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Transposon-Mediated Stable Suppression of Gene Expression in the Developing Chick Retina

Masaru Nakamoto and Chizu Nakamoto

Abstract

The embryonic chick has long been a favorite model system for in vivo studies of vertebrate development. However, a major technical limitation of the chick embryo has been the lack of efficient loss-of-function approaches for analyses of gene functions. Here, we describe a methodology in which a transgene encoding artificial microRNA sequences is introduced into embryonic chick retinal cells by in ovo electroporation and integrated into the genome using the Tol2 transposon system. We show that this methodology can induce potent and stable suppression of gene expression. This technique therefore provides a rapid and robust loss-of-function approach for studies of gene function in the developing retina.

Key words In ovo electroporation, Chick embryo, Retina, Tol2 transposon, miRNA, Gene targeting

1 Introduction

The chick embryo has long been a remarkably important model system in developmental biology. In the field of neural development, the chick retina has contributed to considerable progress in understanding the molecular mechanisms by which cell proliferation and differentiation, morphogenesis, and axon guidance are regulated. The chick retina has significant similarity to the human retina at the anatomical, cellular, and molecular levels. In addition, the practical advantages of the chick retina include its large size, rapid development, and accessibility for visualization and experimental manipulation. The usefulness of the chick embryo as an experimental tool has recently been further expanded by invention and development of the in ovo electroporation technique by Nakamura's group [1, 2].

Despite these advantages, a major technical limitation of the chick embryo has been the lack of a technology that allows for robust and efficient loss-of-function analyses, which have been dependent on the availability of a dominant-negative form of the gene product. Nonetheless, important advances have been made in

this regard in the past decades. For example, RNAi has been successfully used in chick embryos by electroporating small interfering RNAs (siRNAs) [3] or expression vectors for short hairpin RNA [4] in ovo. In addition, an RCAS retrovirus-based siRNA delivery system was shown to efficiently knock down gene expression in chick embryos [5].

Transposons are genetic elements that move from one locus in the genome to another and can be efficient tools for permanently delivering foreign DNA into vertebrate genomes. The Tol2 transposable element, originally identified in the genome of the medaka fish (*Oryzias latipes*), is an autonomous transposon that encodes a functional transposase belonging to the hAT family [6]. The Tol2 transposase can catalyze transposition of a nonautonomous transposon construct, which lacks the transposase coding region but retains the sequences of the left and right ends of the Tol2 element (200 bp and 150 bp, respectively). When a plasmid vector for a nonautonomous transposon construct carrying a gene expression cassette with the Tol2 end sequences is introduced into vertebrate cells with the Tol2 transposase activity, the transposon construct is excised from the plasmid, and the expression cassette is integrated into the host genome (Fig. 1). It has been reported that the Tol2 transposable element can undergo efficient transposition in a wide variety of vertebrate species, including zebrafish, frogs, chick, and mice [7–11].

In this chapter, we describe a loss-of-function approach in which artificial microRNAs (miRNAs) are introduced into the developing chick retina by in ovo electroporation and subsequently integrated into the genome using a Tol2 transposon system [12]. The outline of the procedures is shown in Fig. 2. In this approach, first, DNA oligonucleotides encoding the target pre-miRNA and its complement are designed and generated (Subheading 3.1). The complementary oligonucleotides are then annealed to generate double-stranded oligonucleotides (Subheading 3.2) and cloned into an expression vector for miRNA and the EmGFP marker (Subheading 3.3). Knockdown effects of individual miRNAs can be tested in vitro (Subheading 3.4), and miRNA sequences that show strong activities can be chained to further enhance the knockdown effects (Subheading 3.5). The expression cassette carrying EmGFP cDNA and pre-miRNA sequences is then transferred to the Tol2 transposon vector (Subheading 3.6). Finally, the Tol2 transposon construct is introduced with the transposase expression vector into the embryonic chick retina by in ovo electroporation (Subheadings 3.7–3.9).

This method offers several distinct advantages. First, it supports stable expression of the transgene and thus persistent production of miRNAs. Electroporation of conventional expression vectors permits only transient expression of the transgene, usually lasting only for several days in embryonic cells, because of the inability of the

Fig. 1 Transposition of Tol2-flanked DNA by transposase. A scheme showing transposition of a Tol2-flanked expression cassette for EmGFP and artificial miRNAs (two miRNA sequences are chained) by transposase. When a Tol2 transposon construct containing the expression cassette (pT2K-CAGGS-EmGFP/miRNA) is introduced into cells with a Tol2 transposase expression construct (pCAGGS-T2TP), the Tol2-flanked cassette is excised from the vector and integrated into the host genome by the transposase activity. Expression of EmGFP and miRNAs is driven by the ubiquitous promoter CAGGS (modified from [11, 12])

electroporated plasmid to be integrated into chromosomes. In contrast, the present method involves chromosomal integration of the expression cassette by activity of co-electroporated transposase and therefore can achieve stable expression of the transgenes. Second, the cells that express the transgene can be easily traced because each of the transfected cells is clearly marked by the EmGFP marker. Third, because expression of miRNA and EmGFP is driven by the same CAGGS promoter, the risk of promoter interference is avoided, which is one of the common problems with retroviral vectors containing an additional promoter to attain dual gene expression (e.g., [13]). Finally, the areas of transfection can be controlled by properly choosing the type and positions of electrodes. Because this method does not use replication-competent retroviral vectors, there is no transmission of the transgene to neighboring cells after the electroporation. Within the transfected retinæ, areas that do not express the transgene serve as a good internal control. Therefore, this system provides a stable and efficient loss-of-function option and can contribute to the analysis of gene functions during chick development.

Fig. 2 Outline of the procedures. This diagram shows key steps of the procedures. DNA oligonucleotides encoding the target pre-miRNA and its complement are designed and generated (Subheading 3.1). The complementary oligonucleotides are annealed to generate double-stranded oligonucleotides (Subheading 3.2). Individual double-stranded oligos are cloned into an expression vector for miRNA and the EmGFP marker (Subheading 3.3). Knockdown effects of individual miRNA can be tested in vitro (Subheading 3.4), and miRNA sequences that show strong activities can be chained to further enhance the knockdown effects (Subheading 3.5). The expression cassette carrying EmGFP cDNA and pre-miRNA sequences is then transferred to the Tol2 transposon vector (Subheading 3.6). Finally, the Tol2 transposon construct is introduced with the transposase expression vector into the embryonic chick retina by in ovo electroporation (Subheadings 3.7–3.9)

2 Materials

2.1 Construction of miRNA Expression Vectors

1. *RNAi Designer* (<https://rnaidesigner.thermofisher.com/rnaiexpress/>). An online tool to choose target sequences and design pre-miRNA sequences
2. The BLOCK-iT Pol II miR RNA expression kit with EmGFP (Invitrogen) <https://www.thermofisher.com/order/catalog/product/K493600?SID=srch-hj-K4936-00>

The kit contains the following reagents to be used in the procedures:

- (a) pcDNA6.2-GW/EmGFP-miR vector, linearized
 - (b) 10× Oligo annealing buffer: 100 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), and 1 M NaCl
 - (c) DNase/RNase-free water
 - (d) 5× Ligation buffer: 250 mM Tris-HCl (pH 7.6), 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, and 25% (w/v) polyethylene glycol-8000
 - (e) T4 DNA ligase
 - (f) EmGFP forward sequencing primer: 5'-GGCATGGAC GAGCTGTACAA-3'
 - (g) miRNA reverse sequencing primer: 5'-CTCTAGATCAA CCACTTTGT-3'
 - (h) pcDNA6.2-GW/miR-negative control plasmid
 - (i) S.O.C medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose
 - (j) One Shot TOP10 chemically competent *E. coli*
3. Construction of Tol2 transposon vectors
Tol2 transposon vectors:
 - (a) pT2K-CAGGS vector (Fig. 4): kindly provided by Yoshiko Takahashi (Kyoto University, Japan)
 - (b) pCAGGS-T2TP vector: T2 transposase expression plasmid containing the transposase cDNA under the control of the CAGGS promoter. A generous kind gift of Koichi Kawakami (National Institute of Genetics, Japan). Also available from Addgene.

Primers:

- (a) Forward primer (the 3' end region of the CMV promoter of the pcDNA6.2-GW/EmGFP-miR vector with an artificial EcoRI site): 5'-GGGAATTCTCTGGCTAACTAG AGAAC-3'
- (b) Reverse primer (miRNA reverse sequencing primer with an artificial EcoRI site): 5'-CCGAATTCCCTCTAGATC AACCACT-3'

- 4. Spectinomycin
- 5. Thermal cycler

**2.2 In Vitro
Evaluation
of Knockdown Effects
Using Alkaline
Phosphatase Fusion
Proteins**

- 1. APtag vectors:
Different types of vectors to make alkaline phosphatase (AP) fusion proteins (APtag-1–APtag-5) are commercially available from GenHunter Corp (TN, USA) <https://www.genhunter.com/products/ap-tag-kit-a.html>. We generally use the APtag-2 vector, which is designed for transient transfection and for fusions to the N-terminus of AP.
- 2. 2× AP substrate buffer:

10 M Diethanolamine (pH 9.8)	4 mL
1 M MgCl ₂	10 μL
L-Homoarginine	45 mg
BSA	10 mg
p-Nitrophenylphosphate	63 mg
Add dH ₂ O to total volume of 20 mL.	
The solution can be stored in aliquots at −20 °C. All reagents can be obtained from Sigma-Aldrich (St. Louis, MO)	

- 3. Microplate reader or spectrophotometer

**2.3 Preparation
of Chick Embryos**

- 1. Fertilized chicken eggs
Fertilized chicken eggs (*Gallus gallus*) can be obtained from local farms. Alternatively, pathogen-free chicken eggs can be purchased from commercial vendors (e.g., Charles River). The eggs can be stored at 12–16 °C for up to 1 week before use (*see* **Note 1**).
- 2. Incubation of chick embryos
Chicken eggs are incubated at 38 °C with sufficient humidity. A variety of incubators are available, such as Kuhl—600 Egg Research and Laboratory Incubator (B-Lab-600-110) (<https://www.flemingoutdoors.com/kuhl%2D%2D-600-egg-laboratory-incubator%2D%2D-b-lab-600-110.html>).

**2.4 In Ovo
Microinjection
and Electroporation**

- Equipment for in ovo microinjection and electroporation is presented in Fig. 5:
- 1. Dissecting microscope
 - 2. Gooseneck fiber light source
 - 3. Microinjection apparatus:

Micromanipulator (e.g., Narishige (Japan), MM3), <http://products.narishige-group.com/group1/MM-3/electro/english.html>

18 G Needles

PVC tubing (VWR, Cat. No. 228-3830)

Hamilton syringe (50 μ L, Sigma Cat. No. 20715, Hamilton Cat. No. 80901).

Glass micropipette needles:

Micropipette needles used for microinjection are made by pulling FHC borosilicate capillary tube with omega dot fiber (1 mm O.D. $\times$ 0.75 mm I.D., FHC Inc. ME, USA, Cat. No. 30-30-1) with a micropipette puller (e.g., Sutter Instrument P97, <https://www.sutter.com/MICROPIPETTE/p-97.html>). Pinch off the tip of pulled capillary tubes with fine forceps to generate an opening.

4. Electroporation apparatus:

Electroporator—CUY21 Square Wave Electroporator (Nepa Gene)

Electrodes (CUY611P3-1, Sonidel)

https://www.sonidel.com/product_info.php?products_id=94

Holder (CUY580, Sonidel)

https://www.sonidel.com/product_info.php?products_id=85

Cables:

C117 cables, https://www.sonidel.com/product_info.php?products_id=252

C115CB cables, https://www.sonidel.com/product_info.php?products_id=254

5. Forceps: fine, curved, and straight shape

Scissors: medium and small size

6. Heavy mineral oil (Sigma-Aldrich).

7. 1% Fast Green Solution:

100 mg of Fast Green FCF (Sigma-Aldrich) is dissolved in 10 mL of PBS (with Ca^{2+} and Mg^{2+} ; pH 7.4). Filter the solution through 0.2 μ m syringe filter into 15 mL conical tube. Store at room temperature.

8. Hank's saline solution

3 Methods

3.1 Designing Single-Stranded DNA Oligos for Targeting the Gene of Interest [14]

The design of pre-miRNA oligonucleotides is critical for successful gene silencing. We use an online tool provided by Invitrogen, *RNAi Designer*, which helps choose target sequences and design pre-miRNA oligos. For a given target gene, it may be required to generate multiple pre-miRNA sequences and screen them for

Fig. 3 Design of single-stranded oligonucleotides. The required features of the top- and bottom-strand single-stranded oligonucleotides are shown. The backbone of the miRNA sequence is derived from murine miR-155. The top-strand oligo contains (from 5' to 3') a sequence for 5' overhang, antisense target sequence (mature miRNA sequence), terminal loop sequence (red), and sense target sequence. The bottom-strand oligo represents the reverse complement of the top-strand oligo with additional four nucleotide (CCTG) at the 5' end and without the four nucleotides at the 3' end (AGCA: reverse complement of 5'-TGCT of the top-strand oligo). 5' overhangs are indicated in blue (modified from [14])

knockdown activities. We generally test 5–10 pre-miRNA sequences for knockdown effects in vitro (Subheading 3.4). We then select and chain two most efficient sequences (Subheading 3.5) for using in ovo electroporation.

For each pre-miRNA sequence, two single-stranded DNA oligos are required: one encoding the target pre-miRNA (“top-strand” oligo) and the other its complement (“bottom-strand” oligo) (Fig. 3).

3.1.1 Top Strand Oligo

The top-strand oligo contains the following features (from 5' to 3'):

1. 5' TGCTG: Derived from the endogenous murine miR-155, which is the basis of the pcDNA 6.2-GW/EmGFP-miR vector system [15]. This also provides a four-nucleotide 5' overhang, compatible with a four-nucleotide overhang in the linearized pcDNA 6.2-GW/EmGFP-miR, to clone the double-stranded oligo.
2. Reverse complement of the 21-nucleotide sense target sequence.
3. GTTTTGGCCACTGACTGAC (terminal loop): Derived from miR-155 to form a terminal loop.

4. Nucleotides 1–8 and 11–21 (5′–3′) of sense target sequence. (The nucleotides 9 and 10 are removed to form a short internal loop in the mature miRNA, which results in more efficient knockdown [14].)

3.1.2 Bottom-Strand Oligo

The bottom-strand oligo can be designed as follows:

1. Remove 5′ TGCT from top oligo sequence (new sequence starts with G).
2. Take the reverse complement of the sequence from **step 1**.
3. Add CCTG to the 5′ end of the sequence from **step 2**. The four nucleotides are derived from endogenous miR-155. This also constitutes the four-nucleotide 5′ overhang, compatible with a four-nucleotide overhang in the provided linearized pcDNA6.2-GW/EmGFP-miR, to clone the double stranded oligo.

3.2 Annealing of the Top- and Bottom-Strand Oligos [14]

Anneal the top- and bottom-strand oligos to generate a double-stranded oligonucleotide suitable for cloning into the pcDNA6.2-GW/EmGFP-miR vector:

1. In a 0.5 mL sterile microcentrifuge tube, set up the following annealing reaction at room temperature:

Top-strand oligo (200 μM in TE buffer)	5 μL	(final concentration 50 μM)
Bottom-strand oligo (200 μM in TE buffer)	5 μL	(final concentration 50 μM)
10× Oligo annealing buffer	2 μL	
DNase/RNase-free water	8 μL	
Total volume	20 μL	

2. Incubate at 95 °C for 4 min.
3. Allow the reaction mixture to cool to room temperature for 5–10 min. The single-stranded oligos will anneal during this time.
4. Place the sample in a microcentrifuge, and centrifuge briefly (~5 s). Mix gently. The double-stranded oligo in the annealing mixture can be stored at –20 °C stable for at least a year.

3.3 Cloning the Double-Stranded Oligonucleotides into the pcDNA6.2-GW/EmGFP-miRNA Vector [14]

1. Dilute the double-stranded oligos (50 μM) to a final concentration of 10 nM (i.e., 5000-fold dilution) in two-step serial dilutions:
(Step 1) Using 1 μL of the annealing mixture (from the previous section), dilute the 50 μM mixture 100-fold into DNase/RNase-free water to a final concentration of 500 nM. Vortex to mix thoroughly.

50 μ M double-stranded oligo	1 μ L
DNase/RNase-free water	99 μ L
Total volume	100 μ L

(Step 2) Using 1 μ L of the 500 nM double-stranded mixture (from Step 1), dilute 50-fold into the oligo annealing buffer to a final concentration of 10 nM. Vortex to mix thoroughly. Store the remaining 500 nM double-stranded oligo stock at -20° C.

500 nM double-stranded oligo	1 μ L
10 $\times$ oligo annealing buffer	5 μ L
DNase/RNase-free water	44 μ L
Total volume	50 μ L

Aliquot the 10 nM double-stranded oligo stock, and store at -20° C.

- Set up a 20 μ L ligation reaction at room temperature using the following reagents *in the order* below:

5 $\times$ Ligation buffer	4 μ L
Linearized pcDNA6.2-GW/EmGFP-miR (5 ng/ μ L)	2 μ L
Double-stranded oligo (10 nM in 1 $\times$ oligo annealing buffer) (from the previous step)	4 μ L
DNase/RNase-free water	9 μ L
T4 DNA ligase (1 U/ μ L)	1 μ L
Total	20 μ L

- Mix reaction thoroughly by pipetting up and down (do not vortex).
- Incubate for 5 min at room temperature.
- Place the reaction on ice. The ligation reaction can be stored at -20° C overnight.
- Add 2 μ L of the ligation reaction into a vial of One Shot TOP10 chemically competent *E. coli* on ice, and mix gently by tapping. Do not mix by pipetting up and down.
- Incubate on ice for 10 min (5–30 min).
- Heat-shock the cells for 30 s at 42° C, and immediately transfer the tubes on ice.

9. Add 250 μL of room-temperature S.O.C. medium, and incubate with shaking (200 rpm) at 37 °C for 1 h.
10. Spread 50–200 μL from each transformation on a pre-warmed LB agar plate containing 50 $\mu\text{g}/\text{mL}$ spectinomycin, and incubate overnight at 37 °C.
11. Pick up 5–10 spectinomycin-resistant colonies, isolate plasmid DNA, and confirm the sequence and orientation of the double-stranded oligo insert using the EmGFP forward sequencing primer and miRNA reverse sequencing primer.

**3.4 Evaluation
of the Knockdown
Effects
of pcDNATM6.2-GW/
EmGFP-miR
Expression Clones**

We generally suggest that gene suppression efficiency of individual miRNA sequences is evaluated in transient RNAi analysis in vitro, before applying the miRNA sequences in ovo. Knockdown efficiency can be evaluated by, for example, introducing the pcDNA6.2-GW/-EmGFP-miR plasmids into cell lines that are known to express endogenously the target gene.

Alternatively, individual pcDNA6.2-GW/-EmGFP-miR plasmids can be co-transfected into culture cells with an expression construct for the target gene. If the target gene encodes an extracellular protein (protein “X”), it can be fused with an alkaline phosphatase (AP) tag in the expression construct (“X”-AP fusion protein) by using an APTag vector [16], as used in our previous study [12]. After transfection, the “X”-AP fusion protein will be secreted to the culture media, and its expression levels and knock-down effects of miRNAs can be monitored by measuring the AP activities in the culture media:

1. Plate HEK293T cells in a 24-well plate (8×10^4 cells/well), and culture overnight at 37 °C in a 5% CO₂ incubator.
2. Next day, transfect individual pcDNA6.2-GW/EmGFP-miR constructs with an expression plasmid of an AP-tagged protein, by using a conventional transfection method.
3. 48–72 h After the transfection, collect the culture medium.
4. Put 1 mL of culture medium collected from each well in a 1.5 mL Eppendorf tube, and incubate in a 65 °C water bath for 5 min to heat inactivate endogenous APs.
5. Spin the tubes in a microcentrifuge at maximum speed for 5 min. Collect the supernatant.
6. Take 100 μL of the supernatant, and add an equal amount of 2 $\times$ AP buffer to check the AP activity by measuring OD405 using a plate reader (*see Note 2*). If the activity is high, it may be necessary to dilute the supernatant first, using HBAH or another buffer containing a carrier protein (*see Note 3*).

3.5 Chaining of miRNA Sequence [14]

miRNAs are sometimes expressed in clusters in long primary transcripts driven by RNA Pol II [17]. The pcDNA6.2-GW/EmGFP-miR vector supports chaining of miRNAs to ensure co-cistronic expression of multiple miRNAs. Chaining different miRNAs targeting the same gene or repeating one miRNA can enhance knock-down effects. We usually chain two miRNAs targeting our gene of interest that show highest knockdown activities in vitro (Subheading 3.4) as follows (in this example, miR-1 sequence is cloned into the vector expressing miR-2):

1. Digest pcDNA6.2-GW/EmGFP-miR-1 (insert) with Bam H I and XhoI and pcDNA 6.2-GW/EmGFP-miR-2 (backbone) with Bgl II and XhoI.
2. Run the enzyme-digested insert and backbone on 2% agarose gel, excise the fragments, and purify them using a conventional gel extraction method (e.g., by using QIAEXII gene extraction kit).
3. Ligate the purified backbone and insert fragment at a 1:4 molar ratio, using T4 DNA ligase.
Transform competent *E. coli* (e.g., One Shot TOP10), and analyze resulting clones as described in Subheading 3.3.
4. Test the chained construct for knockdown effects using in vitro assays as described in Subheading 3.4.

3.6 Transferring the EmGFP/Pre-miRNA Expression Cassette to the pTol2 Vector

Finally, the expression cassette containing an EmGFP cDNA and 2× pre-miR sequences is transferred to the pT2K-CAGGS vector (Fig. 4). The expression cassette can be amplified by PCR using primers with an artificial restriction enzyme site (e.g., EcoRI) and cloned into the pT2K-CAGGS vector. We generally PCR-amplify

Fig. 4 The pT2K-CAGGS vector. Expression of ectopic genes inserted into the multicloning site is driven by the ubiquitous promoter CAGGS (including sequences from the cytomegalovirus intermediate early enhancer (CMV-IE) and chicken β -actin promoter). The expression cassette is flanked by the Tol2 sequences

and transfer the expression cassette encompassing from the 3' end of the CMV promoter to the miRNA reverse sequencing primer site:

1. Conduct PCR as follows:

(Reaction setup)	
pcDNA6.2-GW/EmGFP/2x pre-miRNA plasmid or pcDNA6.2-GW/EmGFP-miRNA-negative control plasmid (0.1 µg/µL)	1 µL
10× PFU buffer	5 µL
dNTP mix (10 mM each of dATP, dCTP, dGTP, and dTTP)	1 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
Pfu DNA polymerase (five units/µL)	0.5 µL
dH ₂ O	40.5 µL
Total volume	50 µL

(Thermocycling conditions)	
94 °C	5 mi
30 cycles of the following:	
94 °C	45 s
58 °C	1 min
72 °C	2 min
72 °C	10 min

2. Gel-purify the PCR product (c.1.3 kb), digest with the restriction enzyme (EcoRI), and ligate to the enzyme-digested pT2K-CAGGS vector (pT2K-CAGGS-EmGFP/miRNA). Transform *E. coli* (e.g., ElectroMAX DH10B) cells with the ligated plasmid, culture them overnight, and purify the plasmid by using a conventional Maxiprep method. Confirm the sequence of the expression cassette using the primers used for the PCR.

3.7 Preparation for In Ovo Microinjection and Electroporation

3.7.1 Chick Embryos

For in ovo microinjection and electroporation into the retina, we generally use chick embryos at HH stages 10–11 (HH10: 33–38 h, HH11: 40–45 h). The chicken eggs are incubated at 38 °C, with placing the eggs horizontally (the embryo will be positioned at the top of the egg). Label the eggs with the start date of incubation and their orientation in the incubator (e.g. mark the top side of the egg).

3.7.2 Setting Up of the Microinjection and Electroporation Apparatus

1. DNA cocktail

DNA cocktails are made by mixing the individual pT2K-CAGGS-EmGFP/miRNA plasmids with the pCAGGS-T2TP plasmid (5 $\mu\text{g}/\mu\text{L}$ each) at the ratio of 2:1.

2. Microinjection apparatus (Fig. 5a, b)

The microinjection apparatus consists of (in this order) Hamilton syringe, 18 G needle, PCV tubing (c. 2 cm length), and microglass needle. To set up the apparatus, first, fill the inner space of the Hamilton syringe with heavy mineral oil. Then, attach an 18 G needle, and push the plunger of the Hamilton syringe to fill the inner space of the needle with heavy mineral oil. Repeat the same procedure for the PCV tubing and microglass needle. The tip of the microglass needle must be pinched off by fine forceps to make an opening. Avoid that air bubbles are trapped in the system. Finally, load DNA cocktail into the microglass needle.

Fig. 5 In ovo microinjection and electroporation. (a) Typical work station for in ovo microinjection and electroporation. (1) Dissecting microscope. (2) Light source. (3) Injection apparatus set on a micromanipulator. (4) Electroporation apparatus set on a micromanipulator (electrodes are connected by cables to an electroporator (not shown)). (b) Injection apparatus set on a micromanipulator (5). A pulled glass needle (9) is connected to a Hamilton syringe (6) via PVC tubing (8) and an 18 G needle (7). The inner space of the apparatus is filled with heavy mineral oil. (c) Microinjection into the optic vesicle. DNA cocktail solution with fast green (blue) is injected into the optic vesicle with a micropipette needle. We usually insert the micropipette needle from the medial side of the optic vesicle. DNA solution is injected until the dye is visible throughout the optic vesicle. (d) Electroporation into the retina. Parallel platinum-coated electrodes are placed perpendicular to the long axis of the embryo: One electrode is placed near the anterior (nasal) optic vesicle, and the other is lateral to the hindbrain (posterior (temporal) side of the optic vesicle). The distance between the electrodes is 2 mm

3. Electroporation apparatus

Set a pair of electrodes with an electrode holder on a micromanipulator (the distance between the electrodes is 2 mm), and connect the electrodes to an electroporator with cables (Fig. 5a).

3.8 Microinjection of Plasmid DNA Solution

1. Take out a chicken egg from the incubator, and place it on an egg holder horizontally (in the same orientation as during incubation).
2. Attach a sterile 18 G needle to a 10 mL syringe. Carefully insert the needle through the shell at the blunt end of the egg at a sharp downward angle (be careful not to damage the yolk).
3. Withdraw 3–5 mL of albumin (egg white) from the egg. This will make the embryo and vitelline membrane separate from the shell and thus avoid injury of the embryo or blood vessels when opening a window.
4. Cover the hole generated by the needle with Scotch tape.
5. Confirm that the embryo is detached from the shell, by “candling” the egg with light.
6. Open a window by removing an approximate 2–3 cm-diameter circle of shell from the top of the egg using forceps and scissors (*see Note 4*). Window only up to five eggs at any one time to prevent drying out of embryos during electroporation. A drop of Hank’s solution on the embryo avoids drying of the embryo.
7. Insert the needle into the lumen of the optic vesicle, and inject DNA solution (with fast green) by slowly pushing (or tapping on) the plunger, until the dye is visible throughout the optic vesicle (Fig. 5c) (*see Note 5*). Remove the needle.

3.9 Electroporation

1. Place the electrodes on the vitelline membrane, perpendicular to the long axis of the embryo: one electrode being on the anterior (nasal) side and the other electrode on the posterior (temporal) side of the optic vesicle (lateral to the hindbrain). Position electrodes c. 2 mm apart (Fig. 5d). A drop of Hanks’ solution could prevent damages to the embryo caused by the electroporation.
2. Apply five square pulses (voltage: 15 V, pulse length: 50 ms, pulse interval: 950 ms, pulse number: 5) by the CUY21 electroporator.
3. Remove the electrodes. Seal the window with Scotch tape. Re-incubate the embryos at 38 °C until analyses (Fig. 6) (*see Notes 6 and 7*).

Fig. 6 Stable suppression of gene expression by Tol2 transposon-mediated integration of miRNA transgene. Nel (also known as Nell2) is an extracellular glycoprotein that regulates differentiation, survival, and axon guidance of retinal ganglion cells. During retinal development, Nel is expressed in the pigment epithelium and retinal ganglion cells. A transposon construct containing an expression cassette for EmGFP and miRNA sequences against the Nel gene was co-transfected with a transposase expression vector into the chick retina by in ovo electroporation at HH9-11. (a) Half of the retina (nasal or temporal side) was transfected with the transposon constructs and marked by EmGFP (green) (an example of transfection into the temporal retina is presented). The optic fissure (white arrow) represents the boundary between transfected and untransfected (control) sides. (b–f) Retinal sections were prepared at E4.5 (b–d) or E8 (e, f), and expression of Nel was examined by immunohistochemistry using anti-Nel antibody (red). Nel expression is significantly reduced on the transfection side (in the pigment epithelium (PE) at E4.5 and in the PE and ganglion cell layer (GCL) at E8). Note the complementary pattern of Nel immunostaining and EmGFP expression (b–d). Dotted line indicates the position of the optic fissure (modified from [12])

4 Notes

1. Alternatively, the eggs can be stored at 4 °C for up to 1 week. Extended storage would result in decreased rates of embryonic survival and development.
2. Take 500 µL of the supernatant and add an equal amount of 2× AP buffer, if the AP activity is measured in a spectrophotometer.
3. Do not use buffers containing phosphate, as it is a competitive inhibitor of AP.

4. The entire top of egg can be covered with Sellotape or Scotch tape. This will prevent cracking of the shell during opening a window.
5. Alternatively, DNA injection can be performed using a pressure injection system, such as Picospritzer (Parker).
6. Keep the inside of the incubator clean. Check the eggs in the incubator every day, and discard dead embryos (live embryos have well-developed vasculatures and a clearly visible beating heart).
7. The levels of ectopic expression vary in different embryos. Only the embryos that showed significant levels of expression are to be used for analysis.

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