

Tol2-Mediated Delivery of miRNAs to the Chicken Otocyst Using Plasmid Electroporation

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Abstract

The avian embryo has a well-documented history as a model system for the study of neurogenesis, morphogenesis, and cell fate specification. This includes studies of the chicken inner ear that employ in ovo electroporation, in conjunction with the Tol2 system, to yield robust long-term transgene expression. Capitalizing on the success of this delivery method, we describe a modified version of the Tol2 expression vector that readily accepts the insertion of a microRNA-encoding artificial intron. This offers a strategy to investigate the possible roles of different candidate microRNAs in ear development by overexpression. Here, we describe the general design of this modified vector and the electroporation procedure. This approach is expected to facilitate phenotypic screening of candidate miRNAs to explore their bioactivity in vivo.

Key words microRNA, Inner ear, Tol2 transposon, Vestibular, Auditory

1 Introduction

Gene transfer into the chicken embryo is a valuable tool for the study of development. The ease of access to multiple organ systems, low cost, and similarities to the mammalian system are enticing to researchers. These benefits are especially attractive when studying the development of the inner ear. The fluid-filled otic cup/otocyst gives rise to vestibular and auditory organs and the associated statoacoustic neurons, and is readily accessible to experimental manipulation in ovo on embryonic days 2–3 [1].

Successful overexpression of protein-coding transgenes has been well-documented within the avian inner ear using retroviral infection [2–4], particularly the RCAS system designed by the laboratory of Steven Hughes [5–7]. High-titer retroviruses can produce robust infection and long-term, high levels of transgene expression. However, these viruses can only infect mitotically active cells, and they are limited to transgenes smaller than 2.4 kb if they are to remain replication-competent [6, 8]. Replication-incompetent

retroviral vectors and plasmid vectors can both accept much larger transgenes (on the order of 10 kb for the former).

A further disadvantage of retrovirus-mediated gene transfer was encountered, when we modified RCAS to produce microRNA (miRNA) or short-hairpin RNA (shRNA). Small non-coding RNAs can be used as tools to limit the protein production of their targets by blocking translation or enhancing degradation of messenger RNA [9–11]. On multiple occasions, we inserted into RCAS, an artificial intron containing sequences designed to form short RNA hairpins following reverse transcription, expecting these to be processed into short single-stranded RNAs by the cell's miRNA processing pathway. However, viral titers were often 10–100-fold lower than standard protein-encoding RCAS vectors. We are unsure whether it is the presence of the hairpins, or the extra splice sites that may be interfering with viral replication in these vectors. Even when moderately high viral titers were achieved, subsequent infection of chicken otocysts with RCAS vectors carrying a pre-miRNA intron consistently failed to produce mature miRNAs detectable by *in situ* hybridization methods. In contrast, inserting identical pre-miRNA-containing introns into plasmid expression vectors, and transducing these into the otocyst via electroporation, were successful [12];unpublished data).

While electroporation of standard expression plasmids [13–15] can bypass certain problems encountered with viral vectors, transgene expression typically lasts only a few days [16, 17]. To circumvent this limitation, the Tol2 transposase system can be employed. This method relies on the integrative properties of transposons. When a plasmid encoding the Tol2 transposase is co-electroporated with a vector containing a transgene flanked by Tol2 sites, the DNA flanked by these sites is excised and inserted into the genomic DNA through the activity of the transposase [18–20]. This process ensures strong, long-term expression of protein-encoding genes within the vestibular and auditory organs [21–23].

An important aspect of gene transfer is to combine the detection of transduced cells with cellular phenotyping methods, such as immunofluorescence. This poses an additional challenge for the delivery and detection of small RNAs, because they require *in situ* hybridization to be detected in tissue sections. To identify which cells have been transfected, a separate reporter plasmid, often encoding a fluorescent protein, can be co-electroporated with the small RNA-expression vector [24]. The reporter fluorescence thus serves as a proxy for localization of the transduced small RNAs. This strategy creates a tenuous link between the observation of reporter protein and the assumption that small RNAs are being co-expressed at the same location. A solution to this problem is to use a bicistronic vector design, as is discussed here, where both the small RNA and the reporter gene are processed from the same primary transcript.

In this chapter, we discuss the combined use of a Tol2 transposase system and electroporation to simultaneously deliver a miRNA and a reporter gene under the control of the same

promoter to the chicken inner ear. To achieve this outcome, we modified the Tol2 vector to include an artificial intron [25–27] that houses the genomic sequences for a miRNA of interest. This design exploits the Pol II pathway to produce both a protein-coding transcript from the exon and a small RNA from the intron (Fig. 1).

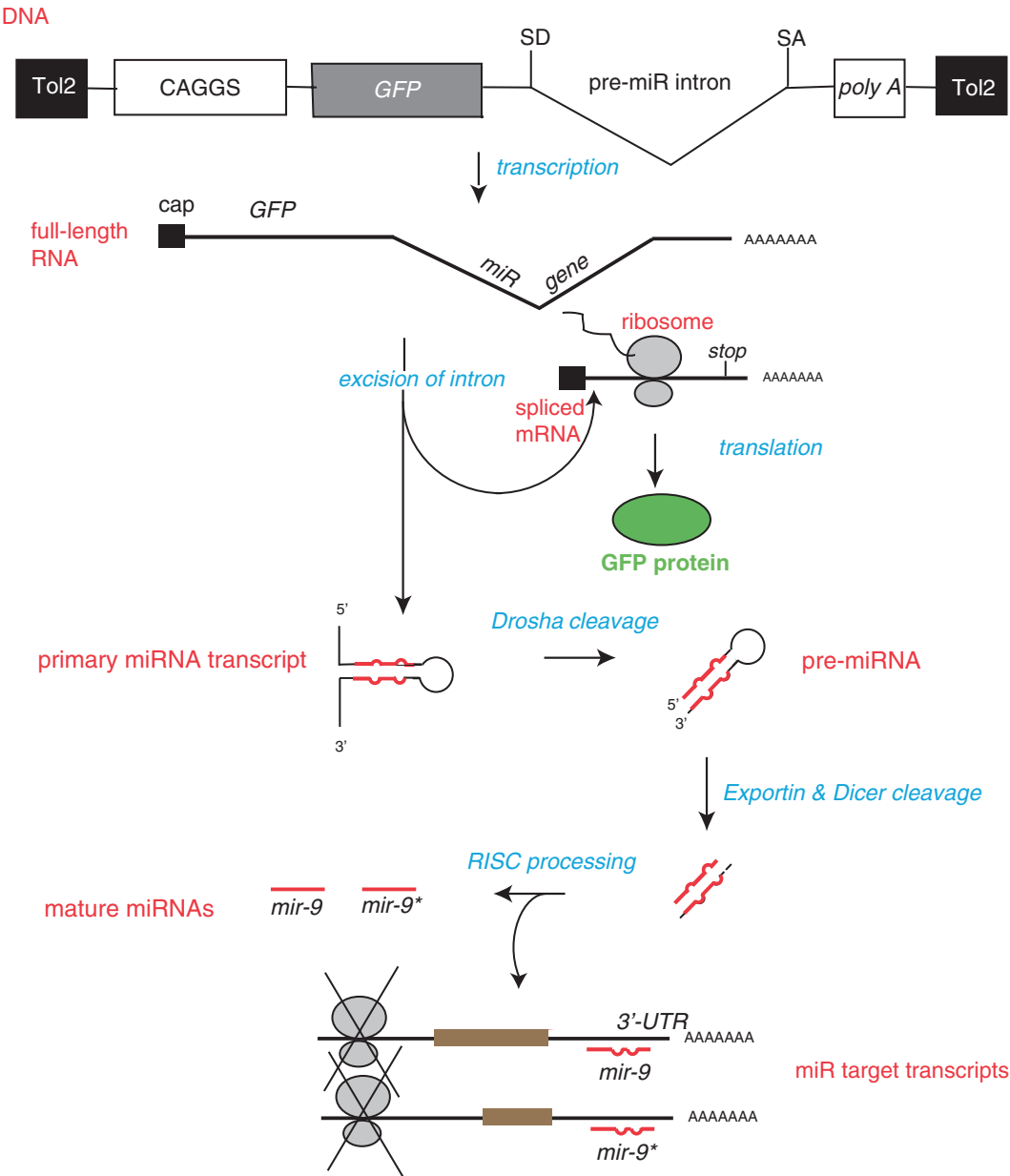


Fig. 1 Modified Tol2-Vector design and processing. When co-expressed with transposase, the DNA region flanked by the Tol2 ends incorporates into the host cell's genomic DNA. The CAAGS promoter drives expression of the exon encoding GFP and the *miR-9* gene from the intron designated by the splice donor (SD) and splice acceptor (SA) sites. Once the DNA is transcribed into RNA, the intron containing the primary miRNA transcript is clipped into a pre-miRNA and exported from the nucleus. It is then processed into mature miRNAs. Separately, the spliced *GFP* transcript is exported from the nucleus and processed as messenger RNA

Similar designs, using different backbones, have been shown to produce both components and provide a more accurate link between the expression of a reporter and distribution of transduced small RNAs [25–30]. Here, we show how this method generates miRNAs at levels that can be detected by in situ hybridization and that corresponds to the localization of green fluorescent protein (GFP) as a reporter on adjacent sections through the inner ear. We describe the overall electroporation method, as well as the construction of an electrode pair specialized for otocyst-stage electroporations. This report complements a previous methods paper describing electroporation at earlier (otic cup) stages, using Tol2 vectors specifically designed for drug-inducible expression of multiple protein-encoding genes [23].

2 Materials

2.1 DNA Plasmid Construction and Preparation

1. pME-MCS-sda (available upon request from the authors).
2. pTol2-GFP-Gateway (available upon request from the authors).
3. Gateway® LR Clonase™ II Plus enzyme mix (Invitrogen; Life Technologies, Grand Island, NY).
4. Genomic DNA from species of interest.
5. Primers and solutions for PCR amplification.
6. Thermal-cycler.
7. Standard reagents and associated buffers for conventional cloning by restriction enzyme digestion and ligation: *Xba*, *Xho*I, ligase, ethidium bromide-agarose gel reagents and running buffers.
8. Commercial maxi-prep kit for preparation of purified plasmid DNA.
9. Primers for DNA sequencing designed to confirm correct cloning: T3 primer.
10. pCAG-T2TP [20].
11. pTol2-GFP-sd-miR(X)-sa.
12. Fast green FCF (Sigma): Prepare a 0.25 % solution by adding 25 mg of Fast Green FCF crystals to 10 mL of millipure or distilled water. Filter using a 0.45 µm syringe filter, make 1 mL aliquots and store at –20 °C until use.

2.2 Electrode Fabrication Supplies

1. Platinum wire, 0.3 mm diameter (Alfa Aesar, Ward Hill, MA).
2. Tungsten wire, 0.5 mm diameter (Alfa Aesar).
3. Heat shrink tubing, 3/64" × 6" (SPC Technology, Blaine, MN).
4. Bunsen burner.
5. Nail polish.

2.3 Microinjection and Electroporation

1. Micropipette puller: Flaming/Brown Model P-97 (Sutter Instrument Co., Novato, CA).
2. Micropipettes: thin wall borosilicate glass capillary with filament, diameter chosen to match electrode holder. We use 1.5 mm OD \times 1.17 mm ID (Harvard Apparatus, Holliston, MA).
3. Manual micromanipulators: $n=2$ (e.g., MM33 roller bearing micromanipulator, Stoelting Co.), each mounted on a magnetic base with articulated arm (Stoelting Co.), and set on an iron base plate (Narishige, Japan).
4. Electroporator (TSS20 Ovodyne) and current amplifier (E21) (Intracel, UK).
5. Pipette holder for tungsten/platinum electrodes (e.g., Tritech Research).
6. Hook cable connector between current amplifier and tungsten/platinum electrodes (Bex Co., Tokyo, Japan).
7. Microinjection apparatus: Pressure injector with nanoliter injection volumes and millisecond resolution, with 60 psi regulator and foot pedal external trigger (e.g., Picospritzer III; Parker Hannifin Corp., Cleveland, OH).
8. Micropipette holder for pressure injection of plasmids: MP Series microinjection electrode holder, straight body with appropriate (male/female) Luer Port, 1.5 mm glass OD (Harvard Apparatus).
9. Manual stereomicroscope with apochromatic correction and magnification to 25 \times or greater.
10. Fiberoptic illuminator, dual gooseneck light guides.
11. Compressed nitrogen tank (>99 % purity).
12. Ice bucket with ice.
13. 1.5 mL Eppendorf tubes
14. Microcapillary pipet tips (Eppendorf MicroloaderTM; Eppendorf, Hauppauge, NY) attached to 10 μ L pipettor to backfill micropipettes.
15. Culture Dishes: two 60 mm dishes and one 35 mm dish.
16. Chick Ringer's Solution: NaCl 123 mM, CaCl₂ 1.53 mM, KCl 5 mM, Na₂HPO₄ 0.8 mM, in 1 L millipure. Bring the pH to 7.4 using NaOH. Once dissolved, filter the solution using a 0.22 μ m Stericup filter unit (Millipore, Billerica, MA). Distribute 100 mL into 125 mL bottles and autoclave and store at room temperature.

2.4 Windowing and Embryo Preparation

1. 3 or 5 mL syringe.
2. 18.5–20-Gauge needle.
3. Scotch tape and clear packing tape.

4. 5 mL plastic disposable transfer pipets.
5. Egg holder: can be fashioned from Styrofoam, plaster-of-paris, or by lining a 60 mm Petri dish with Kimwipes.
6. Dissection tools: one Dumont #55 straight forceps, one Dumont #5 straight forceps with Biology tips, one set of curved dissection scissors (Fine Science Tools, Foster City, CA).
7. Kimwipes.
8. 70 % Ethanol.
9. Humidified egg incubator, benchtop or cabinet (Lyon Technologies, Inc., Chula Vista, CA).

3 Method

3.1 DNA Plasmid Construction and Preparation

1. Figure 2 shows the basic cloning strategy for preparing a bicistronic vector carrying a pre-miRNA of interest. This entails using pME-MCS-sda as a Gateway® donor vector and pTol2-GFP-Gateway as a Gateway® destination vector. As background information, we provide the primer sequences we used to generate the artificial intron cassette found in the pME-MCS-sda Gateway® shuttle vector. The primers, designed based on previous reports [26, 27], are as follows:
2. Forward: 5'gcg gtc gac gta atc tag agg atc cct cga gta cta act ggt acc tct tc 3'.
3. Reverse: 5' gca agc ttc tgc agg ata tca aaa aaa gaa gag gta cca gtt agt act c 3'.
4. We inserted the amplicon containing the artificial intron into a Gateway® shuttle vector to create pME-MCS-sda as described previously [25]. We placed three restriction enzyme sites (*Xba*I, *Bam*HI, and *Xho*I) in the artificial intron to facilitate entry of any miRNA of interest.
5. Amplify your pre-miRNA of interest from genomic DNA, designing the primers to include *Xba*I and *Xho*I restriction site sequences at the ends (*see Note 1*). The resulting amplicon should encompass extra base pairs flanking the pre-miRNA sequence for proper processing [31]. We maintained about 60 nucleotides of endogenous sequence on the 5' and 3' ends of pre-miR-9-1. We also had success with constructs containing a larger number of flanking bases [25].
6. Once the amplicon is generated, insert this into pME-MCS-sda using conventional cloning with *Xho*I-*Xba*I double-digestion and ligation. Confirm that this intermediate construct is correct by DNA sequencing using the T3 primer.

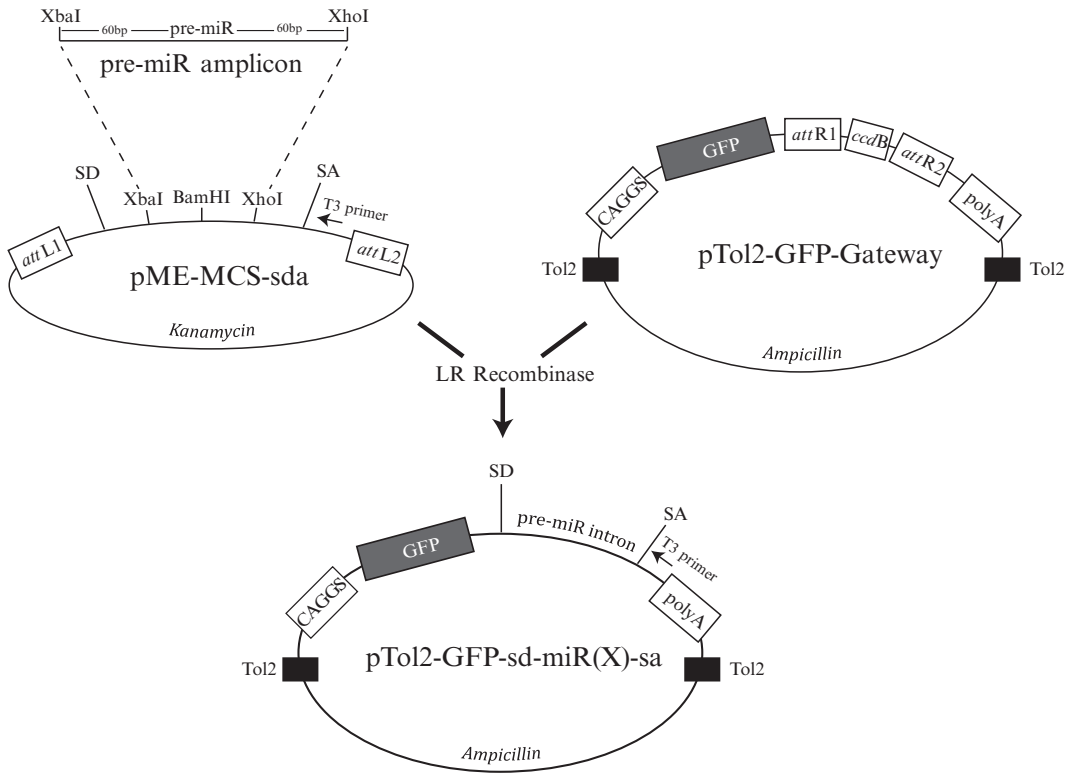


Fig. 2 Cloning strategy for generating a bicistronic Tol2 vector carrying GFP and any pre-miR intron. The pre-miR amplicon is created by amplifying the pre-miRNA of choice from genomic DNA. The amplicon should include genomic sequences predicted to form the pre-miRNA stem-loop (~80 nucleotides) after transcription, along with 60–100 bp of DNA flanking either side of this. Primers should be designed to add *XbaI* and *XhoI* restriction sites to the end of the pre-miR amplicon. These sites facilitate cloning the amplicon into pME-MCS-SDA, which then serves as a Gateway® donor vector (Invitrogen, Life Technologies). The pTol2-GFP-Gateway was created to serve as a Gateway® destination vector. Arrows show the position and direction of T3 primer binding sites that are used to sequence the miRNA intron. SA splice acceptor, SD splice donor

7. Use the LR Clonase™ II enzyme mix (Invitrogen, Life Technologies) according to the manufacturer's protocol to transfer the miRNA-intron into the pTol2-GFP-Gateway vector [12]. This will create the desired miRNA-reporter expression vector, now called pTol2-GFP-sd-miR(X)-sa (substitute miR(X) with the name of your miRNA, e.g., pTol2-GFP-sd-miR9-sa). Confirm the construct is correct by DNA sequencing using the T3 primer and, if desired, a primer located at the 3' end of GFP.
8. Prepare maxiprep plasmid DNA for pTol2-GFP-sd-miR(X)-sa and pCAG-T2TP. Use a standard protocol or commercial kit to yield final DNA concentrations between 3 µg and 5 µg/µL for electroporation (see Note 2).

3.2 Electrode Fabrication

1. Cut a piece of tungsten wire to approximately 4–5 cm in length.
2. Cut a piece of platinum wire 1 cm in length.
3. Trim a piece of heat-shrink tubing to encapsulate the tungsten wire piece, leaving a 0.5 cm segment of wire protruding from the tubing at one end.
4. Insert a 0.5 cm segment of platinum wire into the opposite end of the tubing, ensuring it contacts the tungsten wire.
5. Using the Bunsen burner, wave the insulated portion of the wires, back and forth over the flame, to shrink and secure the insulation surrounding the wires. At this point, the platinum wire should be firmly secured by the insulation.
6. Repeat **steps 1–5** to create second electrode. Once both electrodes are made, place them side-by-side and secure them together by wrapping the insulated portion of the wire closest to the exposed platinum with Parafilm. Joining the two electrodes is also accomplished by holding the insulated portions over an open flame.
7. Bend the final 2–3 mm of exposed platinum by $\sim 45\text{--}60^\circ$.
8. Apply nail polish to cover the exposed platinum, except at the tips, where nail polish is applied only to the outer sides of each electrode, to ensure current flows between the two inner surfaces of the tips (*see Note 3*).
9. Place in electrode holder (Fig. 3).
10. Attach to the current amplifier with hook cable connector.

3.3 Micropipette Production

1. Using a micropipette puller, pull borosilicate glass capillary tubes. A short taper (~ 5 cm) is preferable to minimize clogging.
2. Break the end of the micropipette to create a 10–13 μm opening (*see Note 4*).
3. Pull and break about 10 micropipettes for each batch of embryos, so that clogged micropipettes can be replaced readily.

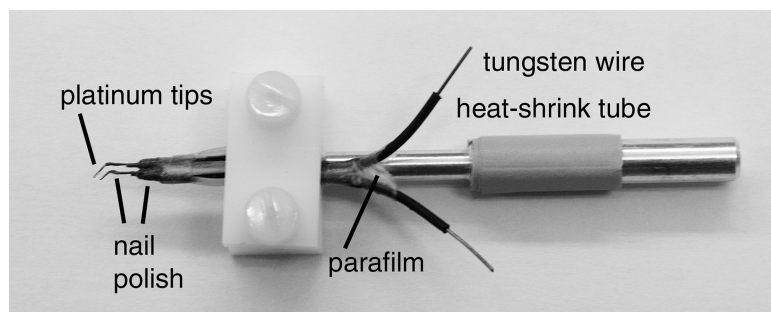


Fig. 3 Platinum-tungsten electrodes secured in electrode holder. See text for details on fabrication

3.4 Embryo Preparation

3.4.1 Egg Windowing

1. Eggs should be placed on their sides in the egg incubator, and the topmost spot indicated with a marker. This is where you can expect to find the embryo on the day of windowing, if the incubator does not rock the eggs.
2. On the second day, place the egg horizontally in an egg holder with the marked position at the top. Create a small hole at the pointed end of the egg using an 18.5–20-gauge needle attached to a 3 or 5 mL syringe.
3. Insert the needle, angled downward as vertically as possible, and remove 1.5–2.5 mL of albumin (*see Note 5*). Cover the hole with Scotch tape.
4. Cover the pre-marked top position of the egg with ~2 cm of Scotch tape. This will minimize dropping broken shell pieces onto the embryo during windowing. Pierce the top portion of the eggshell with the 18.5-gauge needle. Insert the tip of the dissection scissors and remove a circular portion of the eggshell (and Scotch tape) about 1 cm in diameter.
5. Cover the opening with clear packing tape and place back into humidified egg incubator until ready for injection and electroporation.

3.4.2 Injection Day

1. Using the dissection scissors, remove enough of the tape and shell to see the entire chicken embryo.
2. While the egg is in its holder, place it under the stereomicroscope and adjust the fiber optic lighting until it reflects off the chorion.
3. Using #5 forceps, grab a portion of chorion just beyond the dorsal surface of the embryo near the position of the otocyst. Use #55 and #5 forceps to delicately tear the chorion and then the underlying amnion until an area surrounding otocyst is clear. The cleared region must be large enough to accommodate the electrode tips. This is critical for successful electroporation. We have had success using a needle or forceps to pierce the membranes and deliver a small amount of Ringer's solution beneath the amnion to separate it from the embryo before tearing it open, as described previously [23]. The embryonic membranes of each embryo should be pierced immediately before the plasmid injection step as described in Subheading 3.6.

3.5 Preparation of Injection Needle

1. For injection and electroporation of HH16–18 embryos, dilute 9 volumes of plasmid DNA (3–5 $\mu\text{g}/\mu\text{L}$) with 1 volume of 0.25 % fast green. Use a molar ratio of 2:1 for pTol2-GFPsd-miR(X)-sa and CAG-T2TP, respectively. Hereafter, we refer to this mix as plasmid DNA. A simplified version of each plasmid is shown schematically in Fig. 4a. Leave the plasmid DNA on ice.

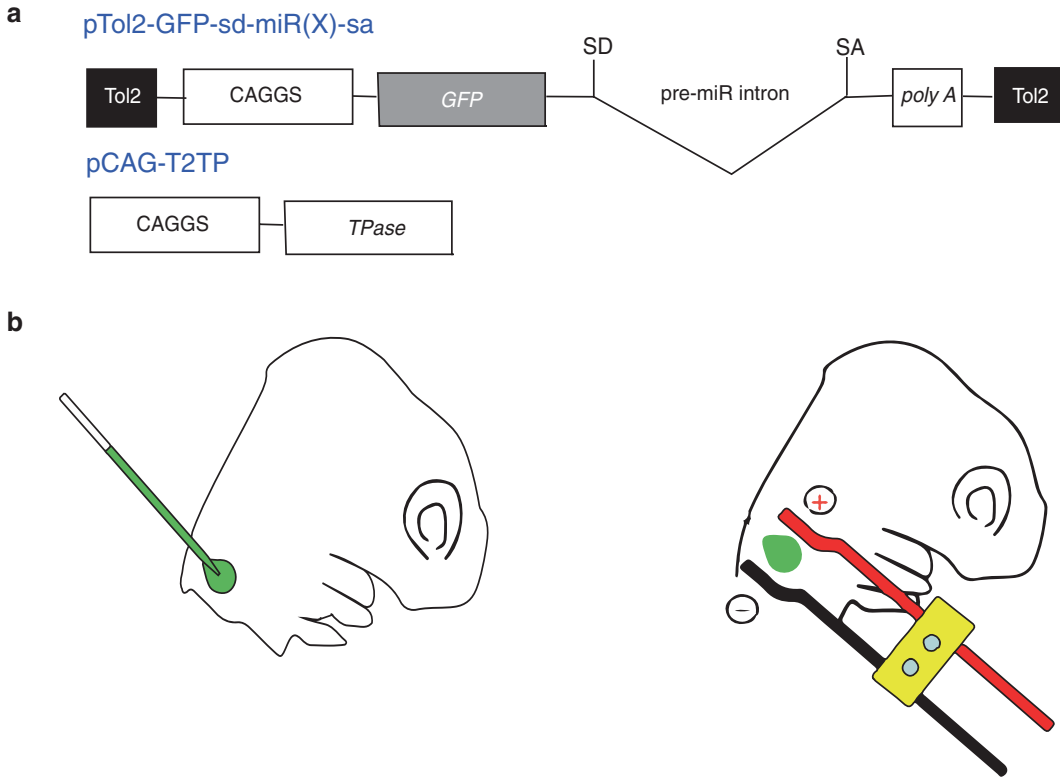


Fig. 4 Electroporation of Tol2-Vectors into the chicken otocyst. **(a)** Schematic representation of miRNA-containing plasmid and transposase-encoding vector. **(b)** A mixture of fast-green and plasmid DNA is injected into the otocyst of an E3 (HH16-18) chicken. **(c)** The platinum tips of the electrode are placed on top of the embryo, with the otocyst in between. The location of positive electrode dictates the direction of DNA migration. This cartoon depicts DNA targeted toward the anterior pole of the otocyst. To target the anterior or posterior poles, the otocyst is approached from either the ventral direction (as shown) or the dorsal direction

2. Backfill the pulled-glass capillary micropipette with 3 μ L of plasmid DNA using microcapillary pipet tips. Place micropipette into the holder connected to the Picospritzer and clamp onto the micromanipulator. Open valve to nitrogen gas cylinder.
3. Ensure that the DNA readily flows out of the tip of the micropipette following a pressure pulse. To accomplish this, place a 35 mm cell culture plate filled with Ringer's solution on top of two 60 mm cell culture plates and view under the stereomicroscope. Lower the micropipette into the solution so its tip barely breaks the surface of the liquid (to avoid excessive backflow of Ringer's solution into the tip). Turn on the Picospritzer and engage airflow until the plasmid DNA is expelled into the surrounding liquid and all air bubbles are removed from the tapered shank of the micropipette.

3.6 Injection and Electroporation of Plasmids into the Otocyst

1. Place embryo prepared in Subheading 3.4.2 under the stereo-microscope. Deposit several drops of Ringer's solution onto embryo using a plastic transfer pipet (*see Note 6*). Adjust lighting obliquely to enhance the visibility of the otocyst. Differences in opacity become more critical as injections are performed on younger embryos because the otic cup or otocyst is more transparent.
2. Use micromanipulator to move the injection micropipette above embryo. The precise injection procedure will differ for otic cup stages (HH11-12) versus otocyst injections (HH13 and beyond). Details for otic cup injections can be found in an excellent methods chapter by Freeman et al. [23]. Here we focus on otocyst stages (*see Note 7*).
3. Slowly lower the micropipette until the tip pierces the epithelium of the otocyst. In some cases, the moment the otocyst is punctured, it can be visualized as a sudden alleviation of resistance applied to the needle tip by the epithelium, causing the embryo to recoil upward.
4. Inject the DNA mixture into the otocyst cavity until it begins to enlarge (Fig. 4b). We typically use pressure settings of 20 psi with 10 ms pulses. Multiple pulses are sometimes required, especially if the micropipette begins to clog after several injections. Take care not to overfill the otocyst to avoid rupturing it. Remove the micropipette. If a delay between injections of more than ~5 min is expected, submerge the tip of the micropipette into the Ringer's solution in the 35 mm plate to avoid it drying out and clogging.
5. Apply two additional drops of Ringer's solution to the embryo. On the opposite side of the embryo, bring into view the electrode holder secured to a second micromanipulator.
6. Place electrodes so the otocyst lies between them, as shown in Fig. 4c. Then, gently lower electrodes until they touch the surface epithelium. Since both electrodes are composed of platinum wire, either side can function as the cathode. Designation of the cathode determines which direction the DNA will travel. In Fig. 4c, the cathode is placed anterior to the otocyst to drive the DNA toward the anterior crista.
7. Deliver two 10-V square wave current pulses, 50 ms long, spaced 10 ms apart using an electroporator and the connected current amplifier. A good indicator of functioning electrodes is a copious amount of bubbles (*see Note 8*).
8. Apply additional Ringer's solution before withdrawing the electrodes. Cover the eggshell window with clear packing tape and place back into the egg incubator until harvest.

9. Clean electrodes after several embryos have been electroporated, by submerging their platinum ends into the liquid contained in the 35 mm plate and delivering several current pulses. Electrodes often become less efficient as tissue debris accumulates on the surfaces. If tissue debris still remains on the electrodes, carefully remove by scraping off using #5 forceps. It may be necessary to reapply nail polish after cleaning electrodes, if they become chipped during the process.
10. When finished with each batch of embryos, clean dissection tools with detergent and a soft bristle brush, then rinse well with water. Clean electrodes as in Subheading 3.6, **step 9**. Dispose of needles and syringes in a sharps container. If any of the DNA mixture remains, it can be stored at 4 °C or -20 °C, and reused. Turn off nitrogen tank.
11. Embryos should be left in a humidified incubator until the desired embryonic stage. Figure 5 shows evidence of gene transfection in sections through the inner ear 4 days after plasmid electroporation into the E3 otocyst.
12. Visualize GFP by immunofluorescence and miR-9 by in situ hybridization. Our lab has successfully collected transgene-expressing basilar papillas 14 days after electroporation, although survival rates decrease dramatically beyond 3–4 days post-electroporation [12].

4 Notes

1. Most pre-miRNA stem-loop sequences can be found using miRBASE (<http://mirbase.org>; [32]. It is also possible to construct an intron that carries multiple pre-miR sequences in series [12]. See manufacturer for further details of Gateway technology (Invitrogen, Life Technologies). Plasmids pME-MCS-SDA and pTol2-GFP-Gateway are available from the authors upon request.
2. When making high concentration DNA, we often resuspend in a lower volume (~25–50 %) than what is recommended by the manufacturer of a commercial maxiprep kit. The resulting solution can be rather viscous or DNA may precipitate out of solution. Placing the solution in a 55 °C water bath for 30 min on the day of injection may help the DNA redissolve.
3. Nail polish application to the outside of the platinum wire is critical. Without the coating, the electric current will emanate from all of the exposed sides of the electrode. This can cause poor DNA transfection and increased embryo death.
4. Tip diameters less than 10 µm can result in clogging, while diameters greater than 13 µm may create too large of a hole in

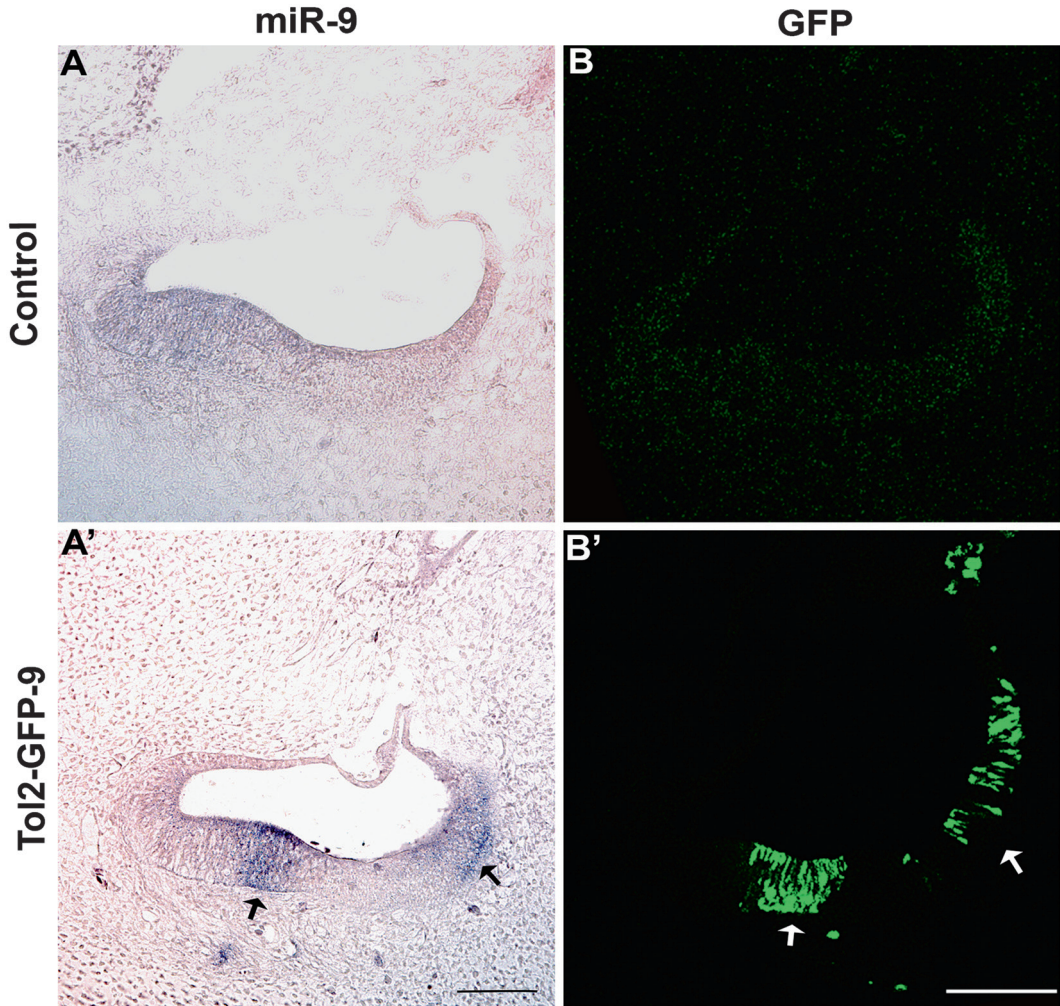


Fig. 5 Tol2-mediated expression of miRNA in the developing chick ear. On E3, pTol2-GFP-sd-miR9-sa and CAG-T2TP plasmids were electroporated into the anterior pole of the otocyst and the embryo was allowed to develop until E7. Alternate sections through the anterior crista were (a') processed by in situ hybridization to detect miR-9 or (b') immunostained for GFP. For in situ hybridization, we used a 5' and 3' double digoxigenin-labeled LNATM microRNA detection probe (Exiqon) and alkaline phosphatase immunohistochemistry. The miR-9 hybridization signal (a' black arrows) is located in areas that correspond to GFP in the sister section (b' white arrows). (a, b) The control consists of the contralateral (*left*) ear with the image flipped to match the experimental (*right*). Scale bar = 100 μ m

the otocyst, leading to excessive leakage of the plasmid DNA. Although not required, we find it helpful to use a student microscope to break the micropipette and immediately judge the tip diameter using an ocular micrometer at low power (10 \times). To do this, we mount the micropipette on the slide holder using molding clay, and watch as we tap the tip head-on against the edge of a stack of glass slides secured to the stage.

5. The amount of albumin removed is based on the size of the egg. It is better to remove too little than too much. A video is available of the egg windowing protocol [33].
6. During the procedure, note the appearance of the embryo. Make sure the embryo remains moist with regular application of Ringer's solution. The embryo will die if dried out.
7. We describe here a protocol to target the anterior or posterior poles of the otocyst, to transfect the anlagen of the utricular macula/anterior crista or the saccule/posterior cristae, respectively, as described previously [15, 34]. It is not uncommon to find the medial wall of the otocyst, including the endolymphatic duct, also transfected with this method. Delivery of plasmids to other sensory organs or to the otic neuroblasts can be determined empirically, and may require transfection into the otic cup at E1.5-2.5 (Hamburger-Hamilton stages 11-13), as described by others [12, 35-37].
8. If bubbles are absent or substantially decreased compared to previous embryos, transfer electrodes to a plate containing Ringer's solution and remove debris by scraping the electrode tips with dull forceps, and wiping with Kimwipes. In some cases, a few pulses in clean liquid will remove the debris without scraping.

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