the dose to 200 ng/μl consistently resulted in 85–90% knockdown. The dose-responsive nature of this effect, taken together with the unchanged expression of the coelectroporated eGFP reporter highlights the specificity of this silencing. Curiously, DsRed silencing seemed to reach a peak of 85–90% that was impervious to further increases in siRNA concentration. It is not clear what determines this ceiling, but it may represent the limit of siRNA electroporation efficiency or the difference in the kinetics between reporter gene expression and the RNAi silencing machinery. Modifying this protocol to use fluorescently tagged siRNAs would allow one to address the issue of electroporation efficiency and draw firmer conclusions about cell-autonomous effects of loss of gene function.

Silencing of endogenous gene expression, in this case of Raldh2, was also dose-responsive but the absolute dose required was much higher than for the reporter DsRed. It is important to note that the pattern of Raldh2 expression in the spinal cord required analysis of knockdown at 72 hr postelectroporation rather than at 48 hr, as was done for the reporter. It is possible that the proliferation of cells during this 24-hr time frame led to further dilution and decreased efficacy of the Raldh2 siRNAs. However, experience with other endogenous genes (M. Rao, B. Zhuang, and S. Sockanathan, unpublished observations) suggests that it is not simply dilution but that there is truly a greater requirement of siRNA for efficient silencing of endogenous genes relative to reporters. This disparity may have to do with inherent structural differences between plasmid and chromosomal gene transcription. Interestingly, at higher doses of siRNA, such as 1.75 μg/μl, we began to note significant variation in the extent of Raldh2 knockdown from one embryo to another as well as across the rostral–caudal axis of the neural tube, suggesting that small variations in local concentration may affect the extent of silencing. Whereas the average loss of target gene expression in our assay with these two particular siRNA sequences was only 50%, we were able to generate a small number of embryos with significantly higher loss, up to 100%. Although 50% silencing may be sufficient to do some functional analyses, the yield of embryos with more dramatic loss can probably be improved by further optimization of target sequence choice and further increases in siRNA concentration. Our findings, thus, highlight the importance of assaying the amount of target gene knockdown in each embryo to be analyzed, as well as titrating RNAi conditions with respect to endogenous genes when designing RNAi protocols.

While siRNA proved to be the most effective substrate for RNAi in our system, it has been argued that dsRNA and esiRNA are more useful for initial screens of gene function because they preclude the variable of siRNA target sequence choice (Calegari et al., 2002). However, in our experience, all of the siRNAs that we designed based on a set of minimal criteria (outlined in the Experimental Procedures section) have been effective at RNA interference. Indeed, when any of the experiments in this study were repeated with a given concentration of one siRNA alone rather than a mixture of

two, the same levels of knockdown were observed (data not shown). Thus, we propose that the requirement for target sequence choice serves as an asset rather than a handicap, because potential siRNA sequences can be screened against EST databases to minimize cross-reactivity with unwanted genes. Recently, several groups have determined empirical guidelines for siRNA target selection, which should further minimize the likelihood of selecting an ineffective siRNA (Reynolds et al., 2004; Ui-Tei et al., 2004).

In summary, we have demonstrated the potential use of RNA interference in combination with in ovo electroporation as a rapid, functional assay for novel gene function in the developing chick embryo. We have shown that siRNA-based methodology is significantly more effective at silencing gene expression than protocols that use long dsRNA duplexes or esiRNAs. This suppression of gene expression is nontoxic and specific to the target gene. Our studies have proven the utility of this technique for the study of genes expressed in the nervous system; however, it is also applicable to any other structures that are accessible to electroporation. The adaptation of emerging plasmid or viral-encoded shRNA technology will very likely allow for further extension of RNAi to the study of all organ systems in the developing chick embryo. It is possible that these techniques could be even more powerful at silencing gene expression in the chick embryo as shRNA more closely resembles the double-stranded, hairpin structure of miRNAs known to be involved in endogenous gene regulation (Bartel, 2004).

EXPERIMENTAL PROCEDURES

siRNA Design

Candidate target sequences were first identified in the cDNA of interest by screening the ORF for 21-bp sequences beginning with the dinucleotide "AA." Candidates with three or more same-letter strings or those located in the first 100 bp of the ORF were excluded to prevent potential interference by secondary structure or transcriptional complexes. Remaining candidates were further screened for GC content (45–60% was deemed acceptable) and subjected to BLAST searches against GenBank's nonredundant (nr) and EST databases to identify the siRNAs most likely to be specific for the gene in question. Finally, RNA duplexes corresponding to two of these final targets were ordered in 2′ deprotected, annealed, and desalted form (Option A4, www.dharmacon.com). Lyophilized RNA duplexes were dissolved in the provided 1X Universal Buffer at a concentration of 5 μg/μl and stored at −20°C. The RNA duplexes used in this study are as follows: DsRed-1: sense 5′-CACCGUGAAGCUGAAGGUGdTdT-3′, antisense 3′-dTdTGUGGCACUUCGACUUCCAC-5′; DsRed-2: sense 5′-GGUGAAGUUCAUCGGCGUGdTdT-3′, antisense 3′-dTdTCCACUUCAAGUAGCCGCAC-5′; Raldh2-1: sense 5′-CAGGAGAGCAGAUCUGUGAdTdT-3′, antisense 3′-dTdTGUCCUCUCGUCUAGACACU-5′; Raldh2-2, sense 5′-UACAACAAGAUCUUGGAACdTdT-3′, antisense, 3′-dTdTAUGUUGUUCUAGAACCUUG-5′.

dsRNA and esiRNA Preparation

For dsRNA and esiRNA preparation, the methods described in Pekarik et al. (2003) and Calegari et al. (2002) were followed as closely as possible to facilitate comparison of the various methods of RNAi. The DsRed ORF was subcloned into pBS II SK+ before transcription of sense and antisense ssRNA from the T3 and T7 RNA polymerase promoters (Ambion MEGAscript kit (Cat no. 1334, 1338)) Transcription reactions were incubated with DNAse I to degrade the template and a small aliquot of the reaction was gel electrophoresed to ensure that there was no remaining DNA contamination. The ssRNA was then subject to acid phenol–chloroform extraction, resuspended in water and quantified by ethidium bromide staining and comparison to standards. Next, the ssRNAs were annealed into duplexes by combining approximately 4 μg each of the sense and antisense ssRNAs in a total volume of 56 μl before denaturation at 95°C for 5 min to remove secondary structure and incubation at 37°C for 30 min. Annealed duplexes were immediately incubated on ice and a 6-μl aliquot was saved for later analysis. The remaining 50 μl of RNA duplex was digested with 33 ng of RNAse A and 10 μl of 1M NaCl at 37°C for 30 min. Another 6-μl aliquot was saved for later analysis, and the remainder ethanol-precipitated and resuspended in 14 μl of ddH_2O. Before electroporation, the aliquots saved from each of the preceding steps were electrophoresed on a 1% agarose gel to confirm the size of the annealed duplex and ensure that the RNAse step had eliminated ssRNA but not the dsRNA duplex. To test the efficacy of dsRNA-mediated RNAi, the annealed duplex was injected and electroporated at a concentration of 200 ng/μl as described below. To generate esiRNAs, 7 μl (~2 μg) of dsRNA was digested for 15 min at 20°C in a 20-μl reaction with 7 U of RNAse ONE ribonuclease (Promega Cat No. M4261). Finally, the digestion reaction was ethanol-precipitated, verified by electrophoresis, and then injected and electroporated as esiRNA. For Raldh2 dsRNA and esiRNA, a 700-bp portion of the Raldh2 cDNA was subcloned into pBS II SK+ and RNA was prepared as described above for DsRed.

In Ovo Electroporation

Fertilized chicken eggs (Charles River) were incubated until embryos reached Hamburger–Hamilton (HH) stage 11–13, and the embryos were visualized as previously described (Nakamura and Funahashi, 2001). RNA solutions for injection were prepared with the relevant amount of siRNA, dsRNA, or esiRNA along with 0.2% Fast Green dye, to allow detection of the solution during injection. One of the reporter plasmids pCAGGS-eGFP or pCAGGS-IresnLacZ was included in the solution at 200 ng/μl to monitor efficiency of RNA/DNA incorporation into the neural tube. For reporter gene experiments, 200 ng/μl of pcDNA3-DsRed was added along with the eGFP plasmid to the injection solution. This cocktail was injected with a drawn-out glass capillary into the neural tube at the caudomedullary junction and electroporated into

half of the neural tube with a BTX Electro Square Porator ECM 830 (Genetronics) configured to deliver 5 × 50 msec pulses of 30 V across the embryo through a pair of gold-plated electrodes 5 mm in length, 0.5 mm diameter (Genetrodes Model 512, Genetronics, Inc.). Similar results were obtained using electrodes constructed from tungsten. A drop of antibiotic solution containing penicillin (1,000 IU/ml) and streptomycin (1,000 μg/ml) in 1× CMF was then placed on the embryo at the site of electroporation. The window was covered with Parafilm, and the egg replaced in the incubator for 2–3 days until analysis at HH stage 21–27.

Loss of Function Analysis

Electroporated embryos were dissected, fixed for 1.5–2 hr in 4% paraformaldehyde/0.1 M phosphate buffer (PB) at 4°C, rinsed once with phosphate-buffered saline (PBS), and subjected to two 10-min washes in PBS. Embryos were allowed to equilibrate overnight in 30% sucrose/0.1 M PB and then embedded in TissueTek O.C.T. Compound (catalog no. 4583). Sections (12.5-μm) were cut on a cryostat, collected onto slides, air-dried for 20 min, and stored at −80°C. eGFP and DsRed fluorescence was visualized directly after slides were rinsed in PBS for 5 min and then mounted with a few drops of Vectashield (Vector Laboratories catalog no. H-1000). In situ hybridization for Raldh2 and NeuroM was carried out as previously described (Sockanathan and Jessell, 1998) according to standard protocols (Schaeren-Wiemers and Gerfin-Moser, 1993). Immunostaining for Raldh2 was done by incubation of slides overnight at 4°C with a rabbit anti-chick Raldh2 antibody diluted 1:500 in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin. For TUNEL staining, slides were immunostained as described above, fixed for 5 min in 4% paraformaldehyde/0.1 M PB at room temperature, rinsed three times in PBS for 5 min per wash, and then subjected to the TUNEL reaction according to the manufacturer's instructions (Roche catalog no. 1684795). Fluorescence and differential interference contrast microscopy images were captured with a ×20 objective on a Nikon Eclipse E800 microscope equipped with a Q Imaging Retiga EX camera.

Quantitative Analysis

At least five sections from each of three to seven embryos were analyzed per experimental condition. Images were scored for DsRed, eGFP, or Raldh2-expressing cells. For the reporter gene experiments, a ratio of $DsRed^+$ to $eGFP^+$ cells was calculated for each section. $eGFP^+$ cells were used in the denominator to rule out embryo stage and electroporation efficiency as variables in the analysis of DsRed silencing. The average DsRed/eGFP ratio was determined for each embryo and these ratios were then compiled into a mean ratio for each condition with an error bar corresponding to the standard error of the mean (SEM). The mean ratios for each condition were then compared with the DsRed/eGFP ratio found in the control embryos, which was normalized to represent 100% to determine the efficiency of reporter gene silencing. The *t*-tests were then done to calculate the significance of these results, and *P* values were noted where applicable. Similarly, in the endogenous gene experiments, three to five embryos were sectioned per condition and subject to in situ hybridization for Raldh2. At least five sections from each embryo were imaged and scored for $Raldh2^+$ cells on the electroporated (EP) and nonelectroporated (control, CL) sides of the spinal cord. The average ratio of EP/CL $Raldh2^+$ cells was determined for each embryo and then combined to calculate the mean ratio for the condition as well as the SEM. The difference between the number of $Raldh2^+$ cells in the EP and CL sides of the neural tube reflects the extent of knockdown achieved by the siRNA. The *t*-tests were done, and *P* values calculated to establish statistical significance.

ACKNOWLEDGMENTS

We thank BinQuan Zhuang, Crystal Falco, and Shengjian Ji for helpful advice and critical reading of the manuscript; and Ben Novitch for the NeuroM in situ probe. M.R. and F.R. are supported by the Medical Scientist Training Program (NIH).

REFERENCES

Bartel DP. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297.

Calegari F, Haubensak W, Yang D, Huttner WB, Buchholz F. 2002. Tissue-specific RNA interference in postimplantation mouse embryos with endoribonuclease-prepared short interfering RNA. Proc Natl Acad Sci U S A 99:14236–14240.

Chestnutt C, Niswander L. 2004. Plasmid-based short-hairpin RNA interference in the chicken embryo. Genesis 39:73–78.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411:494–498.

Elbashir SM, Harborth J, Weber K, Tuschl T. 2002. Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 26:199–213.

Grishok A, Mello CC. 2002. RNAi (Nematodes: Caenorhabditis elegans). Adv Genet 46:339–360.

Heasman J. 2002. Morpholino oligos: making sense of antisense? Dev Biol 243:209–214.

Itasaki N, Bel-Vialar S, Krumlauf R. 1999. 'Shocking' developments in chick embryology: electroporation and in ovo gene expression. Nat Cell Biol 1:E203–E207.

Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS. 2003. Expression profiling reveals off-target gene regulation by RNAi. Nat Biotech 21:635–637.

Katahira T, Nakamura H. 2003. Gene silencing in chick embryos with a vector-based small interfering RNA system. Dev Growth Differ 45:361–367.

Kos R, Tucker RP, Hall R, Duong TD, Erickson CA. 2003. Methods for introducing morpholinos into the chicken embryo. Dev Dyn 226:470–477.

Krull CE. 2004. A primer on using in ovo electroporation to analyze gene function. Dev Dyn 229:433–439.

McManus MT, Sharp PA. 2002. Gene silencing in mammals by small interfering RNAs. Nat Rev Genet 3:737–747.

Nakamura H, Funahashi J. 2001. Introduction of DNA into chick embryos by in ovo electroporation. Methods 24:43–48.

Pekarik V, Bourikas D, Miglino N, Joset P, Preiswerk S, Stoeckli ET. 2003. Screening for gene function in chicken embryo using RNAi and electroporation. Nat Biotech 21:93–96.

Persengiev SP, Zhu X, Green MR. 2004. Nonspecific, concentration-dependent stimulation and repression of

mammalian gene expression by small interfering RNAs (siRNAs). RNA 10:12–18.

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. 2004. Rational siRNA design for RNA interference. Nat Biotech 22:326–330.

Roztocil T, Matter-Sadzinski L, Alliod C, Ballivet M, Matter J-M. 1997. NeuroM, a neural helix-loop-helix transcription factor, defines a new transition stage in neurogenesis. Development 124:3263–3272.

Schaeren-Wiemers N, Gerfin-Moser A. 1993. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labeled cRNA probes. Histochemistry 100:431–440.

Scherer LJ, Rossi JJ. 2003. Approaches for the sequence-specific knockdown of mRNA. Nat Biotech 21:1457–1465.

Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. 2003. Activation of the interferon system by short-interfering RNAs. Nat Cell Biol 5:834–839.

Sockanathan S, Jessell TM. 1998. Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. Cell 94:503–514.

Sui G, Soohoo C, Affar EB, Gay F, Shi Y, Forrester WC, Shi Y. 2002. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc Nat Acad Sci U S A 99:5515–5520.

Swartz M, Eberhart J, Mastick GS, Krull CE. 2001. Sparking new frontiers: using in ovo electroporation for genetic manipulations. Dev Biol 233:13–21.

Ui-Tei K, Naito Y, Takahashi F, Haraguchi T, Ohki-Hamazaki H, Juni A, Ueda R, Saigo K. 2004. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res 32: 936–948.

Zhao D, McCaffery P, Ivins KJ, Neve RL, Hogan P, Chin WW, Drager UC. 1996. Molecular identification of a major retinoic-acid-synthesizing enzyme, a retinaldehyde-specific dehydrogenase. Eur J Biochem 240:15–22.