Preface

In ovo electroporation is an unprecedented achievement in the study of developmental biology. With this method, we can now carry out gain-and-loss-of-function experiments in the desired tissue at any desired stage of development in chick embryos. The introduction of the tetracycline-regulated gene expression system and the transposon system further extended the possibility of this method, which enabled us to obtain long-term expression and to turn on and off a gene of interest at any desired stage. The method is now successfully applied in mice, aquatic animals, and even in plants for the study of developmental biology and for other purposes. Sonoporation is another useful tool, but one that uses ultrasonic waves rather than electric currents, for gene transfers to mesenchymal tissues. In this book, the application of electroporation in the various tissues and organs of embryos is presented, along with chapters that discuss gene transfers in adults.

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Chapter 2
In Ovo Electroporation as a Useful Tool to Pursue Molecular Mechanisms of Neural Development in Chick Embryos

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1 Introduction

In the field of developmental biology, electroporation has become a routine technique for gene transfer because of its simplicity and wide range of application. In chick embryos, the in ovo electroporation technique has enabled molecular analysis of developmental mechanisms in chick embryos and has revived chick embryos as the model animals in developmental biology (Muramatsu et al., 1997; Sakamoto et al., 1998; Funahashi et al., 1999; Nakamura et al., 2000). Especially, many gene analyses were conducted in developing neural tube, because the neural tube is a convenient tissue for application of in ovo electroporation. Here, we show the application of in ovo electroporation to the developmental neural tube in chick embryo.

2 Principle

Living cells can be regarded as a structure consisting of a nonconducting membrane with an aqueous solutions on both sides (BTX, San Diego, CA, USA). Exposure to an electric field leads to charge separation in the membrane, resulting in a transmembrane potential difference. Opposite electrical charges on the membrane attract each other, exerting a pressure on the membrane, which causes thinning of the membrane. Beyond a critical potential difference, small pores are made on the membrane. Through the pores, molecules such as DNA can enter the cells. If the strength of the pulse, length, and duration are appropriate, removal of the field can lead to healing of the pores. Then DNA enters the nucleus and is transcribed under the enhancer and promoter of the vector. Since DNA is negatively charged, DNA moves toward the anode in the electric field. When we target neural tube for

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electroporation, only the anode side is transfected and the cathode side could be used as the control (Funahashi et al., 1999; Nakamura et al., 2000; Odani et al., 2008).

3 Procedure

3.1 Preparation of Plasmids

3.1.1 Gain of Function

Usually, cDNA is cloned into expression vectors such as pMiwIII (which has Rous sarcoma virus enhancer and beta-actin promoter) (Suemori et al., 1990; Wakamatsu et al., 1997; Matsunaga et al., 2000), pRc/CMV (which has cytomegalovirus enhancer; Invitrogen, Carlsbad, CA, USA), or pCAGGS [which has the cytomegalovirus (CMV) and chicken beta-actin promoter] (Niwa et al., 1991). These vectors work well in chick embryos for short-term expression. The plasmid DNA is dissolved in Tris-EDTA (TE) buffer and is injected into the neural tube. Here, we show results of electroporation with pEGFP-N1 (Clontech, Palo Alto, CA, USA) vector for transient expression and EGFP/RCAS vector for long-term expression.

3.1.2 Knock Down

For knock down of certain genes, short hairpin-type DNA (about 20 mer of sense and antisense target sequences connected by a spacer sequence) is inserted in shRNA expression plasmids, which have U6 promoter or H1 promoter that is suitable for generation of small RNA transcripts by RNA polymerase III (Katahira and Nakamura, 2003). A pSilencer1.0-U6 siRNA expression vector (Ambion, Austin, TX, USA) works well in chick embryos. New vector was developed for optimal gene silencing in chick, which uses a chicken U6 promoter to express RNA modeled on a modified chicken microRNA (Das et al., 2006). A strategy for siRNA target sequence has been improved by several companies. We can design shRNA by referring to technical information from companies such as Ambion, NipponBio Service, GenScript, and Invitrogen (Carlsbad, CA, USA).

3.2 Chick Embryos

Fertile eggs are incubated at 38°C, and embryos are staged according to Hamburger and Hamilton (1951). Removal of a small quantity of albumen (4–5 ml) from the pointed pole of the egg helps manipulation in the egg. If the electroporation is carried out after E3, it is recommended that one remove albumen at E2 to avoid injury of the blood vessels. When the embryo is supposed to reach the desired stage, a window

is opened on the top of the egg. Injection of India ink (Rotring) underneath the embryo facilitates visualization of the embryo. A drop of Hanks' solution on the embryo will avoid drying of the embryo.

3.3 Injection of DNA Solution

Cut the vitteline membrane with a sharpened tungsten needle or a microscalpel and inject DNA solution into the lumen of the neural tube with a micropipette made of glass capillary (GD1; Narishige, Tokyo, Japan). The capillary is connected to the silicone tube, and injection of the DNA solution is controlled by mouth. The insertion point of the micropipette should avoid the area where the plasmid will be transfected. Addition of fast green to the plasmid solution facilitates visualization of the solution injected (Fig. 1a, d, g, j). If the DNA solution is injected to the anterior neural tube around E2, making a hole at the anterior tip of the neural tube relieves the pressure of the inside of the tube and prevents the injected DNA solution from coming out (Funahashi et al., 1999).

3.4 Electroporation

A pair of electrodes is set on a micromanipulator (MN-151-; Narishige, Tokyo, Japan) and placed on the vitelline membrane beside the embryo. Then several square pulses (25 V, 50 ms/s each) are charged by electroporator CUY (Bex, Tokyo, Japan; Neppagene, Ichikawa, Japan) or by T820 (BTX, San Diego, CA, USA). Electrodes are available from Neppagene and Unique Medical Imada (Natori, Japan). Hanks' solution prevents damage from occurring on the place where the electrodes are directly attached. The length of exposure part of electrode and distance between the electrodes are adjusted to obtain appropriate expression and avoid injury to the embryo. In addition, adjusting the pulse strength and the number of it also is important to obtain appropriate transfection. After electroporation, the window is sealed with Scotch tape (No. 405, Nichiban, Tokyo, Japan), and the embryos are reincubated at 38°C until the desired stage.

4 Application and Results

4.1 General Application

For transfection to the anterior neural tube in an early developmental stage, we routinely use parallel electrodes of 0.5 mm diameter and 1.0 mm exposure (Fig. 2.1a). A pair of electrodes are placed on the vitelline membrane 4 mm apart,

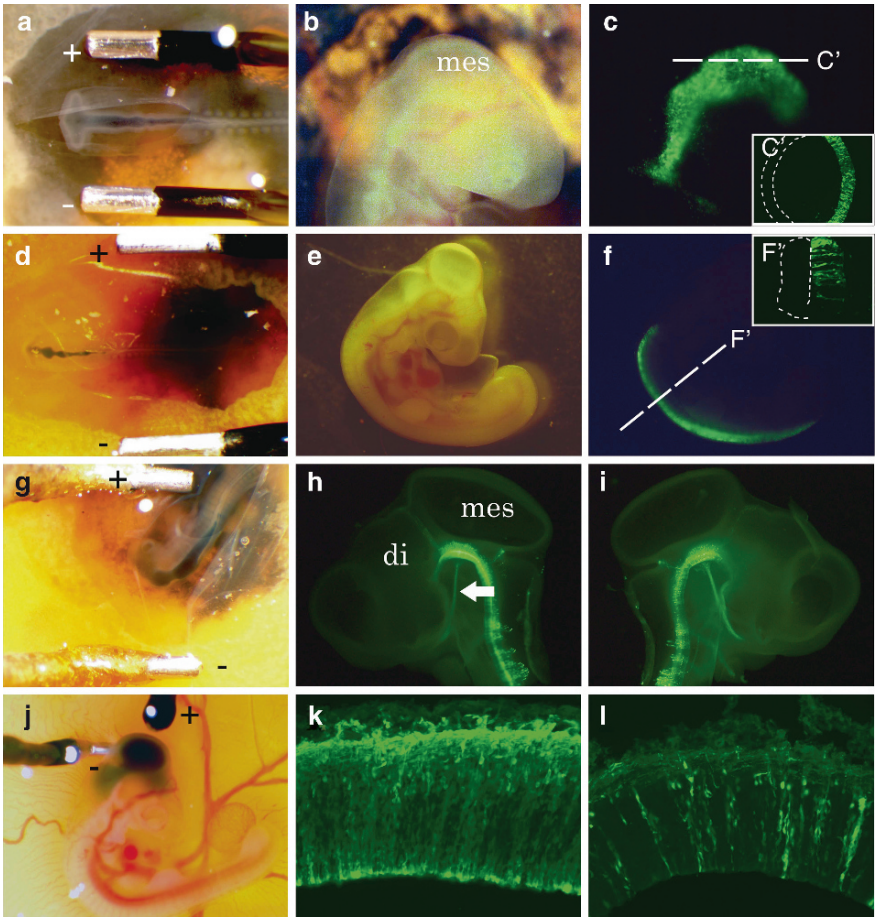

Fig. 2.1 In ovo electroporation. A pair of electrodes held by a manipulator are placed on the vitelline membrane at both sides of embryo (**a**, **d**, **g**). Most of the electrodes are insulated so that only the tip is exposed. For transfection into the mesencephalon at a later stage, a stick-type electrode (2-mm exposure) is placed underneath the left side of the head, and a circular electrode is placed on the right side (**j**). Photographs of the same row except for the bottom row are from the same experiment. The embryos look normal after electroporation (**b**, 24 h after electroporation; **e**, 48 h after electroporation). These embryos express GFP in the transfected side of the anterior brain (**c**) and spinal cord (**f**). The *white dashed lines* in figure c and f indicate the position of the sections (inset **c´**, **f´**). Panel **g** shows a photograph of the electroporation at the stage when the head turns to left side (**g**). Two days after electroporation, GFP fluorescence is seen in the ventral region of the mesencephalon and metencephalon, including the oculomotor nerves (**h**: *arrow*) in both sides of the embryo (**h**: *right side of the embryo*, **i**: *left side of the embryo*). Panel **j** shows a picture of electroporation into the mesencephalon at E4. The stick-type electrode as a cathode is placed underneath the left side of the head, and a circular electrode as an anode is placed on the right side. Then, a rectangular pulse (10 V, 50 ms/s) is charged three times. When we use a RCAS vector for virus-sensitive embryos, widespread misexpression could be obtained (**k**: 48 h after electroporation). On the other hand, when we use a RCAS vector on virus-resistant embryos, the expression is limited to the descendents of the transfected cells (**l**: 48 h after electroporation)

and three to five times rectangular pulse (25 V, 50 ms/s) are charged. With this condition embryos develop normally (24 h after electroporation) (Fig. 2.1b). Since DNA is charged negatively, the anode side is transfected (Fig. 2.1c) and the cathode side can serve as a control so that we can assess the effects of genes by comparing the right and left side of the same embryo (Fig. 2.1c., f.).

For transfection to the spinal cord, electrodes are placed on the caudal part of the neural tube. By using more widely exposed electrodes (2.0 mm, Fig. 2.1d) we can transfect a wider region of the neural tube along the anterior to posterior axis (Fig. 2.1f). At electroporation after vitelline vessels are developed, we must be careful to place the electrodes to avoid these blood vessels.

The direction of the electric field depends on the position of the electrodes, so that the location of transfection can be controlled by the relative position of the electrodes to the embryo. For example, if electroporating at the stage when the head is turning to left side, the ventral region of neural tube is transfected (Fig. 2.1g, h, i).

For electroporation on an embryo around stage 10 (E1.5), it is convenient to use the electrodes of both the anode and the cathode, which are fixed at 4 mm apart (Unique Medical Imada). As development proceeds, it becomes difficult to transfect by fixed parallel electrodes because the size of the embryos has become larger. In that case, it is convenient to use two separate electrodes. We show the figure of electroporation into the mesencephalon of E4 embryos (5–10 V, 50 ms/s, two to three times) (Fig. 2.1j).

For long-term expression, we can use provirus RCAS retrovirus vector. If we electroporate retrovirus vector on virus-sensitive embryos, virus proliferates from cell to cell so that widespread misexpression could be obtained (Fig. 2.1k). On the other hand, if we electroporate on virus-resistant embryos, the cells adjacent to the transfected cells would not be infected, and expression is limited to the descendents of the transfected cells (Fig. 2.1l).

4.2 Knock Down

By electroporation of shRNA-encoding vector, we can knock down the target gene expression effectively. Since mRNA is degraded by siRNA, we can assess the effects of siRNA by in situ hybridization, which is a great advantage to morpholino antisense oligonucleotide method to knock down. Morpholino antisense oligonucleotide interferes with translation so that we need specific antibody to assess its effects. Here knock down of coactosin, an actin-related molecule, is expressed in the cells of the dorsal neural tube and neural crest. Coactosin expression is knocked down at the site of siRNA expression (Fig. 2.2).

Specificity of siRNA was examined by targeting En2 in the mid-hindbrain region (Katahira and Nakamura, 2003). By designing four candidate siRNA sequences (En2-siRNA), efficiency of each candidate was checked. The number of nucleotide mismatch siRNA that could interfere with the target mRNAwas also checked. Among the four candidate siRNA, three worked well as siRNA and knocked down

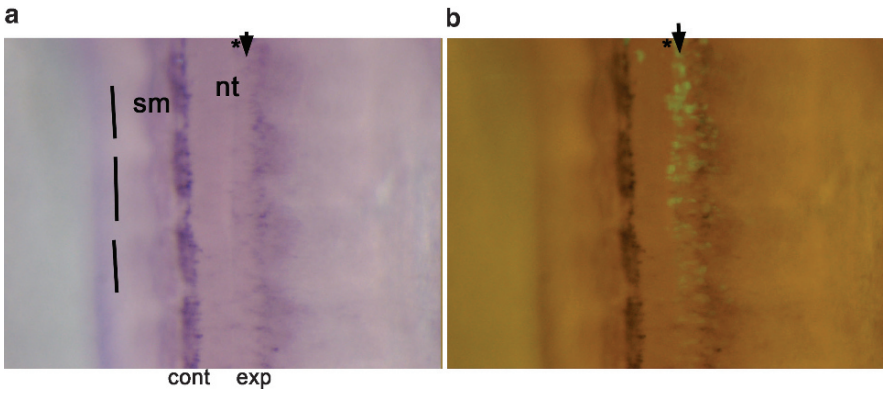

Fig. 2.2 Knock down of coactosin by siRNA. Coactosin-shRNA expression vector and GFP expression vector were electroporated in the neural tube of the trunk level at stage 10 and fixed 24 h later. The dorsal view of the transfected region of the embryo after whole mount in situ hybridization for coactosin (**a**), on which a photo of GFP fluorescence is overlaid (**b**). The GFP expression (*white dots*) represents the site of shRNA expression. Coactosin expression is knocked down on the experimental side in the cells that express shRNA expression (*arrow*). The center of the embryo is shown by asterisk. Abbreviations: sm, somite; nt, neural tube; cont, control side; exp, experimental side. Rostral is to the top. Each somite is represented by vertical bar

En2 expression. Up to two base mismatches, short double-strand RNA could weakly interfere with the target mRNA. Effects on mRNA and the protein were examined by comparison of images of in situ hybridization and immunostaining. A slight reduction of RNA signal could be detected by in situ hybridization by 6 h after electroporation of En2-siRNA expression vector. By 12 h after electroporation, reduction of RNA signal was obvious. Immunostaining with anti-En2 antibody indicated that effects are not detectable at the protein level at 6 h after electroporation of En2-siRNA expression vector. By 12 h after electroporation, reduction of target translation products is discernible.

To conclude, electroporation for misexpression and silencing could be versatile by appropriate setting of a suitable condition of electroporation, such as electrodes, electric parameters, plasmids, and target tissues.

5 Troubleshooting

For effective transfection, appropriate conditions of electroporation should be set. When the embryo is damaged after electroporation, you must alter the conditions such as the position of electrodes, the shape of electrodes, or the electric parameters. In general, lower voltage is used when distance between the electrodes is shorter, and narrower-exposure electrodes make it possible to transfect a narrower region (Sugiyama and Nakamura, 2003).

The expression level of the misexpression gene is dependent on the type of promoter of the expression vector and the concentration of the plasmids (Momose et al., 1999). Recently, the Tet-on/Tet-off system was developed so that strict control of timing of expression has become possible by administration of doxycycline (Dox) (Hilgers et al., 2005; Watanabe et al., 2007). The expression level also is adjustable by this system by changing the dose of Dox. If the translation product is toxic, the Tet system is useful because we can control the expression level and timing by changing the administration volume and timing of Dox.

Since the rational design for siRNA target selection is not perfect, we may have to design plural siRNA to silence a certain gene expression. Some companies (GenScript, Ambion, Invitrogen, NipponBio Service, etc.) support the siRNA design freely (Wang and Mu, 2004).

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