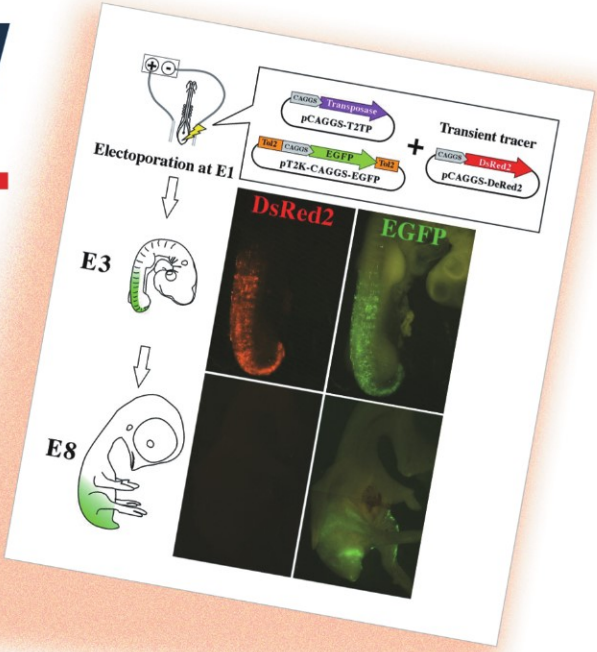
H. Nakamura
Editor

Electroporation and Sonoporation in Developmental Biology



Springer

Electroporation and Sonoporation in Developmental Biology

Harukazu Nakamura
Editor

Electroporation and Sonoporation in Developmental Biology

Springer

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Foreword

At the dawn of the science of animal development, two models were most favoured by embryologists: the amphibian egg and the chick embryo. Both presented the advantage of developing outside rather than inside a mother's womb as we do. They were thus available for the direct and fascinating contemplation of the spectacular changes leading from the egg to the fully developed organism. More importantly for the progress of embryology these two models allowed the transition from a purely descriptive to an experimental approach to emerge. The "causal" analysis of embryonic development was initiated at the end of the XIX Century by Wilhem Roux and later on brilliantly illustrated by Hans Spemann's discoveries. Work by these pioneers was followed by numerous studies.

A new era in developmental biology started when molecular genetics could be applied to the problems raised by the genetic mechanisms controlling embryogenesis. Other models came to the forefront: the fly (*Drosophila melanogastes*), the worm (*Caenorhabditis elegans*), the mouse and the small zebrafish (*Danio rerio*).

These organisms offer assets that make them more suitable for the genetic analysis of development than previous ones: they are easy and relatively inexpensive to raise, have a short generation rate and they can be good models for mutagenesis. Genetically modified embryos could be constructed in these species and the effect of genes on the developmental processes could thus be analysed. The large amount of fundamental results that have been produced by this type of experiments has brought about a conceptual renewal and a considerable enrichment of our view on how development is genetically regulated. The amphibian model could follow the molecular genetic moves since, while allowing embryonic manipulations through microsurgical techniques, the amphibian egg can be genetically modified. In contrast, in the avian model, the genetic analysis was for a long time restricted to the description of the expression patterns of various genes throughout development.

The breakthrough came from Japan, with the innovative adoption of the electroporation method to introduce DNA into cells, of the chick embryo while it is developing *in ovo*.

From 1997, gain and, more recently, loss-of-function of selected genes could be monitored in definite areas and at chosen times of development in the avian embryo.

These techniques were pioneered by Harukazu Nakamura, that I had the pleasure of receiving in my laboratory as a post-doctoral fellow many years ago. Pr. Nakamura recognized the interest of the technique and used it to study important problems concerning brain development.

A step further in the interest of this method has been recently accomplished: the temporal control of expression of the transgene can now be achieved through the introduction of tetracycline inducible systems and transposon mediated gene transfer in combination with electroporation.

Two of the investigators, Drs. Yoshiko Takahashi and Olivier Pourquié who, with Pr. Nakamura, have promoted the chicken model into the modern era of Developmental Biology, have been introduced to the avian embryo when they were in Nogent.

This book, which describes the electroporation technique will be very useful to researchers with a large spectrum of scientific interests. This is particularly true because the technique is now being extended to models other than chick, such as the mouse embryo and various tissues or embryonic rudiments explanted *in vitro*. It is even reaching the world of plants!

One can surmise that transgenesis through electroporation and perhaps also sonication will be applied to a larger spectrum of living organisms nowadays as interest for biodiversity increases in the frame of EVO-DEVO.

Nicole LE DOUARIN
Professeur Honoraire au Collège de France
Secrétaire Perpétuelle de l'Académie des Sciences

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.

Harukazu Nakamura
Sendai, Japan
31 July 2008

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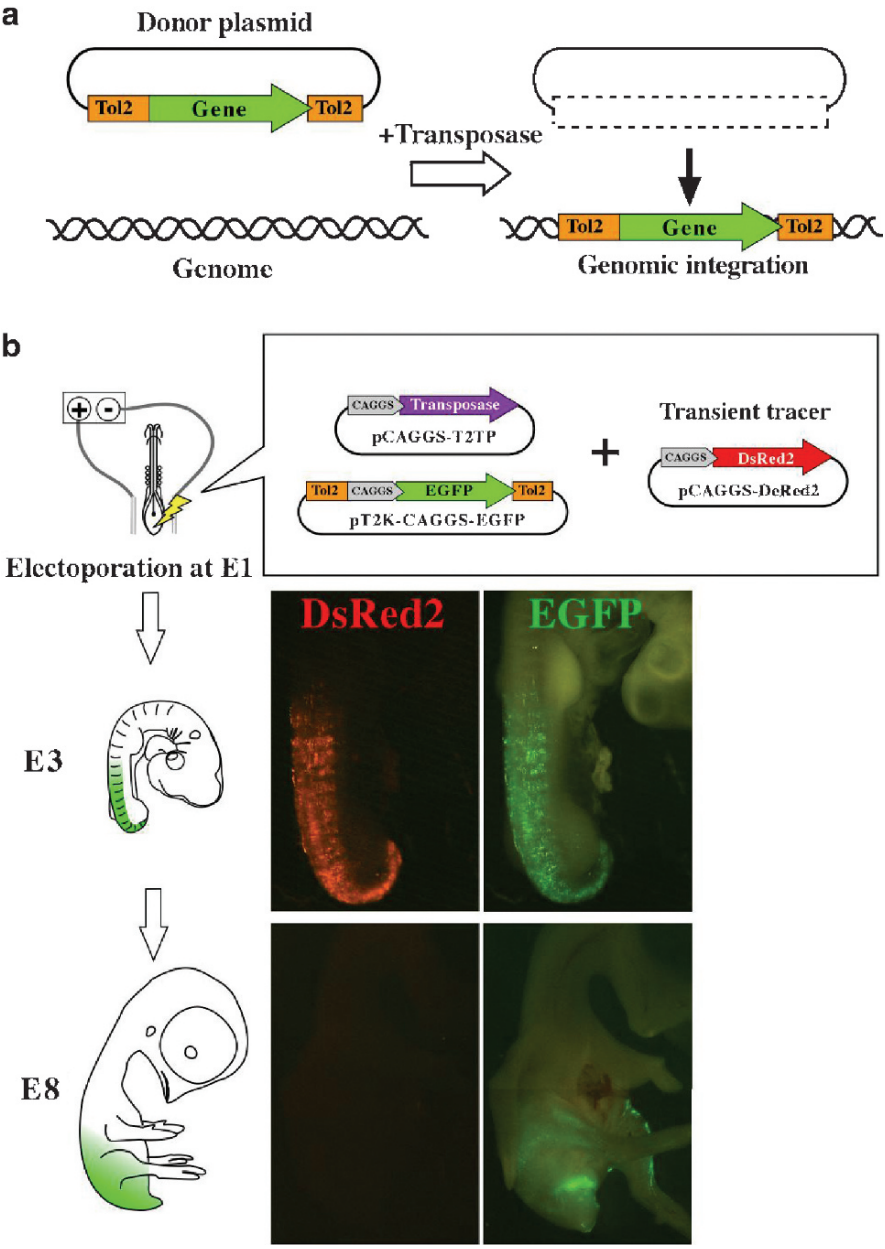
Color Plates



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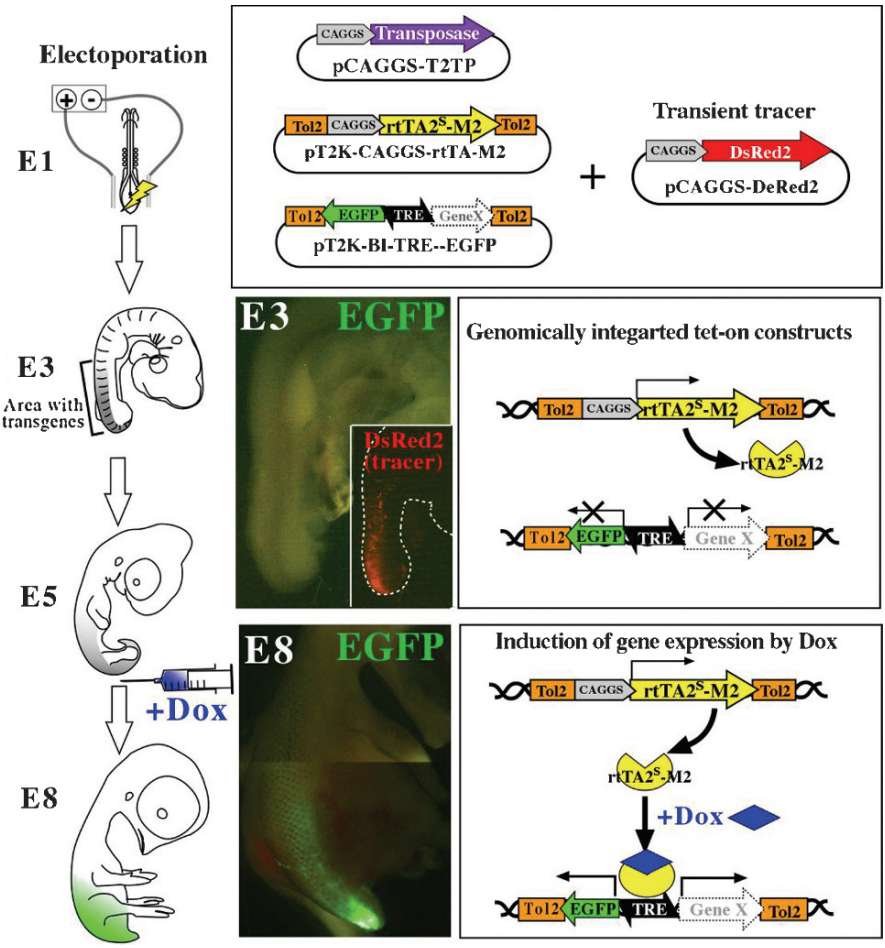
Electoporation
E1
E3
Area with transgenes
E5
+Dox
E8
CAGGS
Transposase
pCAGGS-T2TP
Tol2
CAGGS
rtTA2S-M2
Tol2
pT2K-CAGGS-rtTA-M2
Transient tracer
CAGGS
DsRed2
pCAGGS-DeRed2
Tol2
EGFP
TRE
GeneX
Tol2
pT2K-BI-TRE--EGFP
E3 EGFP
DsRed2 (tracer)
Genomically integarted tet-on constructs
Tol2 CAGGS rtTA2S-M2 Tol2
rtTA2S-M2
Tol2 EGFP TRE Gene X Tol2
E8 EGFP
Induction of gene expression by Dox
Tol2 CAGGS rtTA2S-M2 Tol2
rtTA2S-M2
+Dox
Tol2 EGFP TRE Gene X Tol2

Fig. 3.2 (p. 22)

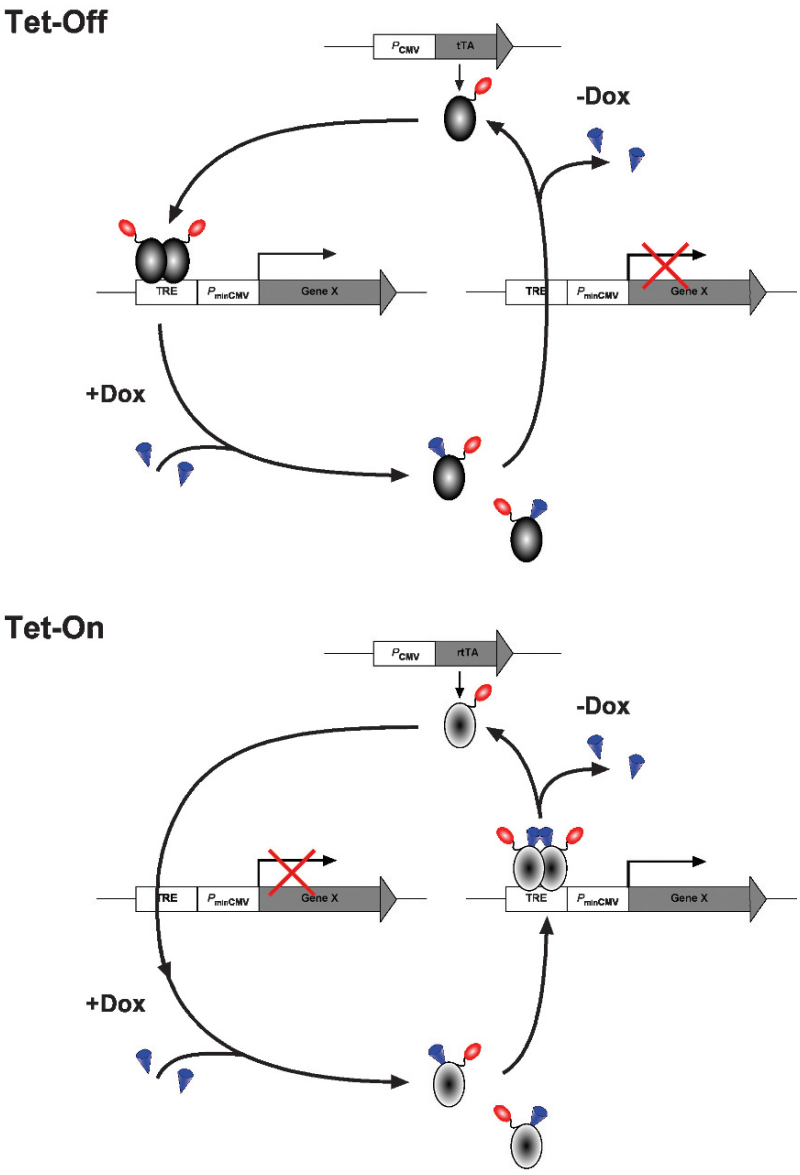
Tet-Off
P_{CMV}
tTA
-Dox
TRE
P_{minCMV}
Gene X
+Dox
Tet-On
P_{CMV}
rtTA
-Dox
TRE
P_{minCMV}
Gene X
+Dox

Fig. 4.1 (p. 27)

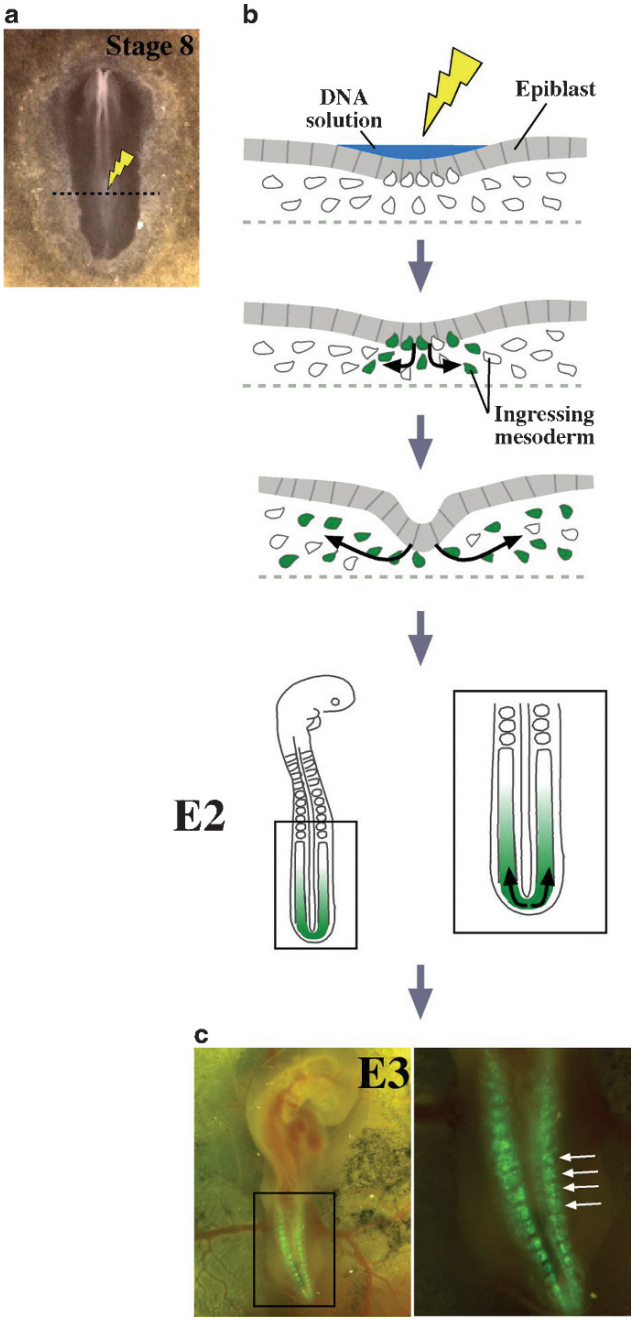
a
Stage 8
b
DNA
solution
Epiblast
Ingressing
mesoderm
E2
c
E3

Fig. 5.1 (p. 38)

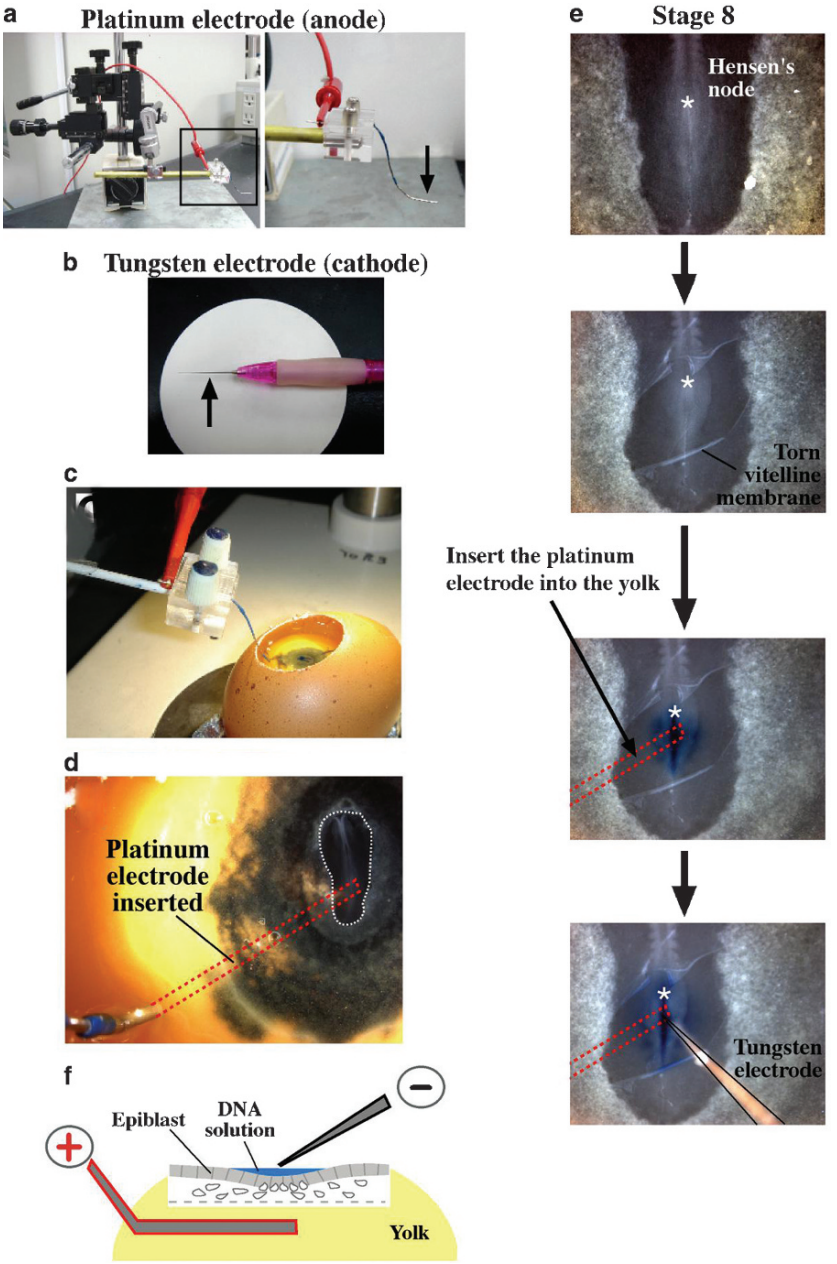
a
Platinum electrode (anode)
b
Tungsten electrode (cathode)
c
d
Platinum electrode inserted
f
Epiblast
DNA solution
Yolk
e
Stage 8
Hensen's node
Torn vitelline membrane
Insert the platinum electrode into the yolk
Tungsten electrode

Fig. 5.2 (p. 40)

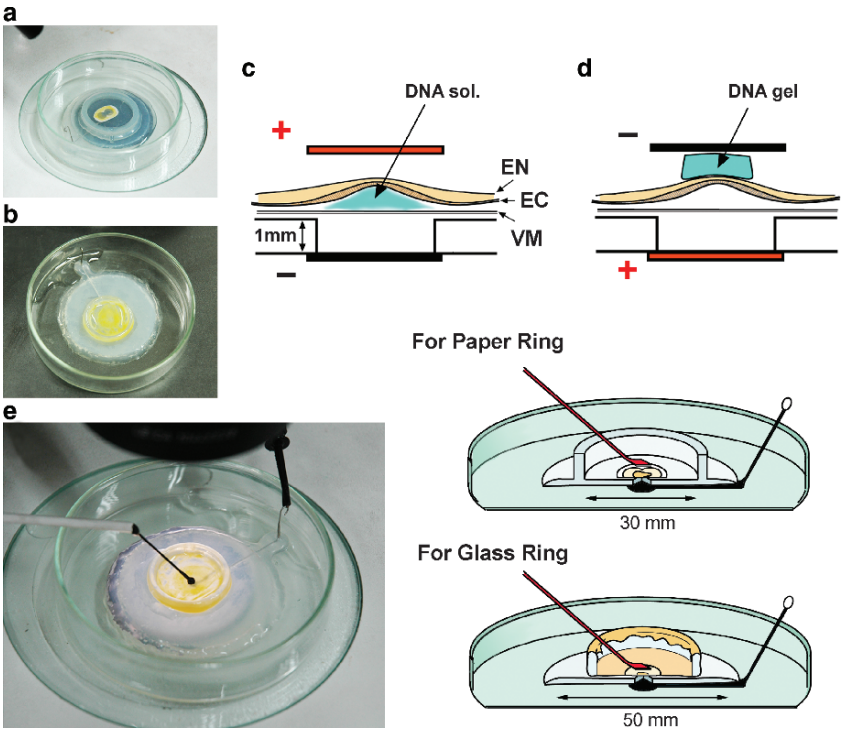
a
b
c
DNA sol.
+
EN
EC
1mm
VM
−
d
DNA gel
−
+
e
For Paper Ring
30 mm
For Glass Ring
50 mm

Fig. 6.4 (p. 50)

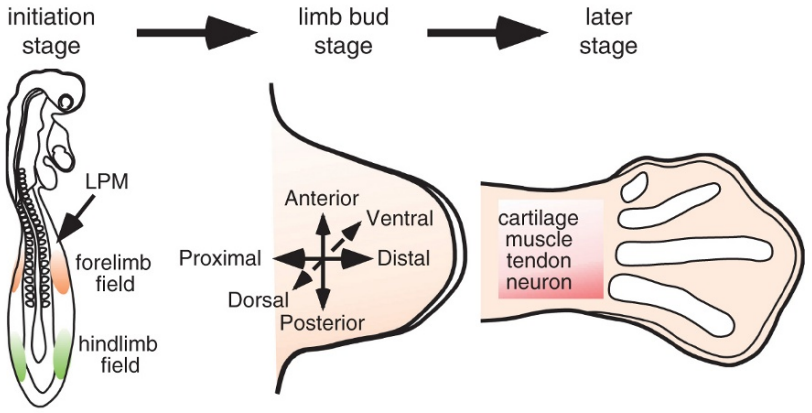

Fig. 9.1 (p. 86)

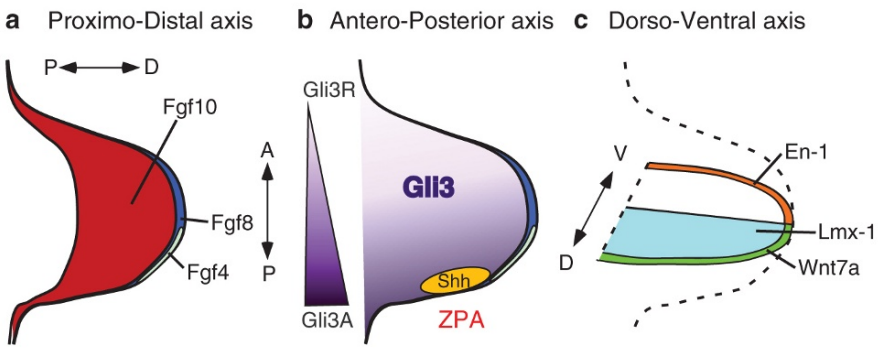

Fig. 9.2 (p. 87)

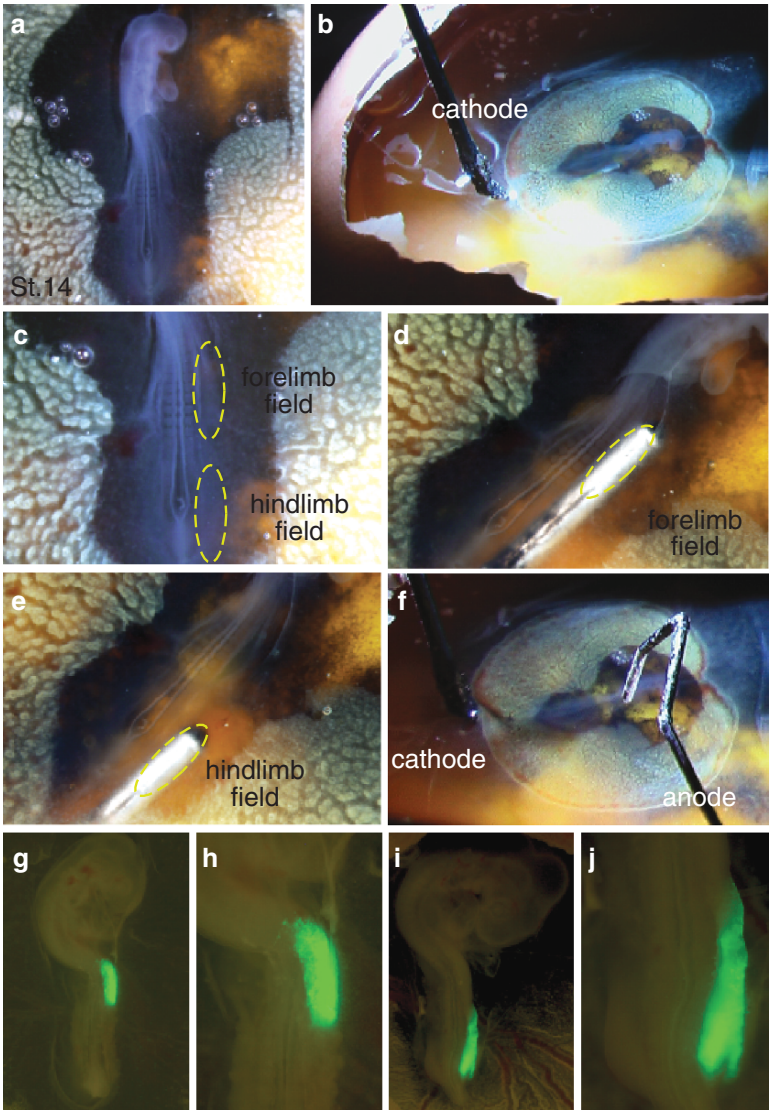
a
St. 14
b
cathode
c
forelimb field
hindlimb field
d
forelimb field
e
hindlimb field
f
cathode
anode
g
h
i
j

Fig. 9.3 (p. 89)

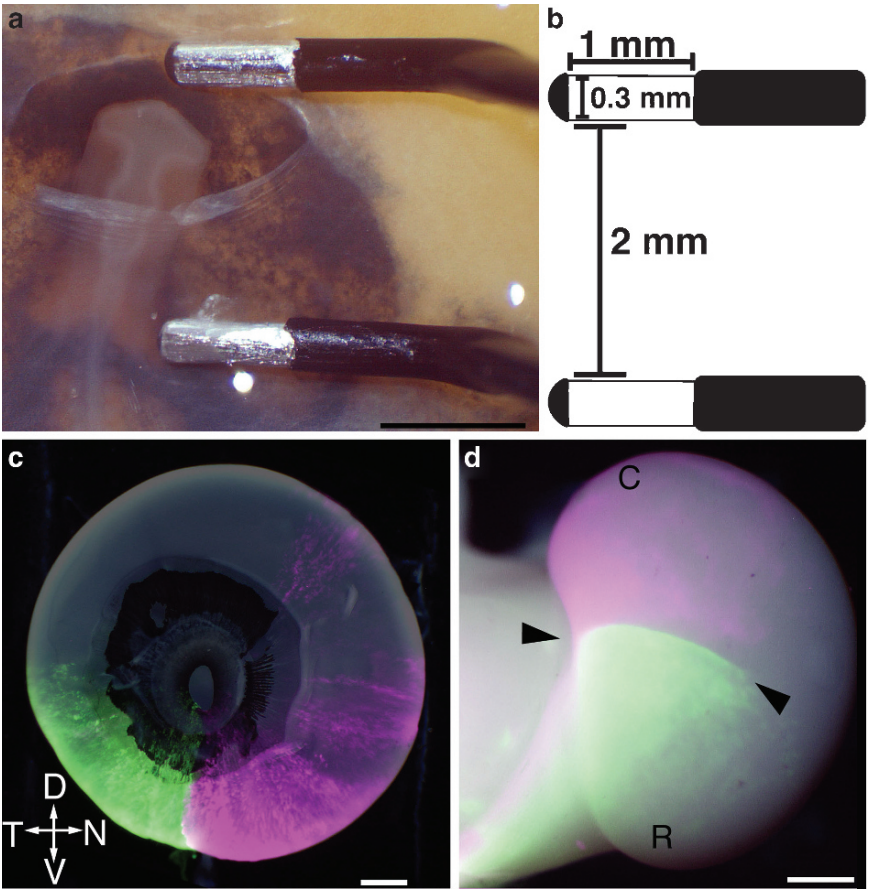

Fig. 10.1 (p. 98)

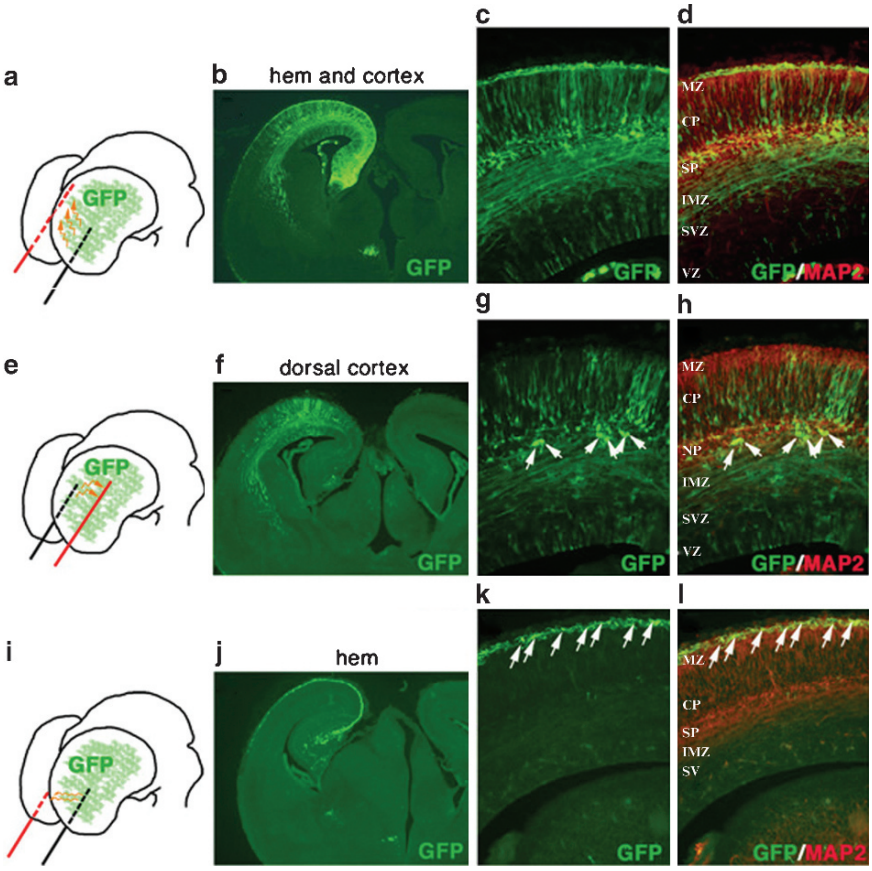
a
b
hem and cortex
c
d
GFP
GFP
MZ
CP
SP
IMZ
SVZ
VZ
GFP/MAP2
e
f
dorsal cortex
g
h
GFP
GFP
MZ
CP
NP
IMZ
SVZ
VZ
GFP/MAP2
i
j
hem
k
l
GFP
GFP
MZ
CP
SP
IMZ
SV
GFP/MAP2

Fig. 15.1 (p. 156)

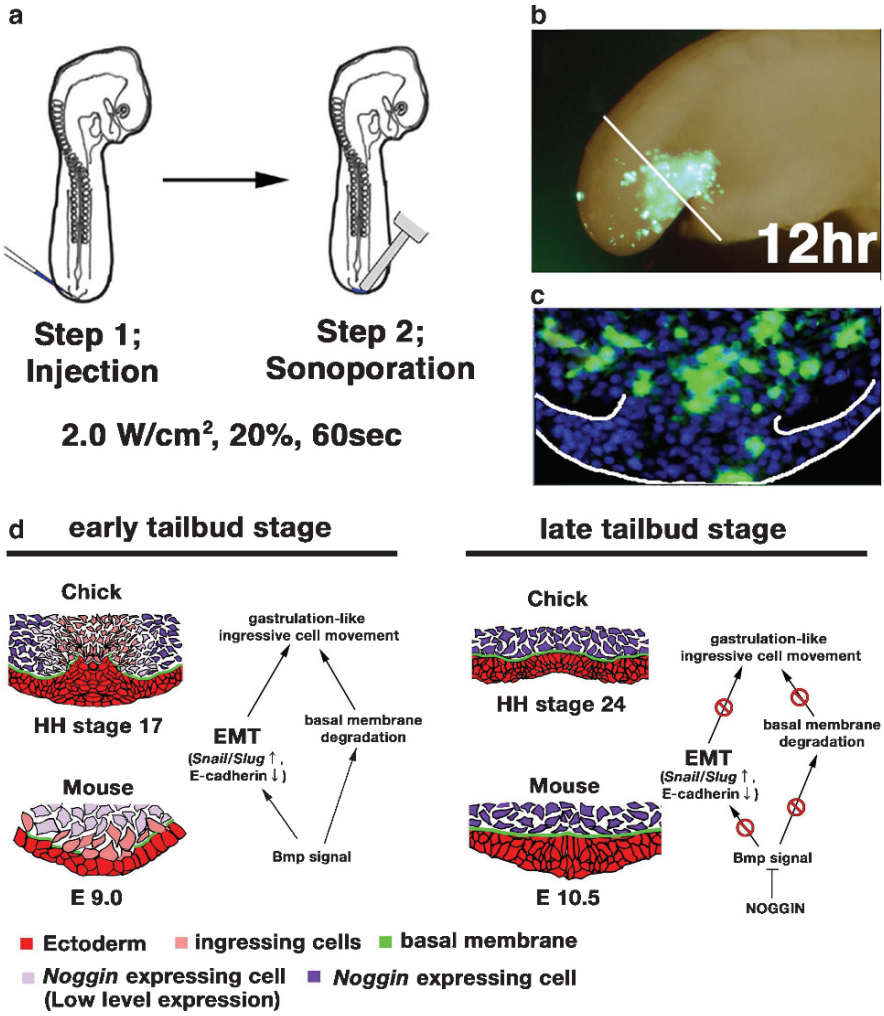
a
b
12hr
c
Step 1;
Injection
Step 2;
Sonoporation
2.0 W/cm², 20%, 60sec
d
early tailbud stage
late tailbud stage
Chick
HH stage 17
Mouse
E 9.0
gastrulation-like
ingressive cell movement
EMT
(Snail/Slug ↑,
E-cadherin ↓)
basal membrane
degradation
Bmp signal
Chick
HH stage 24
Mouse
E 10.5
gastrulation-like
ingressive cell movement
EMT
(Snail/Slug ↑,
E-cadherin ↓)
basal membrane
degradation
Bmp signal
NOGGIN
Ectoderm
ingressing cells
basal membrane
Noggin expressing cell
(Low level expression)
Noggin expressing cell

Fig. 27.3 (p. 323)

Part I
Electroporation

Chapter 1
Short History of Electroporation for the Study of Developmental Biology

Harukazu Nakamura

1 Trial

Electroporation at high voltage has been been applied for transformation of bacteria. Since it damages the tissue of higher organisms, it has been limited to bacteria transformation. Nevertheless, electroporation in higher organisms has been carried out for the transfer of the drugs for treatment of cancer; bleomycin was successfully applied to hepatocellular carcinoma in the Donryu rat (Okino and Mohri, 1987). Mr. Hayakawa (Nepa Gene; Ichikawa, Japan) supplied the electroporator and helped to optimize the condition of electroporation with the BTX electroporator, and Mr. Imada (Unique Medical-Imada, Natori, Japan) designed and prepared electrodes.

Suggested by successful drug delivery by electroporation, gene transfer by electroporation has been attempted by high voltage. First, transfection of *gus* gene (*beta-glucronidase*) by electroporation into plant material was carried out (Hayakawa, personal communication). Then, transfection of lacZ in brain tumor was tried. Electroporation at 300 V/0.5 cm was carried out after injection of expression vector into the carotid artery, and the expression of lacZ was limited to the tumor site, which received an electric charge (Nishi et al., 1996).

2 In Ovo Electroporation

At that time, high voltage, short pulse was applied. Since high voltage damages the tissue, mild conditions had been pursued by the late Dr. Muramatsu, Nagoya University, and Mr. Hayakawa, Nepa Gene, since 1994. They tried electroporation by low voltage, long square pulse, and found that five to eight applications of 20–100 V, 20–50 ms pulses successfully transfected adult chicken oviduct (Ochiai et al., 1998).

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They had also tried and successfully transferred genes to chick embryos in ovo; they presented a poster at the joint annual meeting of the Japanese Biochemical Society and the Molecular Biology Society of Japan in 1996, and published a paper in 1997 (Muramatsu et al., 1997).

I was shocked by such highly efficient transfection in somites, and decided to establish a stable and efficient misexpression of certain genes in ovo. Mr. Imada (Unique Medical Imada, Natori, Japan) helped us to find the best condition to transfect chick embryos in ovo. At first trial, all the embryos electroporated in ovo died, but an embryo that was transplanted into a neural tube that had been electroporated in vitro showed very high efficiency of transfection. To see such high transfection (lacZ expression), I was convinced of this method's success in applications for developmental biology. We came to the conclusion that the best condition for transfection to the mesencephalon of stage 10 embryos is five applications of rectangular pulse of 25 V with electrodes of 0.5 mm of diameter and 1.0-mm exposure (Funahashi, 1997; Funahashi et al., 1999; Nakamura et al., 2000) (now a milder condition is adopted: Odani et al., Chapter 2, this volume). Electrodes are placed on the vitelline membrane 4 mm apart. Mr. Imada prepared many types of electrodes according to our requirements. We were lucky because an effective expression vector, pMiw, had been developed by Kondoh's lab (Suemori et al., 1990). The method is so effective that we could study molecular mechanisms of brain regionalization and analyze mechanisms of isthmus organizing signal (Nakamura, 2001; Nakamura, et al., 2008).

3 Extension to Other Systems

This system was applied to chick embryos in New culture (Kobayashi et al., 2002; Hatakeyama and Shimamura, Chapter 6, this volume) and to many other tissues in chick embryos. Electroporation also has been applied to mouse embryos. It was first adopted to transfect mouse embryos in whole-mount culture (Osumi and Inoue, 2001; Nomura et al., Chapter 13, this volume), and then to trace cell lineages in the mouse brain (Tabata and Nakajima, 2001, Chapter 14, this volume; Saito and Nakatsuji, 2001). By in utero electroporation, transfection of mouse embryos was achieved in an area- and time-specific manner and was applied to the study of telencephalon development (Shimogori et al., 2004, Chapter 15, this volume).

4 Further Extensions

When it was shown that short hairpin RNA can interfere with the target RNA, we tried to knock down the target gene by electroporating shRNA-expressing vector (Katahira and Nakamura, 2003; Odani et al., Chapter 2, this volume). This technique allowed us to knock down the gene of interest at the desired place and time

in chick embryos. Now gain- and loss-of function experiments can be achieved conveniently in chick embryos; chick embryos have been revived as a model animal for the study of developmental biology. Now loss and gain of function of a gene of interest by electroporation has become a routine method in the study of developmental biology.

Recently two revolutionary methods have been developed. Introduction of the Tet-on/Tet-off system to an electroporation-mediated gene transfer system made it possible to control the timing of expression of transferred gene (Hilgers et al., 2005; Sato et al., 2007; Dubrulle and Pourquié, Chapter 4, this volume; Sato and Takahashi, Chapter 3, this volume). Application of transposon-mediated gene transfer by electroporation by Takahashi's group (Sato et al., 2007; Sato and Takahashi, Chapter 3, this volume) made it possible to assure long-term expression of transgene. Furthermore, a combination of Tet-on/Tet-off and a transposon system made it possible to regulate timing of long-term expression in chick embryos. Taking advantage of this, labeling of retinal fibers by electroporation at E2 was developed (Harada et al., 2008, Chapter 10, this volume).

We can electroporate RCAS provirus for long-term expression. We need not elaborate virus particles; we only have to electroporate provirus RCAS plasmid vector (Sakuta et al., Chapter 11, this volume). If we electroporate on virus-sensitive embryos, virus produced in the transfected cells infects adjacent cells and widespread misexpression could be obtained. On the other hand, if we electroporate on virus-resistant embryos, adjacent cells are not infected by virus, and the expression is limited to the descendents of the transfected cells (Sugiyama and Nakamura, 2003; Sugiyama and Nakamura, Chapter 12, this volume).

It is difficult to transfect mesenchymal cells by electroporation, so that transfection to the somites was not successful. Takahashi's group succeeded in transfection to somites by targeting the somite precursor in the primitive streak, which is in an epithelial structure (Ohata and Takahashi, Chapter 5, this volume). Ogura's group successfully transfected limb bud (Suzuki and Ogura, Chapter 9, this volume). Uchikawa et al. have been successful in analyzing enhancers in chicken embryos by electroporation (Uchikawa et al., 2003; Uchikawa, Chapter 7, this volume).

In this volume, electroporation to the mouse hematopoietic system (Kaushik et al., Chapter 18, this volume), to early mouse embryos (Soares and Torres-Paddila, Chapter 17, this volume), to cultured kidney (Haddad et al., Chapter 19, this volume), to neuronal cells in culture (Uesaka et al., Chapter 16, this volume), and to chick endoderm (Fukuda, Chapter 8, this volume) are introduced. Electroporation to the regenerating tissue also has been tried (Mochii et al., 2007; Chapter 21, this volume).

Apart from developmental biology, Prof. Miyazaki has been successfully carrying out gene transfer into adult muscles by electroporation in order to deliver cytokines, growth factors, and other serum factors for basic research and human gene therapy (Aihara and Miyazaki, 1998; Miyazaki and Miyazaki, Chapter 22, this volume; Kishimoto and Watanabe, Chapter 23, this volume). In plants, gene transfer to the seed has become a useful tool to generate transgenic plants (Hagio, Chapter 25, this volume).

5 Sonoporation

Gene transfer by electroporation is quite applicable to the epithelial tissue, but it is difficult to transfect mesenchymal cells by electroporation. Yamada paid attention to the fact that ultrasound exposure can increase cell membrane permeability, and developed sonoporation for gene transfer (Ohta et al., 2003; Ohta et al., Chapter 27, this volume). High ultrasound is necessary for cavitation, which causes temperature increase and damage to the tissue. The problem was conquered by introducing microbubbles, and the method was used as a drug delivery system (Tachibana and Tachibana, 2001). Yamada's group successfully applied this method to gene transfer in ovo chick embryos (Ohta et al., 2003, 2008).

Acknowlegdments We are greatly indebted to Nepa Gene, BTX, Bex and Unique Medical Imada, who have been cooperative in establishing electroporation and sonoporation. Because of the BTX electroporator, we could begin electroporation. After Bex constructed the CUY electroporator, we can control voltage strictly. Mr. Imada has cooperatively supplied electrodes that are suitable for targeting tissue.

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

Noritaka Odani, Xubin Hou, and Harukazu Nakamura

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,

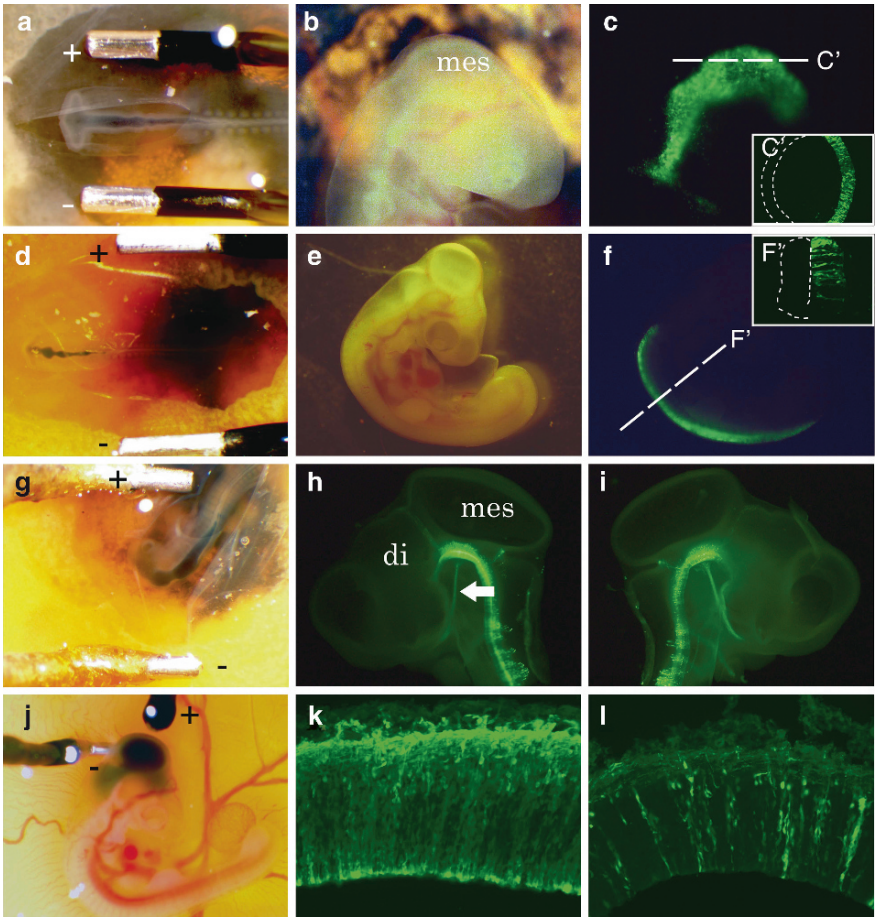

Fig. 2.1 In ovo electroporation. A pair of electrodes held by a manipulator are placed on the vitelline membrane at both sides of embryo (**a**, **d**, **g**). Most of the electrodes are insulated so that only the tip is exposed. For transfection into the mesencephalon at a later stage, a stick-type 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

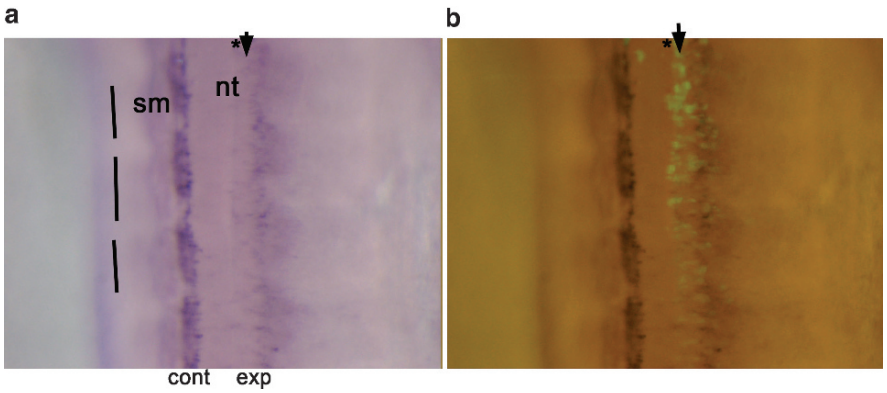

Fig. 2.2 Knock down of coactosin by siRNA. Coactosin-shRNA expression vector and GFP expression vector were electroporated in the neural tube of the trunk level at stage 10 and fixed 24 h later. The dorsal view of the transfected region of the embryo after whole mount in situ hybridization for coactosin (**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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Chapter 3
Applications of *Tol2* Transposon-Mediated Gene Transfer for Stable Integration and Conditional Expression of Electroporated Genes in Chicken Embryos

Yuki Sato and Yoshiko Takahashi

1 Introduction

Because of the high accessibility to developing embryos, avian embryos (chicken and quail) have long been used as a good model animal to study embryogenesis in vertebrates, especially amniotes (reviewed in Wolpert, 2004). The techniques used for "classical" avian embryology included tissue transplantations, tissue ablations, and cell-labeling by vital dye. At the end of the last century, the in ovo electroporation technique was developed by Nakamura and his colleagues, and this modern method opened a way to study the roles of developmental genes directly in living embryos (Funahashi et al., 1999) reviewed in (Nakamura et al., 2004; Yasuda et al., 2000; Yasugi and Nakamura, 2000). This powerful technique allows us to introduce genes (DNA, RNA, morpholino) into embryos in a tissue-specific way by targeting a restricted area of embryonic tissues. Thus, the electroporation technique using chickens has provided numerous novel insights into the understanding of early development in vertebrates, making the chicken a unique model animal.

One of few shortfalls of the original technique has been that expression of electroporated genes does not persist for a long period of time probably because the introduced plasmids, which are not integrated into the genome, degrade or become diluted as embryonic cells undergo massive proliferation. Although a spontaneous genomic integration of electroporated genes could occur, this incidence must be extremely low. Since in most cases the electroporation is performed at embryonic day 1~2 (E1~E2), the "short life" of introduced genes hampers the analysis of the effects by introduced genes at late stages, i.e., from E5 onward, when a variety of organogenesis proceeds. At these late stages, the electroporation is difficult to perform because the embryo is much less accessible.

To overcome this problem and obtain long-lasting expression of electroporated genes, we have recently developed a method of transposon-mediated gene transfer (Sato et al., 2007). Transposons are genetic elements that move from one locus in

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the genome to another. *Tol2* is an autonomous type of transposon, originally found in medaka fish (Koga et al., 1996), and encodes a transposase and transposable elements. Kawakami and his colleagues developed a method of Tol2-mediated gene transfer that enables efficient transgenesis in zebrafish (Kawakami et al., 1998). Since then, the Tol2-mediated transgenesis has also been used in other vertebrate species including frogs, mice, and human (Balciunas et al., 2006; Kawakami, 2004; Kawakami, 2005; Kawakami, 2007; Kawakami and Noda, 2004; Kawakami et al., 1998).

In this review, we first describe the method of the Tol2-mediated stable integration of electroporated genes with early somites as an example. Most of the explanations are with the constitutively active promoter, CAGGS (combination of CMV-IE enhancer and chicken β-actin promoter) (Momose et al., 1999; Niwa et al., 1991). Subsequently, we will show that the expression of stably integrated foreign genes can also be controlled in a stage-specific manner. This is enabled by the tetracycline-induced conditional expression system (tet-on), the method we recently optimized for chickens (Watanabe et al., 2007).

2 Procedure

2.1 Tol2-Meditated Gene Transposition in Chicken Embryo

A background of the Tol2 gene and the Tol2-mediated transgenesis in zebrafish embryo are described in (Kawakami, 2005; Kawakami, 2007). Briefly, when a DNA plasmid that contains a transposon construct carrying a gene expression cassette is introduced into vertebrate cells with the transposase activity, the transposon construct is excised from the plasmid, and the cassette is subsequently integrated into the host genome (Kawakami, 2005) (see also Fig. 3.1a). We here explain the Tol2-meditated stable transgenesis with an example of EGFP and DsRed2 (Clontech).

2.1.1 Plasmids

Two kinds of plasmid vectors are required for the stable integration: pCAGGS-T2TP (T2TP: Tol2 transposase) and pT2K-CAGGS-EGFP (pT2K: a plasmid of transposon construct carrying transposable elements). pCAGGS-DsRed2, which does not contain Tol2-construct, is used as a reference. The above-mentioned three kinds of plasmids, 5 μg/μl for each, are mixed to prepare a DNA cocktail with the following ratio, pCAGGS-T2TP : pT2K-CAGGS-EGFP : pCAGGS-DsRed2 = 1 : 2 : 0.5. The cocktail is then added with Fast green (2% final) prior to the electroporation. Devices and settings for in ovo electroporation should be optimized for a targeting tissue as described elsewhere (Fukuda et al., 2000; Nakamura et al., 2000; Nakamura et al., 2004; Yasuda et al., 2000; Ohata and Takahashi, Chapter 5 in this volume).

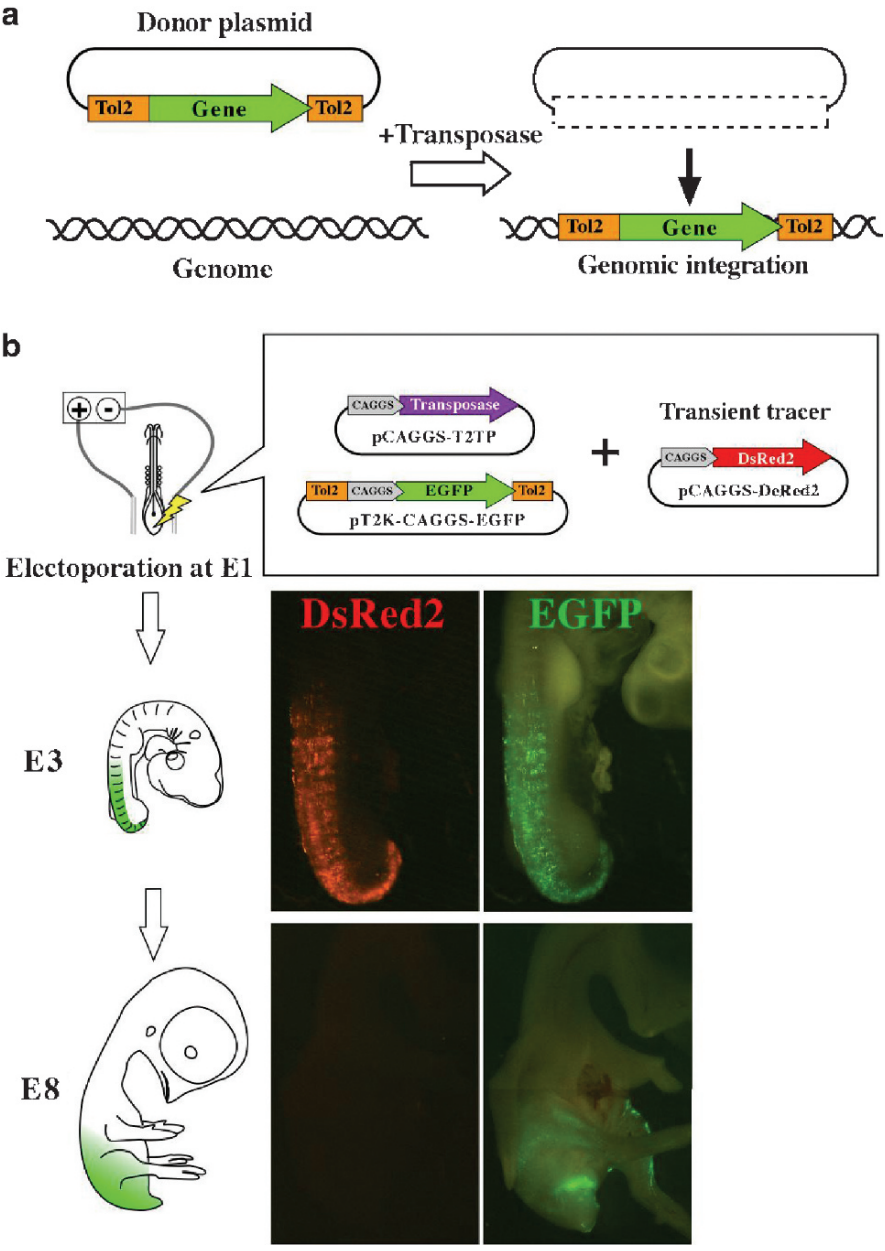

Fig. 3.1 Integration of foreign DNA into the chromosome by Tol2-mediated transposition in chicken embryos. (**a**) When Tol2 transposase is present, a gene cassette (*green*) flanked by the Tol2 transposable elements (*orange*) is excised from a donor plasmid, and becomes integrated into the genome of a host cell. (**b**) Persistent expression of electroporated genes in developing embryos. pCAGGS-T2TP and pT2K-CAGGS-EGFP are co-electroporated along with a transient tracer pCAGGS-DsRed2 into the prospective somitic cells of E1 (HH 8) embryo. Although both DsRed2 and EGFP signals are seen until E3 embryo, DeRed2 signal disappears afterward whereas EGFP signal persists until late stages (*See Color Plates*)

2.1.2 Persistent Expression of pT2K-CAGGS-EGFP-Derived Signals Until Late Stages

To achieve a successful transgenesis in presomitic mesoderm (PSM)/somites, foreign genes are introduced by electroporation into their precursors at earlier stages, i.e., Hamburger and Hamilton stage 8 (HH8), when presumptive somites reside in the anterior end of the primitive streak. The details about the somitic electroporation are described by Ohata and Takahashi, Chapter 5 in this volume (see also Sato et al., 2002). Expression of a gene driven by the CAGGS-promoter, in this case DsRed2, starts to be detected approximately 3 h after the electroporation (Watanabe et al., 2007), and the signals usually disappear by the end of E4 (Sato et al., 2007). In contrast to pCAGGS-DsRed2, EGFP signals generated by pT2K-CAGGS-EGFP are retained for a prolonged period of time because of the Tol2-mediated genomic transposition (Fig. 3.1b) (Sato et al., 2007). The stable expression of EGFP indeed requires the activity of Tol2-transposase because electroporation with pT2K-CAGGS-EGFP without pCAGGS-T2TP did not yield the retained expression. Persistent expression of EGFP in somite-derived tissues was observed until E8, the latest stage examined in our hands. In addition, in vitro analyses using DF1 cells of a chicken fibroblast cell line showed that ~40% of transfected cells retained EGFP signals permanently when transfected with pT2K-CAGGS-EGFP and pCAGGS-T2TP (Sato et al., 2007; Y.T., unpublished data). Therefore, it is expected that the EGFP-positive cells found in the E8 embryo would last much longer until these cells die. For the electroporated embryos to develop in a good condition until late stages, it is recommended that antibiotics (penicillin/streptomycin) are added in HANKS solution to prevent bacterial contaminations.

2.2 Conditional Expression of Stably Integrated Transgenes

It is known that some of developmental genes are repeatedly used during early morphogenesis and organogenesis. Therefore, the technology would be useful that controls stage-specific expression of foreign genes introduced into embryos. The tet-on inducible expression system has widely been used, mainly in vitro, for conditional manipulation of foreign genes. We recently optimized the tet-on expression method for chicken embryology (Watanabe et al., 2007). We have therefore developed a vector set to combine the tet-on method with the Tol2-mediated stable integration so that we can control the onset of expression of electroporated genes at late stages (~E5 onward) (Sato et al., 2007).

2.2.1 Tet-on Inducible Expression

Materials required for the tet-on induction are: the tet-dependent transcriptional activator rtTA2^{S}-M2 (Urlinger et al., 2000), the tet-responsive element (TRE),

and the tetracycline-related drug, doxycycline (Dox, Clontech). rtTA2^S-M2 activates TRE to turn on the transcription of its driving gene only when rtTA2^S-M2 is bound with Dox. Thus, by timing the Dox administration into embryos, one can control the onset of expression of a TRE-driven gene (details described in Watanabe et al., 2007). rtTA2^S-M2 is now commercially available as Tet-on Advanced, Clontech.

2.2.2 Plasmid Preparation for Tol2-tet-on Expression System

Three kinds of plasmids are required. The first one is pT2K-CAGGS-rtTA2^S-M2. The second is pT2K-BI-TRE-EGFP. "BI-TRE" consists of bidirectionally-acting minimal promoters (BI) downstream of TRE. In this article, we explain our methods with a pT2K-BI-TRE-EGFP, which has a vacant slot for the opposite direction. This vector was modified from pBI-EGFP, Clontech. The third plasmid is pCAGGS-T2TP. pCAGGS-DsRed2 is also recommended to ensure a successful electroporation into the embryo because EGFP signal is silent until Dox administration (Fig. 3.2).

These plasmids, 5 μg/μl for each, are mixed to prepare a DNA cocktail with the following ratio, pCAGGS-T2TP : pT2K-CAGGS-rtTA2^S-M2 : pT2K-BI-TRE-EGFP : pCAGGS-DsRed2 = 2 : 1 : 2: 0.5. The cocktail is then added with Fast green (2% final) prior to the electroporation. A Dox solution is prepared at 0.1 mg/ml in HANKS.

2.2.3 Dox-Induced Expression of Stably Integrated Genes

The set of above-mentioned plasmids is electroporated into the presumptive somitic region of embryo at HH 8. Several hours after the electroporation, embryos with successful transgenesis are selected by the signals of DsRed2 (with EGFP signal being silent). To induce EGFP expression, 500 μl of Dox solution is administered into an egg shell using a syringe needle in between the embryo and yolk. The penetration of Dox into embryonic cells may vary from tissue to tissue. In the case of early somites (E2), TRE-driven EGFP starts to be expressed ~3 h post-Dox (Watanabe et al., 2007). For further developed tissues derived from somites of E5 embryos, one shot of Dox administration could start and maintain EGFP signals for a few days. However, from E10 onward, it is recommended that a Dox solution is administered every day with one shot per day because Dox appears to be quickly metabolized in developed embryos (Tanabe et al., 2006).

The Tol2-tet-on-inducible system has been successful for conditional expression of electroporated genes, including the dominant-negative form of N-cadherin in developing neural retina until E12 of chicken (Tanabe et al., 2006), and the constitutively active form of Notch in developing somites of chicken and quail until E6 (Sato et al., 2008).

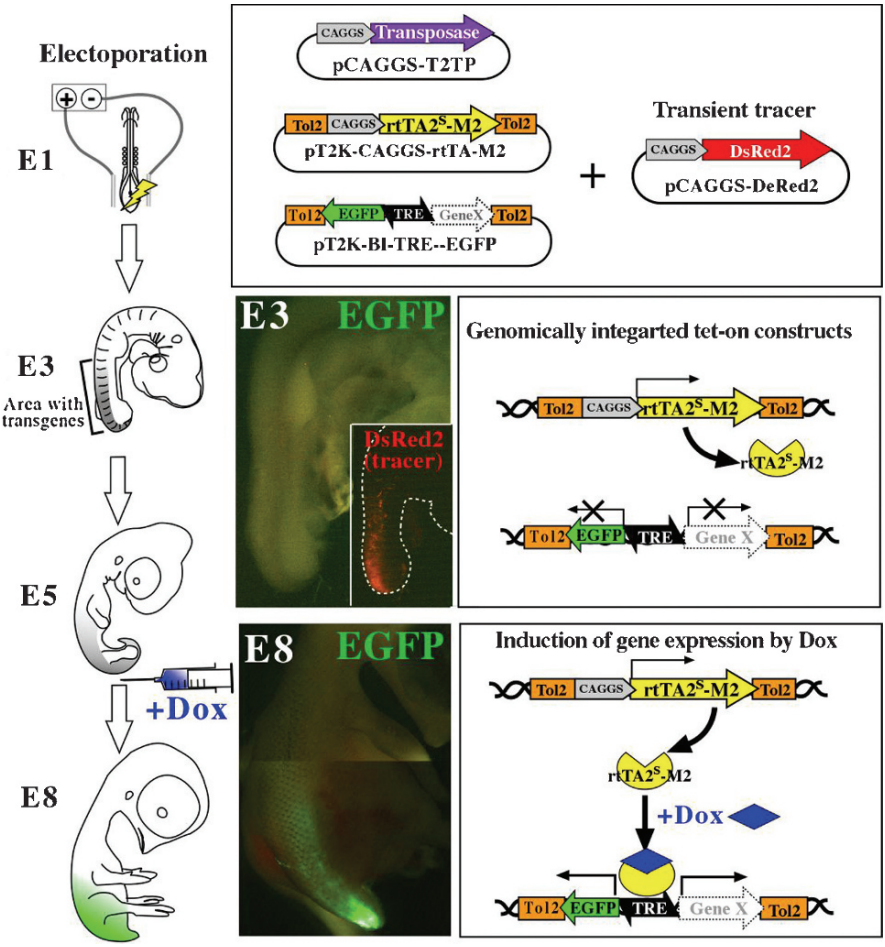

Fig. 3.2 Conditional expression of integrated genes using the tet-on inducible expression system. Three plasmids, pCAGGS-T2TP, pT2K-CAGGS-rtTA2^S-M2, and pT2K-BI-TRE-EGFP, are co-electroporated along with a transient tracer pCAGGS-DsRed2 into the presumptive somite. Even without Dox, early embryos with successful transgenesis can be seen by DsRed2 signals (inset in the image of E3). With a Dox injection at E5, these embryos start to express the genomically integrated EGFP gene in somite-derived tissues. See text for details (*See Color Plates*)

3 Comments

3.1 Future Perspectives

The gene integration method by the Tol2-transposition must be useful in a variety of experimental approaches. These include functional RNAi-knockdown of endogenous gene by short hair-pin type of RNA (shRNA). The shRNA-mediated

knockdown approaches have been attempted for many developmental genes, and some but not all of these are successful, i. e., *Pax2* (Watanabe et al., 2007) and *Ephrin B2* (Sato et al., 2008). Thus, if a shRNA-encoding cassette is manufactured for the use of Tol2-transposition system, a persistent knockdown of gene function could be possible. In addition, by combining with the tet-on inducible expression, conditional knockdown of developmental genes at later stages should be also available.

The stable integration of foreign genes must be also useful if combined with a tissue- or cell type-specific enhancer. Kondoh's group has recently described an efficient *in silico* screening of non-coding DNA elements that are conserved between amniotes. Many of these elements serve as tissue-specific enhancers (Uchikawa et al., 2004). It would be plausible if tissue- or cell type-specific labeling/gene manipulation could be performed for a prolonged period of time until late stages of embryogenesis. Such manipulations using chicken embryos should be much less time-consuming with low cost compared with manipulations in mice. Last, the stable integration method we have described here is expected to be useful for generating transgenic birds, a technology that has not been very successful until present.

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Chapter 4
Temporal Control of Gene Expression by Combining Electroporation and the Tetracycline Inducible Systems in Vertebrate Embryos

Julien Dubrulle and Olivier Pourquié

1 Introduction

The electroporation technique has revolutionized vertebrate embryology. It has greatly contributed to our understanding of how genes and proteins can interact and regulate various aspects of vertebrate development in the last decade. This technique provides an efficient way to transfect embryonic cells in vivo with exogenous DNA by creating transient holes in the plasma membrane with short, squared electric pulses of low voltage (Itasaki et al., 1999; Momose et al., 1999; Muramatsu et al., 1997; Nakamura et al., 2004; Ogura, 2002). It has been particularly well-developed in the chick model since the large size of the embryo and its easy accessibility enables to target specific tissues with great precision. With the electroporation, it is possible to precisely choose which type of cells to transfect by performing a local injection of DNA close to the cells of interest, followed by the application of a small current through the targeted area. To date, all three germ layers – endoderm, mesoderm and ectoderm – as well as an increasing number of differentiated structures have been efficiently transfected (Dubrulle et al., 2001; Grapin-Botton et al., 2001; Itasaki et al., 1999; Luo and Redies, 2005; Scaal et al., 2004) and the continuous improvement in electrode design makes it even possible to aim at sub-populations of cells within a given tissue. In addition to this spatial precision, the technique also allows great temporal precision; any stage of development, ranging from pre-gastrulation stage to adulthood can be reached as long as the cells or structures are accessible for local DNA injection and electrode placement (Bigey et al., 2002; Iimura and Pourquie, 2006).

A drawback of this technique is that such electroporations lead to a sustained overexpression of the transgene until the DNA gets diluted out or degraded, which can take 2 days or even more, depending on the rate of cell proliferation of the targeted

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tissue. This prolonged overexpression may have cumulative effects that can obliterate the primary role of the studied molecule in a given process at the time the phenotype is being assessed. This is especially worrisome, since, during early development, signaling molecules and growth factors are usually transiently expressed and act during short time windows. In addition, signaling molecules or transcription factors are often used repeatedly at multiple steps during morphogenetic events. Artificially prolonged exposure as a result of electroporation may blur their role during a given step or lead to aberrant cellular behaviors. There is therefore definitely a need to control the timing of expression of the transgene in the embryo. Several strategies have been developed over the past decades to control gene expression in time and space. Most of these strategies are based on genetic tools developed in fly and mouse, like the UAS-Gal4 system or the Cre-Lox induced recombination system. However, because manipulating the avian genome is yet unrealistic, those powerful strategies cannot be applied. Alternative inducible systems, based on prokaryotes operons, are available to control gene expression in time without genetic manipulation, and the most versatile one is probably the tetracycline inducible system (Tet-Off and Tet-On) developed by Gossen et al. more than 15 years ago (Gossen and Bujard, 1992). This chapter describes how the tetracycline inducible systems can be applied to avian embryos using the electroporation technique and what kind of applications one can expect from them.

2 Principle

The Tet system is derived from the E. Coli Tn10 tetracycline-resistant operon. In these bacteria, the tet repressor protein (tetR) binds to tet operator sequences (tetO) located in the promoter region of the operon and prevents transcription of the *tetA* gene, which encodes a membrane channel that pumps tetracycline molecules out of the cell. Tetracycline molecules directly interact with the tet repressor protein and presumably trigger a conformational change so that the repressor can no longer bind to the DNA, therefore allowing transcription of the operon (Hillen and Berens, 1994). The tet regulatory system has been engineered for application in eukaryotic cells and has given rise to two potent, very specific inducible systems, the Tet-Off and Tet-On systems (Gossen and Bujard, 1992; Gossen et al., 1995). Both of these systems rely on two components, a tetracycline inducible transactivator, either tTA or rtTA, encoded by the pTet-Off or pTet-On plasmid, respectively, and a second plasmid containing a tetracycline response element (TRE) upstream of a gene of interest (Fig. 4.1). The TRE consists of seven repeats of a 42 bp sequence of the tetO, upstream of a minimal promoter. This minimal promoter alone cannot drive any expression.

In the Tet-Off system, the tetR protein has been fused to the VP16 activation domain (this fusion protein being called tTA for tetracycline Transactivator), converting the strong tetR repressor into a potent activator (Gossen and Bujard, 1992). The tTA constitutively binds to the TRE and therefore, due to the VP16 activation domain, activates the transcription of the downstream gene. Upon addition of tetracycline or its derivative doxycycline, the antibiotic binds to the transactivator and provokes a conformational change that revokes binding to the TRE and therefore silences the transgene (Fig. 4.1).

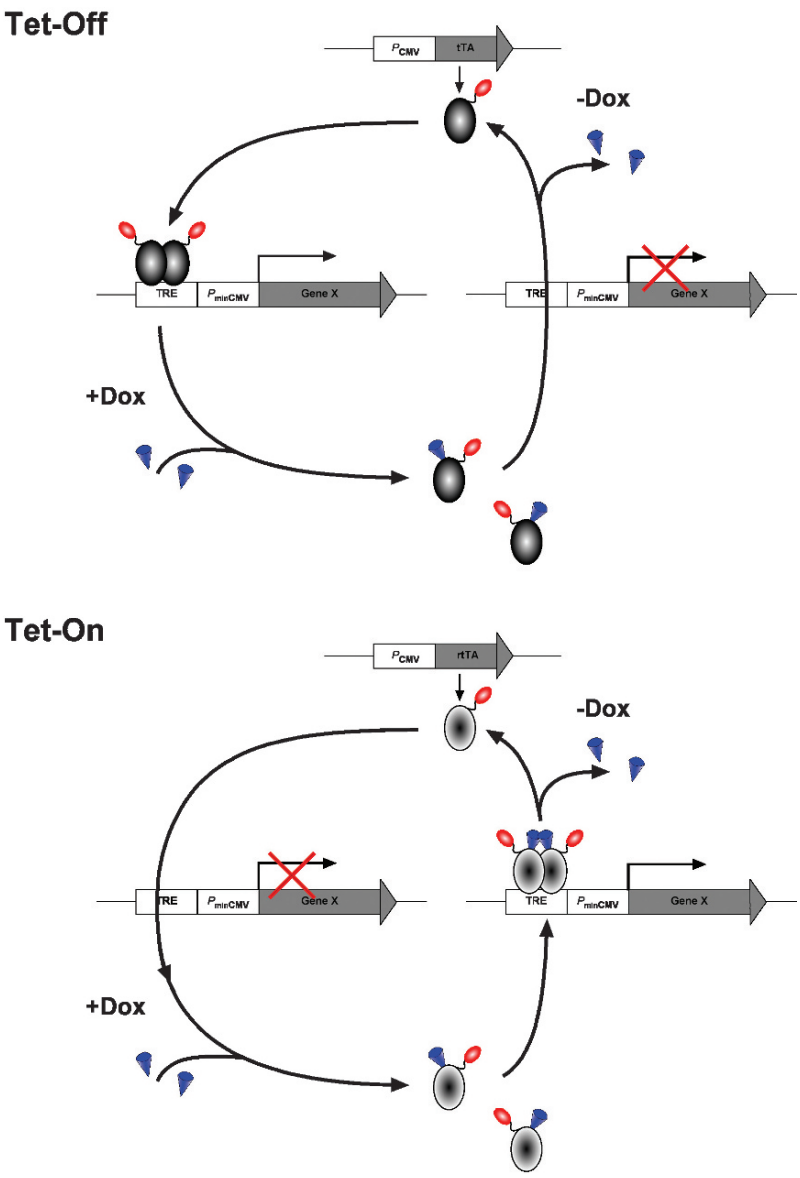

Fig. 4.1 Principle of the tetracycline inducible systems. *Top panel*, the Tet-Off system. In this system, the tTA transactivator is produced by the pTet-Off plasmid under the control of a strong ubiquitous promoter. The tTA binds to the TRE regulatory sequences located upstream of a minimal promoter in the pTRE vector and activates the transcription of the downstream gene. Upon addition of doxycycline, the tTA can no longer interact with TRE sequences, leading to an arrest of transcription. *Bottom panel,* the Tet-On system. The rtTA produced by the pTet-On plasmid is unable to bind the TRE sequences in absence of doxycycline and therefore the transgene is kept silent. After addition of the drug, the rtTA now strongly binds to the TRE sequences, allowing transcription of the transgene (*adapted from "The Tet-Off and Tet-On gene expression user manual", CLONTECH*) (*See Color Plates*)

The Tet-On system has been developed so that instead of repressing transcription like in the Tet-Off system, addition of tetracycline will turn on the transcription of the transgene (Fig. 4.1). To do so, Gossen et al. performed random mutagenesis on the original tetR protein and screened for tetracycline dependence for repression (Gossen et al., 1995). One mutant with four amino acid substitutions shows strong dependence to tetracycline for DNA binding (the opposite of the regular tetR protein). They then translated these mutations to the tTA transactivator to generate the so-called reverse tTA (rtTA), because it exhibits the reverse behavior of tTA: rtTA can bind to the TRE and activate downstream genes only in the presence of tetracycline or its derivatives (Fig. 4.1). In summary, addition of tetracycline blocks transcription in the Tet-Off system, while it activates transcription in the Tet-On system.

These systems have been extensively used in cell culture and stable cell lines containing either the tTA or rtTA regulatory protein have been established. Expression of the pTRE plasmid with the gene of interest in these lines makes this gene susceptible to tetracycline regulation. This system has greatly contributed to the biochemical study of proteins that may become toxic to cells at high levels by reducing the time they are expressed at will. This system has also been extended to transgenic organisms, where the tTA or rtTA regulatory proteins are expressed in specific tissues or cell types using ad hoc promoters. Crossed to transgenic animals expressing the pTRE-Gene X construct, either ubiquitously or under specific promoters, this allows a precise temporal control of gene expression in a given tissue, by simple addition of antibiotics in drinking water or food (Furth et al., 1994). Many tTA or rtTA mouse lines have now been established that express these regulatory proteins in a wide range of tissues, like brain (Mansuy et al., 1998), pancreas (Holland et al., 2002), epidermis (Diamond et al., 2000) and so on. The electroporation technique now makes these systems available to organisms where genome manipulations are very limited, like the chicken embryo.

3 Procedure

3.1 Plasmids

The pTet-Off, pTet-On and pTRE vectors are licensed by CLONTECH Laboratories, Inc and were originally created in the Bujard lab (Gossen and Bujard, 1992; Gossen et al., 1995). The pTRE vector contains a multiple cloning site downstream of the Tetracycline Response Elements to clone the gene of interest. Plasmid DNA is extracted using an Endotoxin Free Qiagen kit.

3.2 Electroporation

Electroporations are performed using established procedures. For an extensive description, the reader can refer to the different chapters of this book. In the case of studying mRNA stability in the neural tube, electroporations are performed as follow:

A window is opened in the shell of 2-day-old embryos (12 to 18 somites). After tearing out the chorion, a mixture of plasmid DNA (pTet-Off and pTRE-reporter, 1:1 ratio, 1 ug/ul each), 0.5% Fast Green in PBS is injected into the lumen of the neural tube. The embryo is then covered with a solution of PBS/Penicillin/Streptamycin and electrodes are placed on each side of the neural tube. Four pulses of current (16 V, 50 ms, 100 ms intervals) are applied using a CUY21 square electroporator (Nepa Gene). Embryos were resealed using transparent tape and reincubated overnight at 38°C in a humidified incubator.

3.3 Doxycycline Treatment

In ovo injection: a stock of 1,000X solution of doxycycline is diluted to 1X for a final concentration of 9 µg/ml in PBS/indian ink 5%. This solution is injected by mouth pipetting in the subblastodermal cavity with a bent, flame-stretched glass Pasteur pipette. Usually, between 100 and 200 µl is injected. The black ink enables to monitor the precision of the doxycycline solution delivery. Eggs are once again resealed and reincubated for the desired period of time.

3.4 In Vitro Culture

Doxycycline can also be delivered using an in vitro embryo culture. This strategy is particularly useful when one wants to combine the Tet systems with realtime imaging. For in vitro culture, we used albumen/agarose plate (50% thin albumen, 0.3% agarose, 66 mM NaCl, 0.15% sucrose). Sucrose, agarose and NaCl are first mixed in ddH2O and the solution is boiled to dissolve the agarose. This solution is then kept at 50°C during the time the thin albumen is collected. The albumen and the agarose solution are then mixed to a 1:1 ratio (V/V) and then doxycycline is added to a final concentration of 9 µg/ml. The solution is then poured into 35 × 10 mm Petri dishes and the agar plates are stored at 4°C for at least a week.

Three-day-old Tet-electroporated embryos are dissected in PBS along with their surrounding, intact extra-embryonic tissue. This extraembryonic tissue will allow a normal growth of the embryo on the albumen/agarose plate and it should absolutely be kept intact. The embryo is transferred to the albumen/agarose plate, dorsal side up. With the help of a plastic Pasteur pipette and forceps, the extraembryonic tissue is gently spread over the plate, and tissue folding or shearing will be avoided. Extra PBS is removed and in vitro cultured embryos are then reincubated in a regular humidified incubator. In these conditions, embryos can be cultured for 24–36 h (up to day 4).

4 Application and Results

This section will discuss some examples of applications using either the pTet-Off or pTet-On systems combined with electroporation in chicken embryos by summarizing three recent publications (Hilgers et al., 2005; Morin et al., 2007; Watanabe

et al., 2007). Along with the work of Sato and colleagues, who used the Tet-On system driven by retrovirus infection to conditionally express GFP (Sato et al., 2002), these are the first papers, to the best of our knowledge, that use tetracycline inducible systems to perform functional studies in the chicken embryo. This section will also highlight the numerous modifications that can be introduced into these systems to generate sophisticated regulatory strategies.

In the first paper, we used the pTet-Off system to analyze the turnover rate of different mRNA species in the developing embryo (Hilgers et al., 2005). We used the neural tube as a reference tissue because it is easy to electroporate, with high reproducibility and low lethality. The strategy consists of electroporating a cocktail of 2 plasmids – the pTet-Off plasmid encoding the tTA protein and the pTRE containing a reporter gene upstream of different 3′ untranslated regions (3′UTR), since mRNA stability is known to be mainly dictated by consensus sequences located in the 3′UTR of the transcript (Garneau et al., 2007). Once the system has reached a steady state, doxycycline is applied to the embryo to block tTA-dependent transcription. The dynamics of mRNA decay is then monitored either directly by in situ hybridization or indirectly by following the reporter activity over time.

Two mRNA species have been studied, *Fgf8* and *Lunatic Fringe*. Previous studies suggested that these two mRNA have different half lives: *Fgf8* was thought to be very stable (Dubrulle and Pourquie, 2004b) while *Lunatic Fringe*, a gene expressed cyclically in the presomitic mesoderm, was believed to be very unstable (Aulehla and Johnson, 1999; McGrew et al., 1998). To test these assumptions, the coding sequence of the unstable GFP reporter gene containing either the 3′UTRof *Fgf8* or *Lunatic fringe* was cloned in the pTRE vector. Each construct was co-electroporated with the pTet-Off plasmid in the neural tube. In these conditions, the tTA protein is efficiently transcribed and translated and transactivates genes downstream of the TRE sequences. After overnight incubation, electroporated embryos were transferred to an agar plate supplemented with doxycycline and the GFP intensity was monitored by epifluorescence microscopy and recorded in every single embryo over time. Because addition of doxycycline blocks tTA-dependent transcription, the decay of GFP intensity is directly function of the speed at which mRNAs are cleared off. It was found that 50% of the original GFP intensity was lost in 5 h in constructs bearing the 3′UTR of *Lunatic Fringe* and in 10 h with the 3′UTR of *Fgf8*. These results support the idea that *Lunatic Fringe* mRNA is very labile, which is expected for a "cyclic" gene and that that of *Fgf8* is very stable. These findings have been corroborated by looking directly at the expression of these transgenes by in situ hybridization. While the amount of mRNA seems globally to be the same with both of these constructs 24 h after electroporation, no mRNA containing the 3′UTR of *Lunatic Fringe* can be detected in the neural tube 1 h after addition of doxycycline. In contrast, mRNA containing the 3′UTR of *Fgf8* can still be detected up to 6 h after addition of the antibiotic in cells of the neural tube (Hilgers et al., 2005).

Another recent study also took advantage of the Tet-Off system combined to electroporation. Morin and coworkers looked at the role of LGN, a G-protein regulator, during the development of the neural tube (Morin et al., 2007). They found that this protein is involved in controlling spindle movements and orientation and regulates planar division of neural progenitors located in the ventricular zone.

By overexpressing a dominant-negative form of LGN (CtLGN) in the neural tube, the plan of division of the progenitors is no longer regulated and some daughter progenitor cells leave the ventricular zone and colonize the mantle zone. There, they undergo multiple rounds of cell division, leading to an aberrant hyperproliferation in normally unproliferative regions. To show that the proliferation of these mislocated progenitors is not due to continuous blocking of LGN, they transiently expressed CtLGN by the Tet-Off system. They allow expression to occur for 24 h and then blocked the production of CtLGN by injected a doxycycline solution in the egg. When they analyzed proliferation 3 to 4 day after doxycycline injection, they still found an overproliferation in the mantle region of the electroporated side, suggesting that the proliferation of these mislocated progenitors is not due to sustained expression of CtLGN (Morin et al., 2007).

The second approach used the Tet-On system to study the role of genes involved in somitogenesis (Watanabe et al., 2007). Somites form periodically from the anterior tip of the presomitic mesoderm (PSM), which originates from mesoderm precursor cells located in the anterior part of the primitive streak (Dubrulle and Pourquie, 2004a). Overexpressing constructs in the PSM is usually done by electroporating the anterior part of the epithelial primitive streak. This strategy is powerful since it targets the precursors of the paraxial mesoderm, leading to a sustained mesodermal overexpression all along the AP axis of the embryo. However, some overexpressed genes may directly play a role or interfere with the ingression of mesodermal cells out of the primitive streak, thus making the analysis of their function later during somitogenesis impossible. This is the case for genes like *c-meso-1* or *Pax2*, which are normally expressed in the anterior region of the PSM (Buchberger et al., 1998; Suetsugu et al., 2002). Primitive streak cells expressing either of these genes fail to migrate properly and get stuck in the tailbud. To circumvent this issue, Watanabe et al. took advantage of the Tet-On system (Watanabe et al., 2007). They co-electroporated the rtTA encoding plasmid with the pTRE plasmid containing either *c-meso-1* or *Pax2* in the primitive streak, along with a neutral reporter, dsRed, to assess the efficiency of the electroporation. In this situation, pTRE-driven transgenes are kept silent, since the rtTA protein needs doxycycline for transactivation. They then waited for 18 h to allow electroporated cells to colonize the whole PSM. They finally turned on the expression of the transgenes by simply injecting a doxycycline solution in the egg. By doing so, they managed to avoid the deleterious effect of these genes during gastrulation and could therefore analyze their role during somitogenesis. They have been able to show that *c-meso-1*, the chicken homolog of *Mesp2* in the mouse, acts upstream of *Pax2* in mediating cell aggregation, in part via the upregulation of the adhesion molecule N-CAM (Watanabe et al., 2007).

The three examples described above highlight some of the possibilities that the tetracycline inducible systems offer to study gene function in the chicken embryo. The primary strength of these systems is obviously to tightly control the duration of gene overexpression (tet-Off system, Fig. 4.2) and to define the time at which a gene is overexpressed, independent of the stage at which the electroporation is performed (tet-On system, Fig. 4.2). Now that the chicken genome has been sequenced (Wallis et al., 2004) and that regulatory regions of genes are being

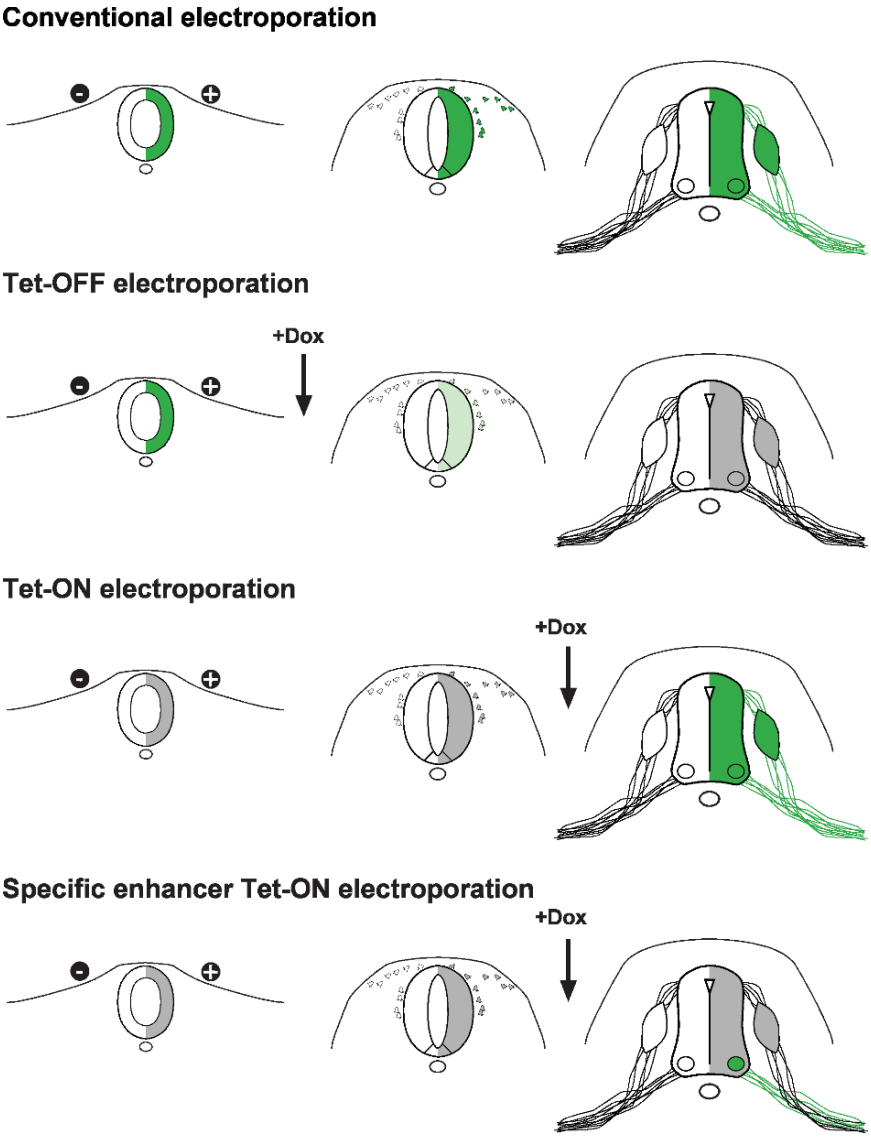

Fig. 4.2 Examples of control of gene expression in time by combining electroporation and tet systems during neural tube development. The development of the neural tube is summarized here by three different states for simplicity: undifferentiated (*left*), patterned (*middle*) and fully differentiated (*right*). With conventional electroporation, the expression of the transgene is sustained from the time of electroporation throughout later development. Analyzing the role of a gene supposedly involved in neural tube patterning and the consequence of its misexpression in the terminal differentiation of neural cells is made difficult, because this gene may have side effects on the long term, like affecting proliferation or neural crest cells migration for example. These effects may not be related to the patterning activity of this particular gene. On the same line, if one wants to study the role of a gene during motoraxon pathfinding without altering neural tube

identified, one can even specifically express the transactivators in discrete subpopulations of cells (Fig. 4.2). Moreover, combined with the recently developed Tol2/transposase technique, which permits stable integration of the exogenous DNA into the genome (Kawakami, 2007; Kawakami and Shima, 1999), it should be feasible to target populations of cells late during development, which are nowadays hardly accessible for electroporation without the risk of DNA dilution or degradation (Sato et al., 2007). One can for example design a cassette containing the rtTA gene under the control of specific enhancers, say that drive expression in osteoblasts, followed by the TRE sequences and a gene of interest, the whole cassette being flanked by tol2 sequences. This construct, as well as the transposase, can then be electroporated in the paraxial mesoderm or in somites, which contain the precursors of the osteoblasts. After integration, the rtTA will be only expressed in osteoblasts, but its activity is kept silent without doxycycline and the TRE-gene of interest cassette is therefore shut down. Simple injection of a doxycycline solution in the egg at any stage of development will then specifically turn on this gene in osteoblasts.

The control of gene expression by the Tet systems combined to electroporation is a promising tool to study precise events during development. The combination of these two techniques has just started to be developed, and it is almost certain that it will rapidly expand in the scientific community and that the continuous improvement of new strategies will enhance the opportunities to address biological questions that were unreachable so far.

5 Comments

5.1 Tet-Off Versus Tet-On

The two systems are complementary and it is up to the experimentator to decide which one is the most suitable for his/her experiment. In our hands, we found that the Tet-Off system displays a strong and tight dependency to doxycycline even at very low concentration, while we experienced leakiness with the Tet-On system,

Fig. 4.2 (continued) patterning and differentiation, one needs to electroporate late during development where motoneurons are hardly accessible. The Tet systems make it possible to circumvent these issues. With the Tet-Off system, one can analyze for example the consequence of misexpressing a gene interfering with neural tube patterning on the terminal organization of the neural tube. To do so, the gene is misexpressed by using the Tet-Off system during the patterning period of the neural tube and is then shut off with addition of doxycycline. Putative defects seen during late differentiation will therefore more likely be due to a perturbation in neural tube patterning rather than an artefact of prolonged expression. With the Tet-On system, on can study the role of a molecule during terminal neural differentiation or during axon pathfinding. The gene of interest is overexpressed in the neural tube at a time where the electroporation is easily amenable and is turned on at the required moment by addition of doxycycline in the egg. The system can be finely tuned to specifically target a subpopulation of neurons by driving the expression of the rtTA with specific enhancers

i.e. the transgene being expressed even in the absence of doxycycline. However, a new rtTA transactivator has been engineered since, called rtTA2^{S}-M2, which has been reported to show strong dependency to doxycycline for gene transactivation with no detectable background (Forster et al., 1999; Urlinger et al., 2000).

One will favor the Tet-Off system when cells of interest at the desired stage are directly accessible by targeted electroporation. This will ensure a rapid transactivation that can be easily stopped by addition of doxycycline (Fig. 4.2). The Tet-On system will be preferred when one wants to hit late differentiated cells that cannot be accessed by DNA injection and local electroporation, say for example, fully differentiated motoneurons buried in the ventral spinal cord (Fig. 4.2). It has to be noted that, once the transgene has been activated using the Tet-On system, it is quite difficult to shut it down. This will require to wash away the doxycycline from the egg, or to explant the embryo to an agar plate devoid of doxycycline. In both cases, the clearing of residual doxycycline molecules may take a while.

5.2 Rapid Shut Down of Gene Expression by Adding Destabilizing 3′UTR Sequences

When using the Tet-Off system, it is important to reach a rapid shut down of the transgene. Suppression of the transactivation by the tTA protein is achieved within minutes after addition of doxycycline. However, the complete loss of activity of the protein depends on its degradation rate, which is inherent to its biochemical properties, but also to the full clearance of its mRNA (Garneau et al., 2007). Most of the expression vectors often contain stabilizing 3′UTR (like the beta globin's) to ensure sustained overexpression by massive accumulation of mRNA overtime. In the case of the Tet-Off strategy, such constructs will slow down the speed at which the transgene is switched off. As described above, addition of a destabilizing 3′UTR efficiently clears out the remaining mRNA after addition of doxycycline within 1 h, without affecting the global levels of gene expression. mRNA stability is known to be context dependent and it is important to choose and to test first by in situ hybridization the properties of the 3′UTR one wants to add. Those from genes cyclically expressed in the PSM are good candidates to confer strong instability to the transgene's mRNA and therefore a rapid shut down of its expression.

5.3 Doxycycline Delivery: In Ovo Injection Versus In Vitro Culture

There are two main ways to deliver doxycycline to the embryo: by direct injection of a doxycycline solution into the egg, between the embryo and the yolk, or by culturing the embryo in vitro on an agar plate supplemented with doxycycline. Doxycycline is a small, highly diffusible molecule and its effect is very fast, probably within few minutes. The easiest way is certainly to inject a small volume

(around 100–200 µl) of a doxycycline solution (at 9 µg/ml) directly into the yolk. However, if one wants to perform time-lapse microscopy for example, leaving the embryo in the egg may complicate the imaging procedure. One alternative way is to explant the embryo and culture it on a small agar plate supplemented with doxycycline, which can be placed under the objective of the microscope. When extra-embryonic tissues are left intact and the embryo carefully spread onto the plate, a 2-day-old embryo can be cultured for at least 48 h without deleterious effects. The efficiency of doxycycline is the same in both conditions.

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Chapter 5
In Ovo Electroporation for Targeting the Somitic Mesoderm

Emi Ohata and Yoshiko Takahashi

1 Introduction

The somite is a transient structure present in early vertebrate embryos, giving rise to a variety of essential tissues including skeletal muscles, dermis, axial bones and blood vessels. The term "somite" refers to a tissue of spherical structure that forms by pinching off from the continuous tissue called presomitic mesoderm (PSM, also called segmental plate in avian embryos). The PSM is recognized as a pair of longitudinal stripes along the midline of the body. Thus, each somite forms at the anterior end of PSM, and this process recurs periodically in time and space, generating the segmented pattern of the body along the antero-posterior axis.

Soon after the invention of the in ovo electroporation technique that was originally applied to the neural tube of chicken embryos (Funahashi et al., 1999; Momose et al., 1999; Nakamura et al., 2004), somites were also challenged for electroporation-mediated transgenesis. However, as long as the PSM was targeted, transgenesis was not successful for unknown reasons. Several years ago, we achieved somitic transgenesis by targeting PSM precursors (presumptive somitic cells) of earlier embryos, the cells residing in the epiblast of the anterior primitive streak (Nakaya et al., 2004; Sato et al., 2002). When development proceeds, these cells ingress and migrate anteriorly beneath the ectoderm to form the PSM on either side of the neural tube (Fig. 5.1). We will describe the methods of electroporation-mediated transgenesis of early somite/PSM by taking advantage of the dynamic morphogenetic movement of the presumptive somitic cells.

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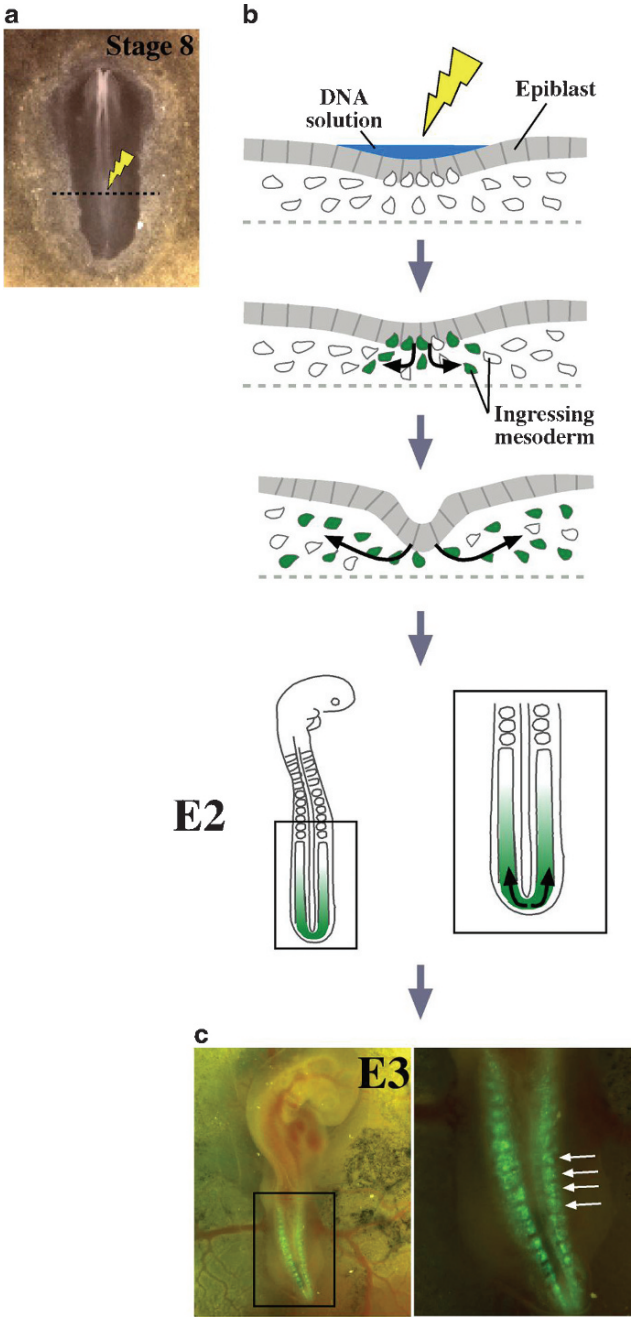

Fig. 5.1 (**a**) A chicken embryo of stage 8 is visualized with black ink injected in between the embryo and yolk. (**b**) Diagrams demonstrate DNA electroporation into developing somites by taking advantage of mesodermal ingression from the epiblast. Electroporation is carried out with a stage 8 embryo wherein presumptive somitic cells reside in the epiblast. As development proceeds, the electroporated cells ingress as a mesoderm inward the embryo, and migrate anteriorly to comprise the PSM by E2. (**c**) An E3 embryo displays successful transgenesis with electroporated EGFP cDNA (*See Color Plates*)

2 Procedure

2.1 Embryos

Cell fate studies showed that in chicken embryos of stage 8 (Hamburger and Hamilton, 1951), the presumptive PSM/somite cells are located in the anterior-most region of the primitive streak just posteriorly to Hensen's node (Catala et al., 1996; Psychoyos and Stern, 1996). We here describe the method using stage 8 embryos as an example. In our experiences, embryos younger than stage 6 are very sensitive to electric pulses and prone to be easily damaged.

2.2 Devices and Settings for Electroporation

A positive electrode is prepared with a piece of platinum wire with a 'Z' shape (diameter: 0.3–0.5 mm). The electrode is held by a micromanipulator (NARISHIGE), and is connected to the red lead (anode) (Fig. 5.2a). A negative electrode is made from tungsten, sharpened either by being immersed in hot melting sodium nitrite or by electrolysis with a solution of sodium hydroxide (2M) so that a diameter of the tip becomes around 40 μm. The prepared electrode is encased in a plastic holder for insulation, and connected to the black lead (cathode) (Fig. 5.2b). To generate electric pulses, we use ELECTRO SQUARE PORATOR T820 or ECM830 (BTX) or ELECTROPORATOR CUY21 (BEX). The condition for applying pulses is 4–5 V, 25 ms, five pulses with 975 ms intervals. These parameters can be optimized for each experimental situation.

2.3 DNA Preparation

The preparation of DNA plasmids and DNA solution is essentially the same as that used for other tissues. A DNA cocktail needs to be prepared freshly. Prior to the electroporation we add Fast Green FCF (Wako) dye with the final concentration of 2% to visualize the DNA solution in embryos.

2.4 Procedures for In Ovo Electroporation

Eggs are incubated horizontally at 38.5°C. When embryos develop to stage 8, 2–3 ml of thin albumen is removed by a syringe needle from the pointed end of egg, and a window is made on the top of the shell. The embryo is visualized by injecting black ink into the yolk underneath an embryo. HANKS' saline is useful to keep the embryo and electrodes wet.

A piece of vitelline membrane is removed by a tungsten needle to expose the anterior primitive streak (Fig. 5.2e). Subsequently, the platinum electrode (anode) is inserted

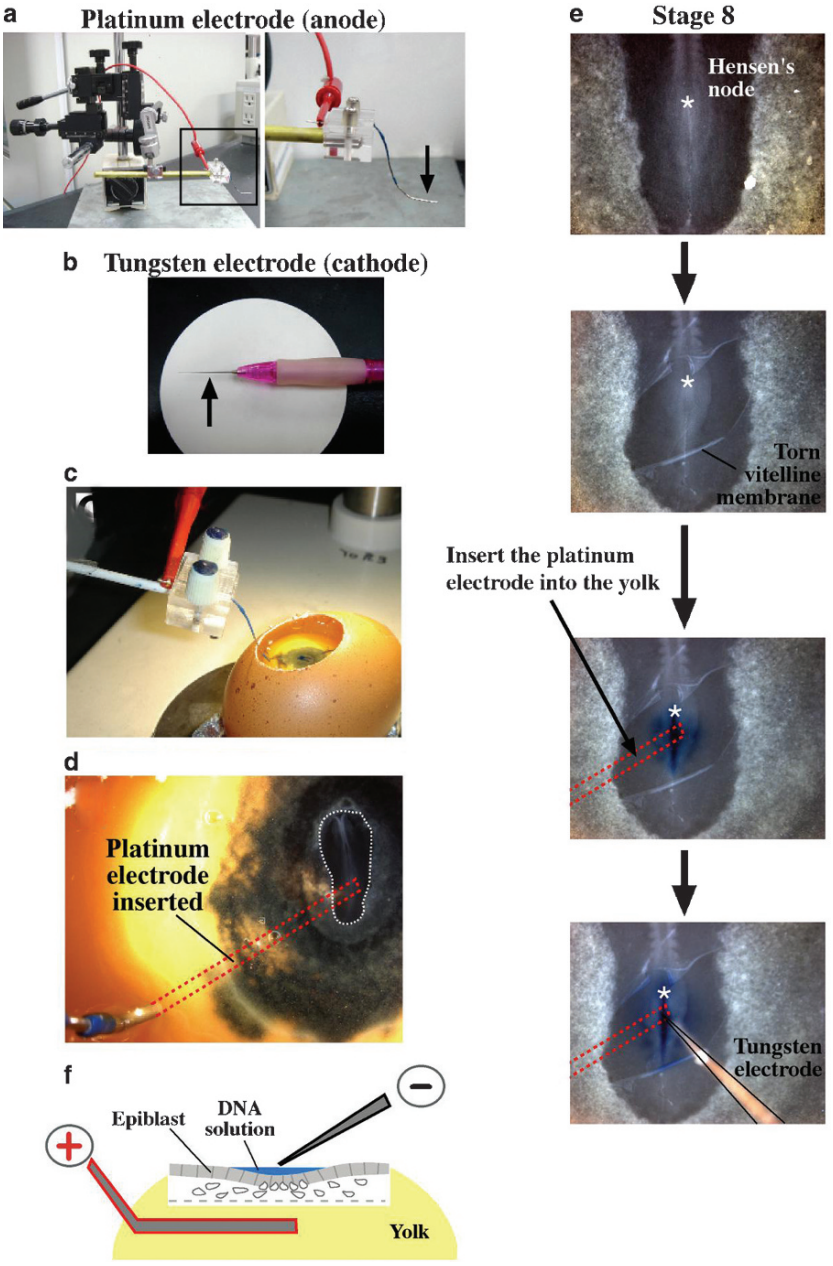

Fig. 5.2 A procedure for in ovo electroporation to target presumptive PSM/somite in chicken embryos of stage 8. (**a**) A setting for in ovo electroporation. A platinum electrode is held with a manipulator and connected to the anode. (**b**) A sharpened tungsten electrode is encased in a plastic holder, and connected to the cathode. (**c, d**) A platinum electrode is inserted into the yolk, and adjust the manipulator to locate the tip of the electrode underneath the anterior primitive streak. (**e**) After a DNA solution is laid on top of the area to be targeted, a tungsten electrode is positioned nearby, followed by application of electric pulses. (**f**) Relative positions between the DNA solution, embryo, and two electrodes. * Hensen's node (*See Color Plates*)

into the yolk (Fig. 5.2c) with the tip of the electrode positioned underneath the anterior primitive streak (Fig. 5.2d). Using a glass capillary, a DNA solution is laid on the epiblast of the anterior primitive streak. Immediately, the tungsten electrode (cathode) is carefully placed on the area to be targeted, and electric pulses are applied (Fig. 5.2f). Bubbles come out near the tip of the tungsten electrode, and it is a sign of the electric current generated. Importantly, both electrodes should be positioned closely to but not in a direct contact with the target since a direct contact would cause severe burn or malformation of tissues. It is also important to correctly locate the area of presumptive somite in the primitive streak because targeting more anterior or posterior regions would result in transgenesis of the neural tube or lateral plate, respectively. The anterior primitive streak used for somitic transgenesis also contains presumptive neural tube cells (Catala et al., 1996; Psychoyos and Stern, 1996). Therefore a successful transgenesis in the somites is often accompanied with some signals electroporated in the forming neural tube.

Following the electroporation, the platinum electrode is pulled out, and the window on the shell is sealed with plastic tape. Manipulated embryos are re-incubated until they are sacrificed.

When we use pCAGGS-EGFP, signals of EGFP become detectable ~4 h after electroporation, and the signals persist for three to four days at most (E4 or E5) (Fig. 5.1c). The electroporated cells are mosaic in developing somites with 30%–50% of the total population of cells.

3 Comments

We have described the method for somitic transgenesis that takes advantage of the ingression of presumptive mesoderm from the epiblast. However, if a product of electroporated gene affects the ingression process, one cannot investigate the role of the gene at later stages including somitogenesis. To circumvent such problems, we have optimized the condition of tetracycline-inducible expression of electroporated genes as described in Sato and Takahashi in this book (see also Watanabe et al., 2007).

For somitic electroporation, there is another method reported by Scaal et al., which is useful for targeting a specific region within a formed somite, such as dermomyotome. In this case, a DNA solution is injected into a somitocoele of formed somite and electric pulses are applied with electrodes positioned laterally (Scaal et al., 2004).

4 Troubleshooting

4.1 High Lethality of Electroporated Embryos

- Decrease voltages and/or the number of pulses to a milder condition.
- Check whether tungsten or platinum electrodes are in a direct contact with an embryo. They should not touch the targeting tissue.

- Sharpen the tip of a tungsten electrode to have a smaller diameter.
- Insert the platinum electrode more deeply in the yolk. This would increase a resistance between two electrodes.
- Avoid using embryos earlier than stage 6.

4.2 Electroporated Cells Are Seen More in the Neural Tube than in PSM

- Target a region more posteriorly further from Hensen's node. The region around Hensen's node contains more of neural tube precursors than the region used for the somitic transgenesis.
- The efficiency of somitic transgenesis decreases if embryos older than stage 9 are used.

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Chapter 6
Method of Electroporation for the Early Chick Embryo

Jun Hatakeyama and Kenji Shimamura

1 Introduction

Chick embryos have long been one of the favored model systems in the field of embryology and developmental biology. Recent advances in the gene manipulation technologies (Muramatsu et al., 1997; Nakamura et al., 2004) make this model system even more attractive for the developmental biologists (see review by Stern, 2005). Thanks to its two dimensional geometry, easiness in accessibility and observation, and well-established fate maps (e.g. Couly and Le Douarin, 1988; Garcia-Martinez et al., 1993; Hatada and Stern, 1994; Psychoyos and Stern, 1996; Sawada and Aoyama, 1999; Cobos et al., 2001; Lopez-Sanchez et al., 2001; Redkar et al., 2001; Fernandez-Garre et al., 2002; Kimura et al., 2006; Matsushita et al., 2008), it has great advantages especially for studies at the early embryonic stages, such as the processes of gastrulation, neural induction, left-right patterning, etc. For such purposes, a whole embryo culture system, originally invented by Dennis A. T. New (New, 1955), and its derivatives (Flamme, 1987; Sundin and Eichele, 1992; Stern, 1993; Chapman et al., 2001) have been widely used.

Here we describe a method of electroporation for the early chick embryos using the *in vitro* whole-embryo culture. This method is applicable for some modified version of the New culture, by choosing an appropriate type of electrode. It can be applied for the stage 4 to stage 8 embryos (Hamburger and Hamilton, 1951), and the embryos can be cultured up to stage 17. For a long term study, the tissue of interest may be transplanted to the host embryo in ovo to let it survive for the longer period. This also allows precise positional control of the transgene expression in the host embryo. It should be noted that the younger embryos are the more sensitive to the electric stimuli in general, such that marked deformation of the embryos, even though they are alive, are frequently observed. Therefore, the voltage, pulse duration and numbers, and electrode distance, as well as DNA concentration should be optimized in each actual experimental condition. Cells in either the

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epiblast/ectoderm from the early stages (stage 4~), or the endoderm at relatively later stages (stage 6~) can be electroporated essentially in a similar way, except for the polarity of the electrodes and administration of the DNA solution. As an example, we previously introduced exogenous genes broadly into the early anterior neural plate to demonstrate that the specific responsiveness for the inductive signals and the regional properties was defined by the homeodomain transcription factors (Kobayashi et al., 2002; Lagutin et al., 2003).

2 Principle

The blastoderm of chick embryo exhibits very simple organization, a relatively large disc-like structure floating on top of the yolk, enveloped by a translucent vitelline membrane. The chick embryo has been best utilized for experimental embryology, in which various kinds of manipulation including microsurgery and transplantation have been well carried out in ovo. This is difficult or very much restricted to perform for the mammalian embryos due to their development in the mother's body. The gastrulation is perhaps the most intriguing step in animal development through which not only the three germ layers have formed but also basic pattern formation fundamental to the various organogenesis takes place concomitantly. Yet it is still difficult to perform precise and consistent experimental manipulation even for the chick embryos at the early stages. Besides their fragile and sensitive nature, a tension of the vitelline membrane that physically supports the embryo in every direction is essential for its proper development. When the vitelline membrane is harmed excessively upon manipulation in ovo, the yolk flows out and the internal pressure of the yolk sack is reduced, which leads to abnormal morphogenesis and ultimately death of the embryo. To overcome this problem, an *in vitro* whole embryo culture system was invented originally by New (1955) and has been modified by several researchers for easier operation and/or better viability and morphogenesis of the embryo (Flamme, 1987; Sundin and Eichele, 1992; Stern, 1993; Chapman et al., 2001). The principle of these methods is to provide a tension to an isolated embryo either by holding the vitelline membrane by glass or metal rings or to support the embryo on the surface of a semi-solid substrate. Moreover, since the embryos are set the ventral side up in these cultures, the bottom layer of the embryo (the hypoblast/endoderm) becomes accessible for the manipulation, which is very difficult to achieve by in ovo operation.

When one wants to introduce genes into the early chick embryo by electroporation, two major points should be considered: how to administrate exogenous DNA or small nucleotides, and how to set the electrodes. Because of the flat nature of the blastoderm and the early somite stages of embryo, one of the electrodes should often be located beneath the embryo (thus inside the yolk sack). One way to achieve this is to use a fine platinum wire penetrating inside the yolk sack so that the damage of the yolk sack should be minimal, preventing an excess spillage of the yolk (Sato et al., 2002; see Chapter 5). The egg as well as the electrode

devices therefore should be handled with a particular care during the operation. An easier alternative is to cultivate the embryo *in vitro* by methods introduced above, providing much more flexibility in choice of size, shape, and position of the electrode. Moreover, as noted above, the endoderm can also be targeted by applying a DNA solution onto the inverted embryo in the culture. We will provide a detailed description of required apparatus and protocols to introduce exogenous genes by electroporation using an *in vitro* culture system for the early chick embryos in the following sections.

3 Materials

- Electrode set consisting of the upper and base electrodes (Fig. 6.1a, b; CUY701P2L and CUY701P2E, respectively; commercially available from NEPA Gene, Japan, Unique Medical Imada, Japan). The base electrode has a square or circle platinum plate in the center of a Petri dish, over which 50 mm diameter, 1 mm thick silicone rubber disc (called platform thereafter) with a window (called electrode pocket) exactly same size and shape of the platinum plate and a dam (I.D. 30, 7 mm deep) is glued (Fig. 6.1b). For cultures with a glass ring, the base electrode with a flat platform may be better (Fig. 6.1c) to utilize the ring as a chamber. The upper electrode is used commonly for both methods (Fig. 6.1a). An appropriate size and shape of the electrode plate should be designed or chosen depending on the target tissue. Various types of the platinum electrodes are commercially available from the manufacturer. Platinum is the best material for the electrodes and their surface should be kept clean.
- Square pulse generator (electroporator) (e.g. CUY21SC, NEPA Gene, Japan, Bex, Tokyo, Japan). It is ideal that the voltage can be set by 1 V, and the total electric current may be monitored for consistent operation. It is desirable that the pulse intervals can also be set by millisecond to provide wider variation for the electric stimuli.

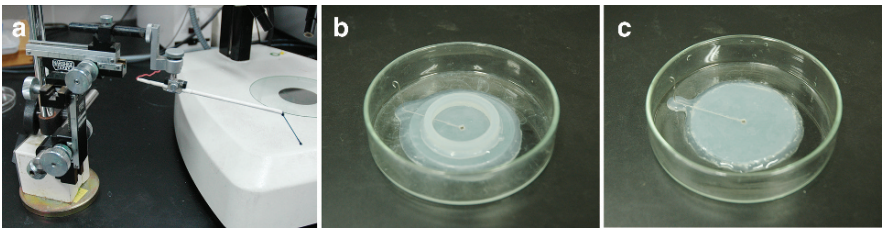

Fig. 6.1 Devices for electroporation for the early chick embryos. (**a**) Upper electrode with 2 mm-square platinum plate (CUY701P2L, NEPA Gene, Japan) set at the mechanical manipulator with a magnetic stand. (**b**) Base electrode with 2 mm-square platinum plate for the culture with a paper ring (CUY701P2E, NEPA Gene, Japan). (**c**) Base electrode for the culture with a glass ring. The platform is simply flat. From Hatakeyama and Shimamura (2008) with permission

- Three dimensional mechanical manipulator mounted on a stable base (e.g. Model MMN-3, Narishige, Japan) (Fig. 6.1a). A scale at milli-meter at least for the Z axis is required to adjust the electrode distance precisely.
- Dissection microscope equipped with a transmitted light base.
- Two pairs of fine forceps (No. 5).
- Two pairs of blunt forceps (for the glass ring method).
- Two pairs of sharp curved scissors for cutting the egg shell and the vitelline membrane, respectively.
- Glass needles pulled out of a glass capillary (borosilicate, O.D. 1.0 mm, I.D. 0.75 mm) and a silicone tubing (O.D. 3/32″, I.D. 1/32″) with a mouth piece.
- Fire-polished Pasteur pipette with a rubber nipple (for the glass ring method).
- Watch glass about 60 mm diameter (for the glass ring method).
- Glass bowl or container about 70 mm deep (for the glass ring method).
- Rings cut from a glass tube, approximately O.D. 27 mm, I.D. 24, 4 mm thick. The edges of the glass ring should be smoothened by a fine file or a piece of sandpaper.
- Sterile plastic tubes of appropriate size.
- Thirty-five millimeter plastic dishes with lids.
- Saline solution (Tyrod's, Ringer, phosphate buffered saline, etc) with calcium and magnesium (1 mM, each).
- Incubator set at 39°C.
- Humidified container with lid for incubating culture dishes pre-incubated at 39°C.
- Fertilized hen's eggs incubated for an appropriate period.

4 Procedure

4.1 New Culture

These have been modified from the originals. See the references for details.

4.1.1 With a Paper Ring

4.1.1.1 Preparation of Agarose/Albumen Plate

1. Collect the thin albumen from fresh eggs by a blunt end pipette.
2. Add 10% (W/V) glucose/water to 0.3% (0.6 ml/20 ml).
3. Stir for 10 min at room temperature.
4. Boil 0.6% agarose solution containing 123 mM NaCl.
5. Incubate dissolved agarose solution and the albumen at 50°C for 10 min.
6. Mix them in equal volumes and quickly pour it to 35 mm dishes (about 2 ml each). Antibiotics (penicillin streptomycin) can be added for longer preservation (up to 1 week at 4°C).
7. Solidify the agarose/albumen solution.

4.1.1.2 Embryo Explantation

1. Crack the egg onto a 100 mm plastic Petri dish. A skill is needed not to damage the vitelline membrane by the edge of the egg shell and locate the blastoderm disc on top of the yolk sack (Fig. 6.2a).
2. Remove the thick albumen covering the top of the embryo by tissue paper, where a paper ring will be placed. Avoid touching the embryo, especially at early stages. They are very fragile. Remaining albumen causes rapid detachment of the paper ring from the vitelline membrane.
3. Gently place the paper ring so that the embryo is located at the center of the ring and its anterior-posterior axis should be parallel to the long axis of the ring (Fig. 6.2b).
4. Cut the vitelline membrane with a sharp scissors along the outer edge of the paper ring (Fig. 6.2b).
5. Pinch the paper ring by a forceps together with the vitelline membrane, and remove it gently from the egg. Be careful not to detach the embryo from the paper (through the following steps).

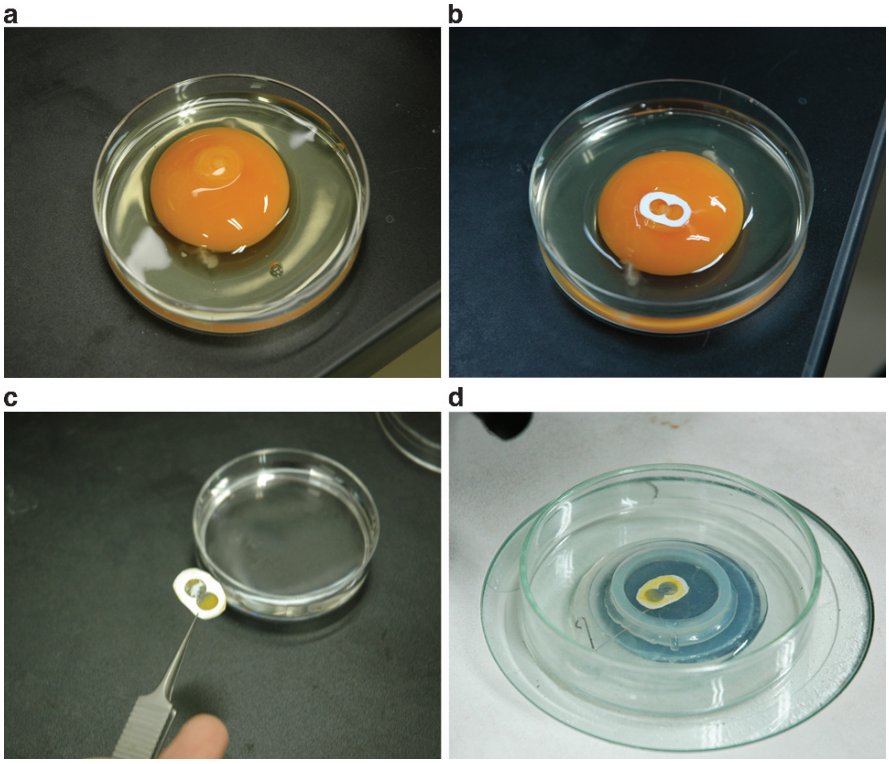

Fig. 6.2 Snap shots of preparation of the paper ring culture. A 3 mm filter paper ring with a gourd-shaped hole is shown in **b** and **c**. See text for details

6. Gently dip the embryo with the paper ring into a saline to wash out an excess yolk (Fig. 6.2c).
7. Transfer the embryo with the paper ring onto the platform of the base electrode, being subjected to electroporation (Fig. 6.2d). Make sure the ventral side of the embryo facing up.

4.1.2 With a Glass Ring

1. Fill a glass bowl with a saline as deep as the yolk ball is completely submerged and floating in the middle of the saline.
2. Open a small window on top of the egg shell. Collect the thin albumen to the tube for culture. Remove the thick albumen from the yolk ball by sucking with a fire-polished blunt-ended Pasteur pipette. Be careful not to damage the vitelline membrane.
3. Transfer the yolk into the bowl. Clear residual thick albumen attaching to the yolk (Fig. 6.3a).
4. Cut the vitelline membrane with a pair of sharp scissors just below the equator all the way around the circumference of the yolk ball (Fig. 6.3b).
5. Carefully peel up the upper half of vitelline membrane by grabbing its cut edge with two pairs of fine forceps (Fig. 6.3c). Minimize the attaching yolk but do not separate the blastoderm disc from the vitelline membrane.
6. Transfer the embryo with the membrane on a watch glass, inner face pointing up.
7. Take out the embryo on the watch glass out of the bowl. Spread the membrane on the center of the watch glass, and place a glass ring such that the embryo disc comes in the middle of the ring (Fig. 6.3d). Make sure that the ventral side is up.
8. With two pair of blunt end forceps, wrap the ring all the way around its edge with the vitelline membrane (Fig. 6.3e). Work carefully not to tear the membrane.
9. Drain the liquid inside the ring using the fire-polished Pasteur pipette. Now it is ready to transfer onto the base electrode (Fig. 6.3f).

4.2 Electroporation

1. Place the embryo on the platform of the base electrode (Fig. 6.4a, b; the ventral side up) which should be pre-rinsed with the saline to avoid sticking. In the case of the paper ring, the base electrode chamber should be filled with the saline in advance. For those with a glass ring, gently pour the saline with a pipette inside the glass ring at about a few millimeters in depth, thereby using the ring as a chamber (Fig. 6.4b). Air bubbles in the electrode pocket should be cleared prior to embryo transfer.
2. Inject a DNA solution (~10 mg/ml) dyed with Fast Green® at the site of interest according to the fate maps. For the epiblast or ectodermal cells, inject the DNA solution between the vitelline membrane and the epiblast/ectoderm by

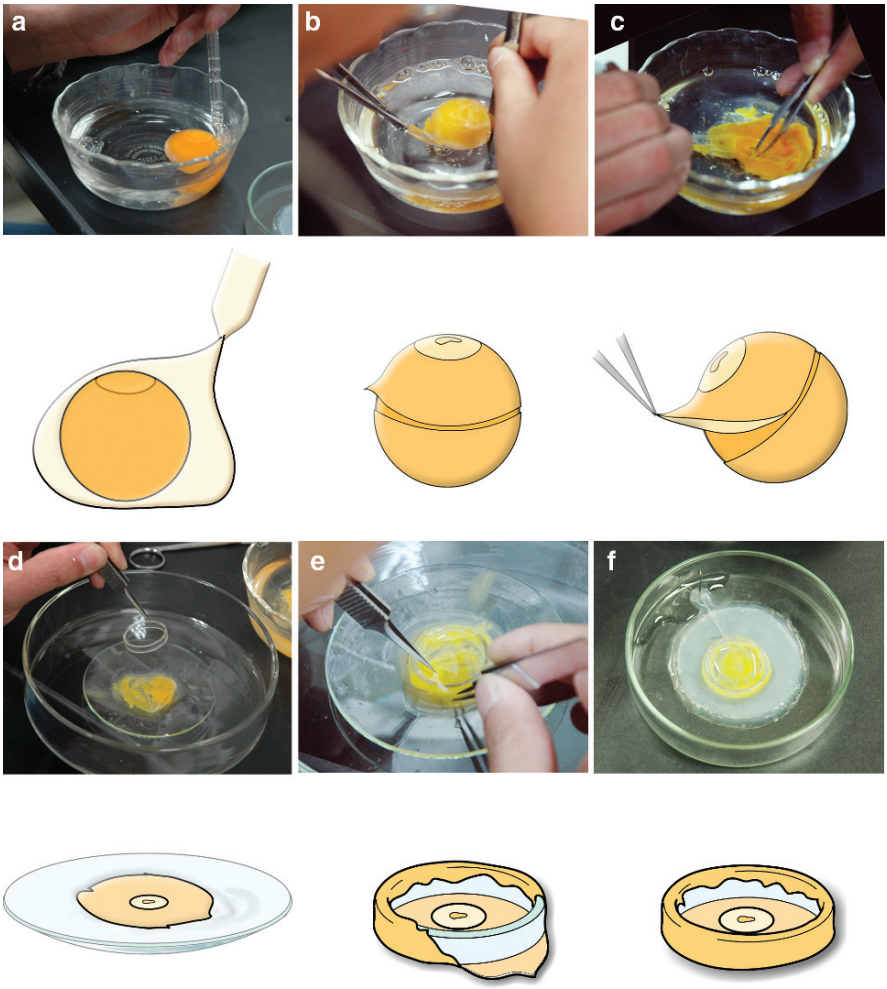

Fig. 6.3 Snap shots and schematic drawings of each step making the glass ring culture. See text for details

penetrating the embryo from the ventral side carefully not piercing the vitelline membrane (Fig. 6.4c). For the endodermal cells, the DNA solution should be solidified with low melting agarose (~0.7% w/v). A small block of the agar/DNA is placed onto the endoderm layer (Fig. 6.4d). To introduce into the mesodermal cells, target the epiblast located at a certain anterior-posterior level along the primitive streak according to the fate maps before they delaminate from the streak (see Fig. 6.5e, f).

3. Set the embryo carefully so that the target field is precisely above the electrode plate. Then place the upper electrode attached to the manipulator right on top of the

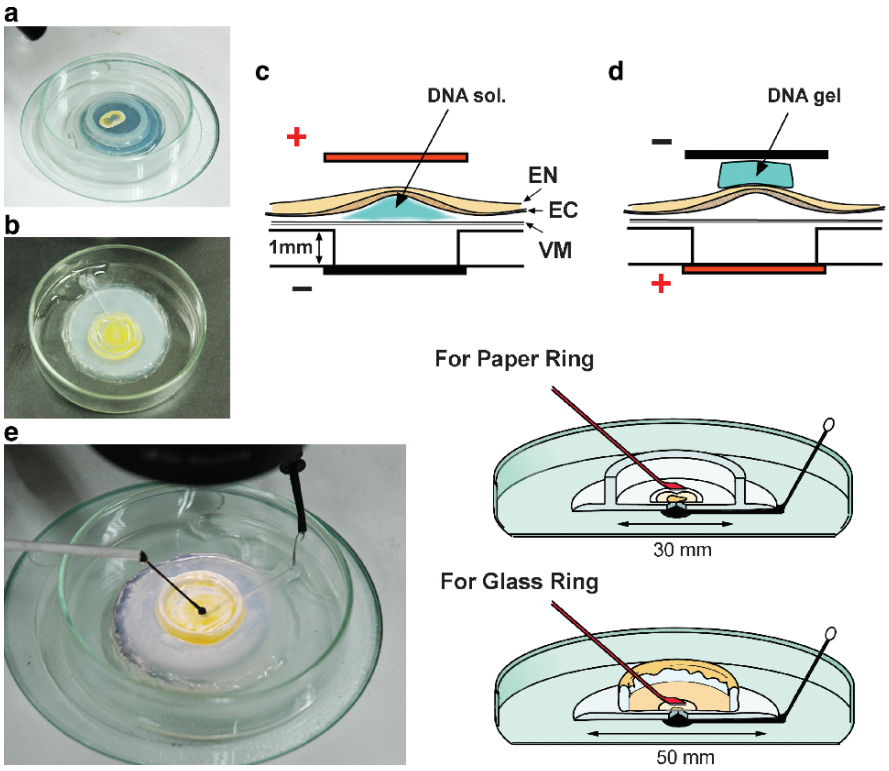

Fig. 6.4 Settings of electroporation for the explanted chick embryos. The paper ring (**a**) and the glass ring culture (**b**) on the base electrodes. (**c, d**) Schema for the polarity of the electrodes and DNA administration for targeting the ectoderm/epiblast (**c**) and the endoderm (**d**). Cross sectional views are illustrated. (**e**) Delivery of electric pulses for the glass ring culture. The upper electrode is placed over the target field of the embryo. See text for details. Abbreviations: EC, ectoderm; EN, endoderm; VM, vitelline membrane. From Hatakeyama and Shimamura (2008) with permission (*See Color Plates*)

target field (Fig. 6.4e). Adjust the height of the upper electrode using the scale of the manipulator, so that the distance between the electrodes to be consistent (usually 5 mm). Make sure that the upper electrode plate touches the liquid surface.

4. Check the polarity of the electrodes. For the epiblast/ectoderm targeting, the base electrode should be minus and the upper one should be plus (Fig. 6.4c). It should be reversed for the endoderm (Fig. 6.4d).
5. Deliver five square electric pulses of 10 V, 50 ms duration with 100 ms intervals.
6. Remove the upper electrode, pick up the embryo with a glass or paper ring. In the case of the paper ring, remove excess saline by carefully touching to a paper towel, and place it on the agarose/albumen plate. Place another well-dried paper ring on the specimen so that the embryo is sandwiched by the paper rings. This prevents the embryo from detaching from the paper ring. In the case of the glass ring method, drain the saline within the ring again, and then place the ring with

an embryo over the large drop of the collected thin albumen in the center of a 35 mm dish. Press the ring lightly to attach to the dish, making the vitelline membrane bulging up. This is important in order to provide a tension to the vitelline membrane that physically supports the embryo. Transfer the dish with the ring to a humidified container incubated at 39°C.

7. Keep the upper electrode wet. Remove the air bubbles generated around the base electrode pocket by flushing to prepare for the next round of operation. The saline in the electrode chamber should be changed periodically to clear the disposals

5 Application and Results

1. pCAGGS-GFP plasmid (Momose et al., 1999) was electroporated to the presumptive neural plate of stage 5 embryo. The targeted area is boxed. The expression of GFP was monitored during the course of culture. A broad region of the CNS shows transgene expression. (Fig. 6.5a–d).

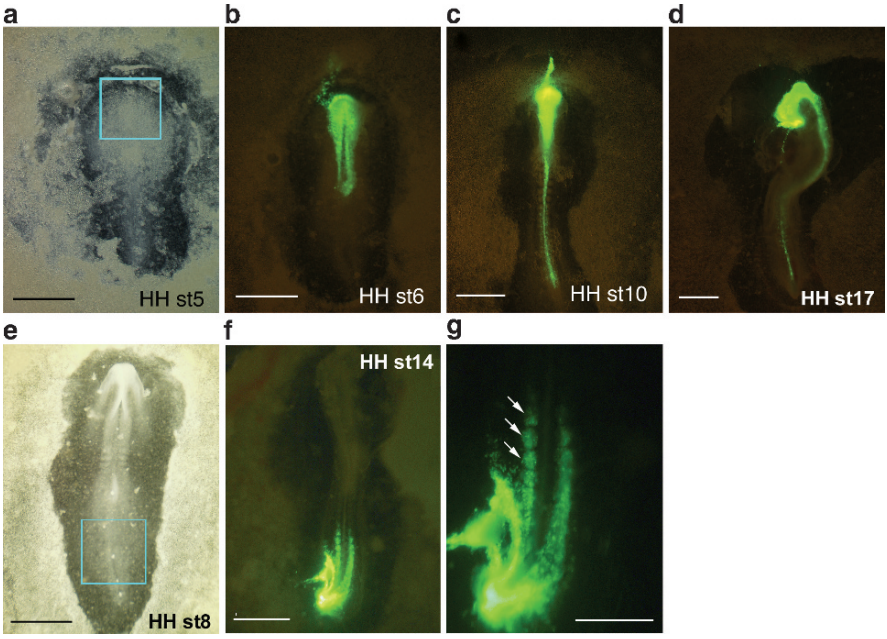

Fig. 6.5 Experimental examples. (**a**) A stage 5 chick embryo electroporated with pCAGGS-GFP plasmid in vitro. (**b–d**) The embryo under an epi-fluorescent dissecting microscope at stage 6, 10, and 17. The neural tube and some head ectoderm express GFP. (**e**) pCAGGS-GFP plasmid was electroporated into the posterior portion of the primitive streak at stage 8. The targeted area is boxed. (**f–g**) GFP fluorescence is observed in the presomitic mesoderm and some somites (arrows) at presumptive stage 14. **g** is magnification of **f**. Scale bars, 1 mm for **a–f**, 0.5 mm for **g**. From Hatakeyama and Shimamura (2008) with permission

2. The posterior region of the primitive streak was targeted at stage 8 and cultured for 24 h. The target field is boxed. The surface ectoderm, presomitic mesoderm, and some somites express GFP (Fig. 6.5e, f).

6 Comments

1. The process of injection and electroporation should be done as quickly as possible so that the embryo does not come off from the paper ring.
2. The injected DNA solution is often swept away to the periphery of the neural folds due to tension generated by attaching to the paper ring. To avoid this, make the solution viscous, and create a space between the vitelline membrane and the ectoderm by gently lifting the embryo with the glass needle upon DNA injection.
3. It is generally said that the paper ring method is easier to operate and yields relatively consistent results, but the embryos often exhibit abnormal morphogenesis especially at the cephalic region.
4. Detailed precise fate maps for given stages are essential for successful transfection especially for the mesodermal tissues (Hatada and Stern, 1994; Psychoyos and Stern, 1996; Sawada and Aoyama, 1999; Redkar et al., 2001). It is recommended that initial sites of electroporation should be recorded and analyzed well with the actual site of transgene expression afterwards. It sometimes happens that a very slight difference of targeted fields resulted in big difference in tissues introduced.
5. Similar sets of the electrode (the upper electrode should be designed accordingly depending on the situation) and the protocols can be applied to various tissue explants or slices cultured on floating filters to introduce exogenous genes or small nucleotides (e.g. Stuhmer et al., 2002; Hashimoto-Torii et al., 2003). It was also reported that primordia of the internal organs, such as liver, can be electroporated at relatively late stages (stage 12~) using a metal ring culture (Tatsumi et al., 2007).

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Chapter 7
Enhancer Analysis

Strategies for Locus-Wide Identification and Systematic Analysis of Enhancers Using Chicken Embryo Electroporation

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1 Introduction

During embryonic development, genes are expressed under a strict spatial and temporal order in cells and tissues. This regulation is governed by regulatory regions in the genome, usually identified as enhancers (Kondoh, 2008). The identification and mapping of a set of enhancers allow clarification of essential regulatory elements involved in the enhancer action and their interacting protein factors. Enhancer analysis also determines upstream signaling cascades that regulate interacting protein factors. If the regulatory regions do not function properly, spatio-temporal order of the gene expression will be disrupted, and this may cause abnormal development and diseases (Kleinjan & van Heyningen, 2005; Sabherwal et al., 2007). Thus, identification of the regulatory regions provides an important entry point to clarify regulatory mechanisms underlying embryonic development.

In the investigation of regulatory regions associated with a gene, various animal systems and tissue-cultured cells are widely employed, each having unique advantages and drawbacks. We find that electroporation of chicken embryos is most advantageous for systematic survey of development-associated regulatory regions (Uchikawa et al., 2003, 2004; Uchikawa, 2008). The chicken embryo has advantages in developmental studies (Stern, 2004), for its amenability to live observation and tissue manipulation, and availability of simple culture system (New's culture), and application of in ovo electroporation (Muramatsu et al., 1996; Funahashi et al., 1999) certainly enhances its merit.

Comparative genomics indicate that important regulatory sequences tend to be conserved across a wide range of vertebrate species, but the extent of sequence conservation varies in a way dependent on the phylogenetic distance between species (Hardison, 2000; Woolfe et al., 2005). We find that the phylogenetic distance between the chicken and mammals, both belonging to the same amniotes,

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is appropriate for predicting potential regulatory sequences as conserved sequence blocks (Uchikawa et al., 2003; Izumi et al., 2007). The chicken genome is one third as large as mammalian genomes, despite possessing an equivalent number of genes, providing another advantage of the chicken system in the genome analysis.

Here, we will detail strategies used for locus-wide identification and systematic analysis of genetic regulatory regions and their practices using chicken embryo electroporation. While the identification and analysis of the regulatory regions require substantial effort, chicken embryo electroporation technique is simple enough and very efficient, greatly facilitating analysis. In addition, information provided by the genome comparison between chicken and other vertebrates will be discussed. These in combination provide a straightforward avenue to identify and analyze regulatory regions, in particular enhancers.

2 Procedure

2.1 Construction of Reporter Vectors

A substantially large genomic region surrounding a gene needs to be investigated to enumerate the major regulatory regions associated with the gene (Fig. 7.1). BAC clones are usually useful to generate subfragments of the genomic region of interest (Matsumata et al., 2005). Subfragments can be prepared by various means, such as restriction enzyme digestions, amplification of the PCR reactions using specific primer sets of your choice and polymerases with high proofreading activity. Overlapping subfragments need to be prepared to span the genomic region of the study, in order not to overlook a regulatory region at the border of a subfragment. Practically, overlapping fragments of 4–5 kb length are useful, if you test enhancer activities in electroporated chicken embryos using reporter vectors such as ptkEGFP.

The reporter vectors of our routine practice are constructed based on pCAT3-basic vector (Fig. 7.2a) (Promega). A promoter sequence was first inserted into the *Hin*dIII site of polylinker sequence in the vector. The Herpes simplex virus thymidine kinase (tk) promoter of 253 bp *Pvu*II-*Bgl*II fragment (position 4–256; GenBank accession number V00470) was found to be useful in detecting enhancers, for its low background and sharp response to insertion of exogenous enhancers. The native promoter of the gene of interest may also be used, if the promoter satisfies the above conditions. The CAT (chloramphenicol acetyltransferase) coding sequence of the original vector was then replaced with various fluorescent protein genes for EGFP, Venus, mRFP1, mCherry or AmCyan1 (Campbell et al., 2002; Nagai et al., 2002; Shaner et al., 2004). A destabilized form of these proteins carrying a PEST sequence, for example, of mouse ornithine decarboxylase may be employed to gain a high temporal resolution of enhancer activity (Li et al., 1998). Luciferase, β-galactosidase or alkaline phosphatase genes are also used for quantitative assays.

Resent versions of ptkEGFP vector of our use have polylinker sequence with two pairs of restriction sites for *Sal*I and *Xho*I, and *Bam*HI and *Bgl*II that generate compatible cohesive ends. These combinations of restriction sites are useful in the

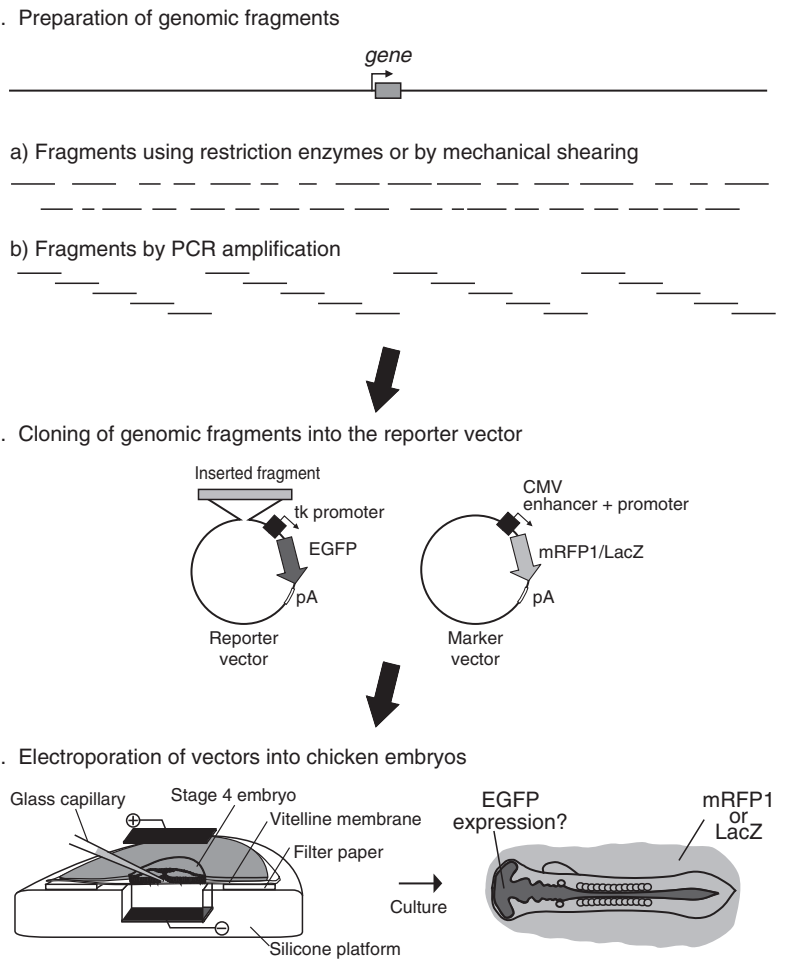


Fig. 7.1 Strategy for identifying enhancers using chicken embryo electroporation. The overlapping genomic fragments covering a gene locus of interest are cloned, and individually inserted into the reporter vector. The reporter vector, together with marker vector, are injected between the embryo and the vitelline membrane with the glass capillary, and electric pulses are applied immediately. Enhancer activity of the inserted genomic fragment is detected by expression of EGFP. The area of successful electroporation in the chicken embryo is monitored by the expression of mRFP1 or LacZ. Modified from Fig. 1 in Uchikawa, M. 2008. Dev. Growth Differ., 50, 467–474, with permission from Blackwell

production of multimers of an inserted fragment (Fig. 7.2b), which is a technique taken to enhance the activity borne by a DNA fragment. A fragment to be multimerized is usually inserted into the *Sma*I site placed between the restriction sites for shared cohesive ends. To be used for electroporation, the plasmid DNAs should be prepared in a large-scale and of a high purity, using a large-scale plasmid preparation kits (e.g., Qiagen), or, preferably, two rounds of CsCl-gradient centrifugations.

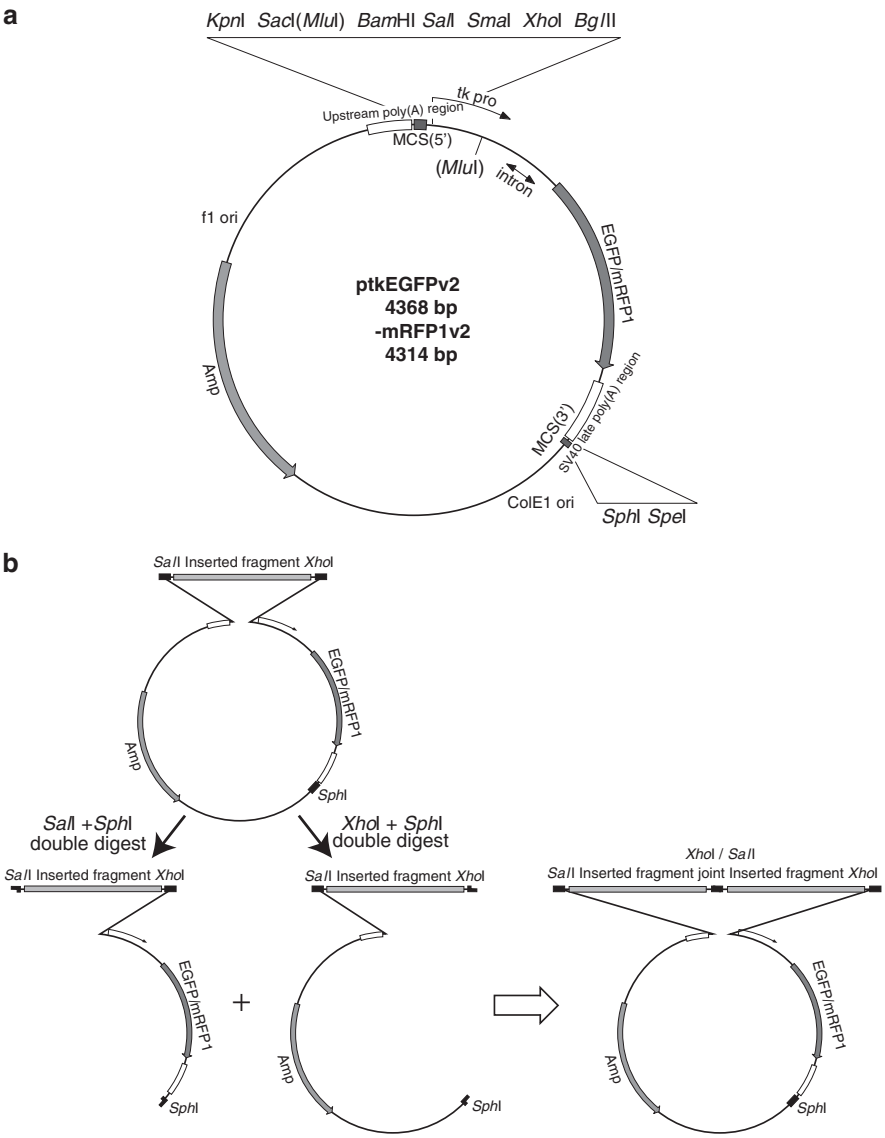


Fig. 7.2 Basic structure of ptkEGFP and ptkmRFP1 (version 2) and procedure for multimerization of an inserted DNA sequence using restriction sites for compatible cohesive ends. (**a**) Structure of ptkEGFPv2/ptkmRFP1v2. The backbone is based on pCAT3-basic vector (Promega). The tk promoter is inserted in *Hin*dIII site. EGFP/mRFP1 sequence replaced the CAT gene. The 5′ multiple cloning sites (MCS) includes sites for *Kpn*I, *Sac*I, *Bam*HI, *Sal*I, *Sma*I, *Xho*I and *Bgl*II, and the 3′ MCS, sites for *Spe*I and *Sph*I. (**b**) Multimerization of inserted fragment utilizing a pair of enzymes generating compatible cohesive ends. The fragment inserted in *Sma*I site is multimerized using a restriction enzyme pair, generating compatible cohesive ends, as *Sal*I and *Xho*I, or *Bam*HI and *Bgl*II. The plasmids are digested by *Sal*I [GTCGAC] plus *Sph*I, and *Xho*I [CTCGAG] plus *Sph*I, and the fragments including the insert of the two different digestions are prepared and ligated.

2.2 Electroporation of Chicken Embryo

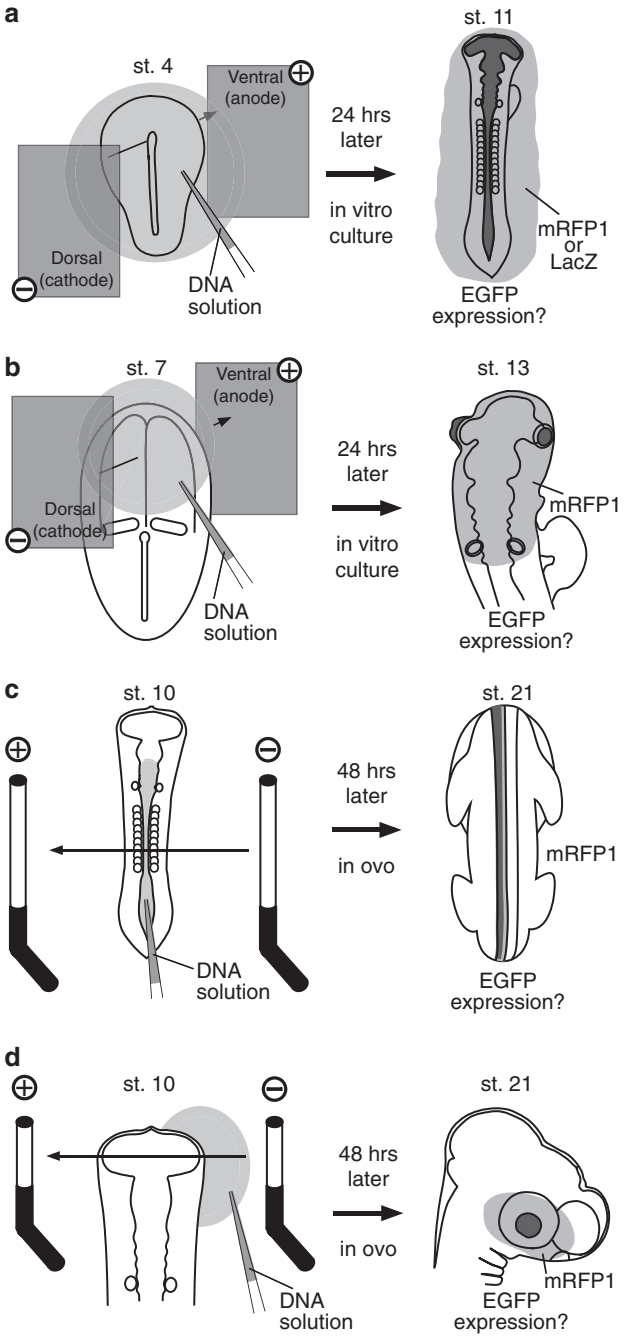
Electroporation is a simple and efficient method to introduce DNA into a chicken embryo, and can be applied to a variety of developmental stages and tissues using various setups (Fig. 7.3). Details of the methods are described in other chapters. To identify and analyze regulatory regions active in early chicken embryos, electroporation of disk-shaped stage 4 embryos placed in culture using modified New's technique is advantageous (Figs. 7.1 and 7.3a). DNA solution used for electroporation typically includes the reporter vectors at 2 μg/μl plus marker vector for successful electroporation at 1 μg/μl, carrying a strong and non-specific enhancer/promoter and encoding mRFP1 or LacZ. The DNA solution is injected between the embryo and the vitelline membrane using a glass capillary. The electric pulses should be immediately applied after DNA injection to minimize the diffusion of the solution. Addition of non-electrolytes such as sucrose and glucose increases the viscosity of solution and reduces diffusion of DNA solution to some extent. Conditions for electroporation should be optimized in each laboratory environment.

Electroporation of disk-shaped embryos is performed by placing an embryo between electrodes (Fig. 7.1). Two 2 × 2 mm platinum plates connected to wires are used as anode and cathode. Cathodal electrode is placed at the bottom of a centrally engraved 2-mm deep well in a silicone platform made of Silgard 184 (Dow Corning). Anodal electrode is held precisely 4 mm above the cathode. The anodal electrode is cleaned by a stream of Hank's solution and the cathodal electrode using an interdental brush each time after electroporation of an embryo, in order to prevent accumulation of electric resistance at the surface of the electrodes.

2.3 Detection of Enhancer Activity

The fluorescence of reporter protein is usually detectable 2–3 h after it is electroporated, if enhancer activity is strong enough. If the activity of enhancer persists, the fluorescence may be followed over a 48-h period after electroporation of an embryo. To reproducibly detect the enhancer activity borne by a DNA fragment, a marker gene should be expressed in a wide tissue area. Under this condition, reproducible expression of reporter fluorescent protein in a specific tissue indicates an enhancer activity associated with the DNA fragment inserted in the reporter vector.

Fig. 7.2 (continued) The constructed plasmid has the dimerized insert in the form *Sal*I-insert-GTCGAG joint-insert-*Xho*I sites at the MCS. As the GTCGAG joint is not cleaved by *Sal*I or *Xho*I, repeating the same step yields tetrameric inserts separated by the GTCGAG joint. Arbitrary multimers can be produced by combination of the number of inserts in the two different digestions. Reprinted from Fig. 1 in Kondoh, H. and Uchikawa, M. 2008. Methods Cell Biol., 87, 313–336, with permission from Elsevier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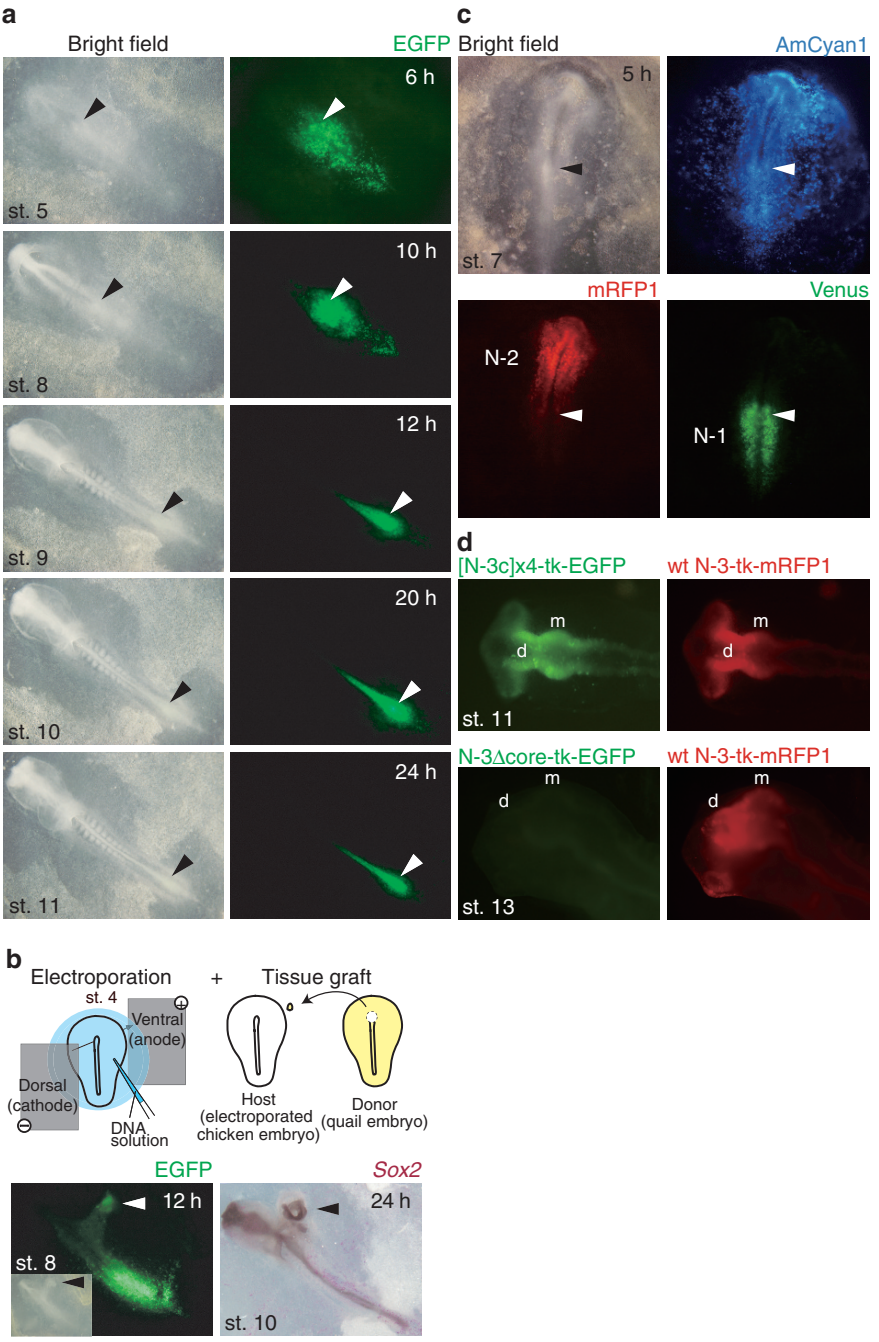
As a circular form of vector DNA is introduced into cells by electroporation, it is not integrated into the genome. Expression of reporter genes therefore diminishes after two days. An advantage, however, is that the reporter expression pattern is highly reproducible, being free from effects of chromosomal integration sites often encountered in transgenic mice.

Once an enhancer activity is detected, time-lapse analysis is highly informative in characterizing the enhancer (Fig. 7.4a): the temporal change and tissue distribution of enhancer activity, in comparison with expression pattern of the endogenous gene.

2.4 Analysis of Enhancers for Regulatory Elements

To clarify the mechanisms underlying the regulation of a gene, the most straightforward approach would be to determine regulatory elements comprising the enhancer (Fig. 7.5). Typically, an enhancer consists of several elements, some are essential and determinative to the specificity of enhancer action, often called core elements, and others are less-specific and act to augment the enhancer activity (Goto et al., 1990; Takemoto et al., 2006; Inoue et al., 2007). To determine the regulatory elements, a DNA fragment showing an enhancer activity is divided into subfragments, which is further dissected into smaller subfragments. The subfragment may be deleted from either ends to map the span of essential elements. DNA sequences of the genomic regions conserved among different animal species are, of course, candidates of important regulatory regions, but unbiased analysis of the entire genomic region for the enhancer activity is required to enlist and map the full repertoire of enhancers involved in the regulation of a gene.

Fig. 7.3 Schematic representation of chicken embryo electroporation. (**a**) Electroporation of stage 4 embryos under New's culture. The reporter constructs are electroporated into the epiblastic/ectodermal side of stage 4 chicken embryo which is cultured using a modified New's technique. EGFP expression that is elicited in response to an inserted enhancer sequence is detected after 6–48 h of electroporation during neurulation stage. mRFP1 or LacZ driven by CMV enhancer/promoter is introduced to monitor the embryonic area of successful electroporation. Electroporation is performed with five square pulses of 10 V for duration of 50 ms and with 100 ms intervals using a pair of 2 × 2 mm platinum plate electrodes with an interelectrode distance of 4 mm, usually causing ~18 mA current. Modified from Fig. 1 in Uchikawa, M. et al., 2003. Dev. Cell, 4, 509–519, with permission from Elsevier. (**b**) Electroporation of stage 7 embryos under New's culture. The reporter constructs are electroporated into the cranial region including the presumptive sensory placodes. Electroporation is performed using same electric conditions as in (**a**). (**c**) Electroporation of neural tube of stage 10 embryos in ovo. The vectors are electroporated into one side of neural tube. Electroporation is performed with five square pulses of 20 V for duration of 50 ms and with 100 ms intervals using a pair of platinum wire electrodes with an interelectrode distance of 4 mm. (**d**) Electroporation of head ectoderm of stage 10 embryos in ovo. The reporter constructs are electroporated into one side of head ectoderm including the presumptive lens. Electroporation is performed using same electric conditions as in **c**. (**b–d**) Reprinted from Fig. 3 in Uchikawa, M. 2008. Dev. Growth Differ., 50, 467–474, with permission from Blackwell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EGFP
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st. 5
10 h
st. 8
12 h
st. 9
20 h
st. 10
24 h
st. 11
c
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mRFP1
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N-2
N-1
d
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wt N-3-tk-mRFP1
m
d
st. 11
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Electroporation
+
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Ventral
(anode)
Dorsal
(cathode)
DNA
solution
Host
(electroporated
chicken embryo)
Donor
(quail embryo)
EGFP
Sox2
12 h
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24 h
st. 10

In general, a core element has more-or-less the same spatio-temporal specificity of the larger fully equipped enhancer, but exhibits much weaker activity. The activity of the core element of the enhancer can be enhanced to the level of full-length enhancer by multimerization of the core element sequence (Fig. 7.4d, upper panel). Deletion of the core element from the full-length enhancer inactivates the enhancer activity (Fig. 7.4d, lower panel).

Determination of the individual regulatory elements, usually corresponding to transcription factor binding sites, in the core sequence helps understanding the spatio-temporal regulation of the enhancer operating through the core sequence. Introduction of block-wise mutations scanning the core sequence is useful to determine the regulatory elements. Mutations may be designed based on in silico prediction of potential transcription factor binding sites.

To reliably assess the effect of mutations, wild-type enhancer and a mutated enhancer activating fluorescent reporter proteins of different colors are co-electroporated in the same embryo, e.g., EGFP/Venus vs. mRFP1/mCherry (Fig. 7.5b).

Once regulatory elements of the core sequence are determined, action of potential transcription factors interacting with the elements can be experimentally assessed. Overexpression of a transcription factor by co-electroporation of expression vector with a reporter vector carrying the enhancer to be tested (gain-of-function), and shRNA-based knockdown of the transcription factor expression (loss-of-function) are the most popular experimental maneuver. The effect of manipulating a transcription factor on the enhancer activity is assessed by the expression level

Fig. 7.4 Various enhancer analysis using chicken embryo electroporation. (**a**) Time-lapse observation of enhancer N-1 activity in same live embryo under New's culture. The activity of enhancer N-1 was always surrounded Hensen's node (*arrowheads*). Durations after electroporation (hours) are indicated in the top right corner of the panels, and the developmental stages of embryo in the lower left corner. (**b**) An example of the combination of electroporation and tissue manipulation of chicken embryo, demonstrating Hensen's node tissue grafted in an extra-embryonic region can ectopically activate the enhancer N-1. This also causes ectopic *Sox2* expression in the same extra-embryonic tissue, as shown in the right. The embryo electroporated with enhancer N-1 reporter vector received graft of a tissue piece containing Hensen's node of a donor embryo in an extra-embryonic area of stage 4 host embryo. The fluorescence representing the enhancer N-1 activity was observed surrounding the grafted node (*arrowhead*) in addition to the normal site of as shown in (**a**). The same embryo was fixed after 24 h and examined for the *Sox2* expression by in situ hybridization. (**a**, **b**) Reprinted from Fig. 3 in Uchikawa, M. et al., 2003. Dev. Cell, 4, 509–519, with permission from Elsevier. (**c**) The spatial relationship of the activities of enhancer N-1 and N-2 in the same embryo labeled by the ptkVenus and ptkmRFP1 respectively. The enhancer N-2 is active in the anterior neural plate, and the enhancer N-1 in the area posterior to the Hensen's node (*arrowhead*). The AmCyan1 activated by a non-specific promoter marks the electroporated area. The fluorescence of Venus, mRFP1 and AmCyan1 proteins were observed with use of YFP (SZX-FYFPHQ, Olympus), RFP2 (SZX-FRFP2, Olympus) and CFPHQ (SZX-FCFPHQ, Olympus) filters respectively. (**d**) Characterization of the essential element of an enhancer. The enhancer N-3 core tetramer ([N-3c]x4) showed similar activity to the full-length enhancer N-3 (wt N-3) in the same electroporated embryo (*upper panel*). Removal of the enhancer N-3 core sequences from the full-length enhancer N-3 abolished the enhancer activity (*lower panel*). In these embryos, the full-length enhancer N-3-dependent activation of mRFP1 was used to control the mutational effects. Reprinted from Fig. 3 in Inoue, M. et al., 2007. Genes to Cells, 12, 1049–1061, with permission from Blackwell

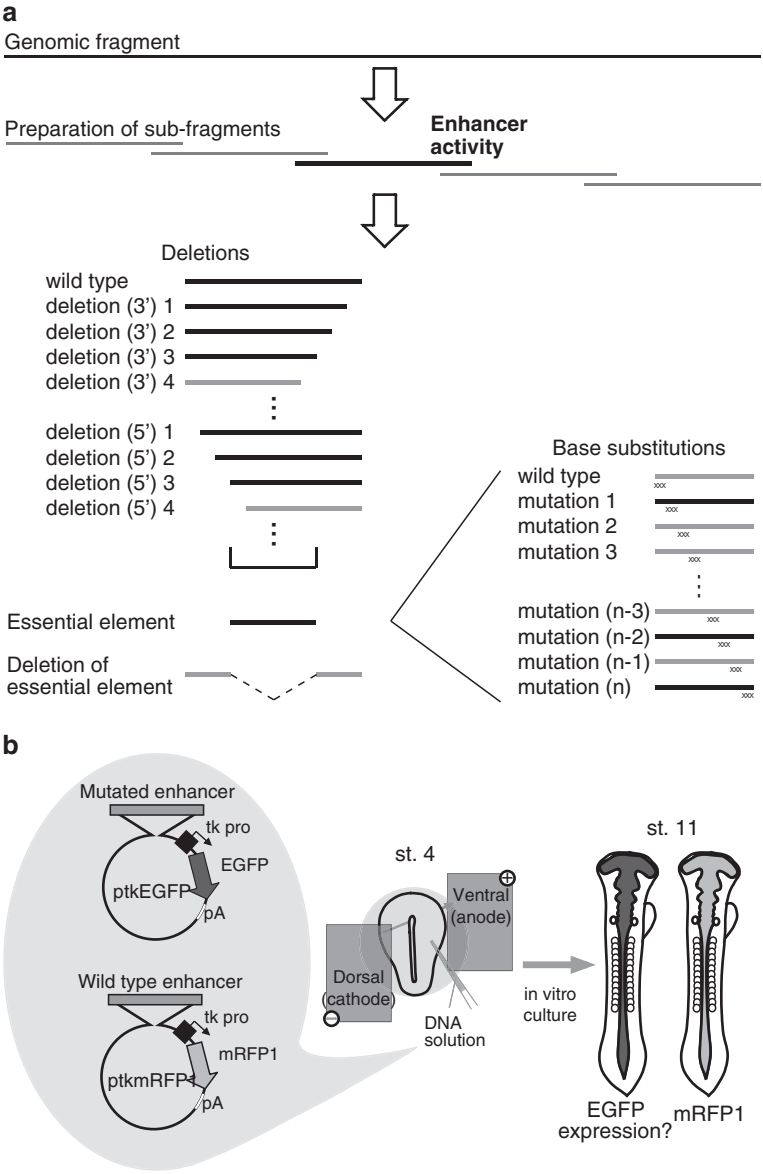

Fig. 7.5 Strategy for analysis of an enhancer. (**a**) The fragment which has activity of enhancer is dissected into sub-fragments step by step. The minimal sequence showing the enhancer activity is subjected to finer analysis using deletions. Removal of essential element (core sequence) from the entire enhancer inactivates the enhancer activity. The genetic elements in the essential element are scanned by introduction of block-wise mutations. In this hypothetical case, black lines indicate the DNA sequences having the enhancer activities and gray lines lacking an enhancer activity. (**b**) The combination of two color reporter vectors is useful to compare the activities between wild type and mutant enhancers in the same electroporated embryo. Reprinted from Fig. 5 in Uchikawa, M. 2008. Dev. Growth Differ., 50, 467–474, with permission from Blackwell

of the reporter fluorescent proteins. The combined action of multiple transcription factors and their synergism can also be demonstrated.

The contribution of cell-exogenous signaling factors that act upstream of the transcription factors can be investigated, by administration of the factor itself or reagents activating or inhibiting the intercellular signaling cascades to the chicken embryo electroporated with the reporter vector. Administration of reagents to embryonic tissue can be done in various ways, placing reagent-soaked beads in an area of cultured embryo, placing a pellet of cells expressing the reagent, grafting embryonic tissue producing the factors, or even bathing the entire embryo in the solution of the reagent (Fig. 7.4b).

Thus, combination of mutational analysis of the enhancer core, analysis of gain-/loss-of function effects of transcription factors, and analysis of the impact of signaling factors on enhancer activity reveals the overall regulatory system operating through the enhancer core sequence.

3 Application and Results

3.1 Before Analysis of Gene Regulation Using Chicken Embryo Electroporation

Endogenous expression pattern of the gene of interest in chicken embryos should be carefully investigated in the first instance. To investigate enhancers of a gene derived from other animal species, in silico analysis of non-coding regions for sequence conservation between the species, plus expression analysis using the original animal species are important. These analyses assist with determination of the developmental stages, embryonic domains and tissues for the reporter vector electroporation and subsequent analysis.

We have investigated the regulation of *Sox2* gene for the interest of its expression associated with the genesis of neural and sensory primordia (Uchikawa et al., 2003). The stage 4 disk-shaped chicken embryos are employed for reporter electroporation, where the DNAs can be introduced in a broad area of a side of the embryo. Numerous enhancers, each having distinct spatio-temporal specificity, have been identified (see below). Many of these enhancers are conserved in their DNA sequences among mouse, chicken, frog and fish, reflecting the similarity of *Sox2* expression pattern in neural and sensory development among species (Mizuseki et al., 1998; Wood & Episkopou, 1999; Uchikawa et al., 2003; Okuda et al., 2006).

3.2 Genomic Distribution of Regulatory Sequences

There is no a priori measure of the potential genomic range of distribution of regulatory regions of a gene. Regulatory regions could be anywhere: 5′ side, 3′ side of

the gene, or transcribed sequences including exons and introns (Kleinjan & van Heyningen, 2005). Thus, unbiased survey for regulatory sequences through the genomic region including the gene is recommended. The *Sox2* gene is located in a genomic region sparse in the gene distribution (sometimes referred to as the "gene desert"), allowing analysis of distribution of regulatory regions without interference by other genes. We find that majority of regulatory regions are located within the 50-kb region centered by the *Sox2* gene, but additional enhancers are also found outside of this 50-kb region, mostly within 100 kb from the gene in the chicken genome (R. Okamoto, unpublished data). This is one ground to recommend the use of a BAC clone to start enhancer analysis. It should be borne in mind that chicken genome is more compact than mammalian genomes, and twice as large genomic area should be investigated using a mouse genomic sequence. Conserved regulatory sequences of the *Sox2* gene are distributed twice to three times as widely in the mouse genome, and half as narrow in fish genomes, roughly reflecting their genome sizes.

3.3 Key Elements in the Experimental Design

To construct reporter vectors for enhancer activity to be electroporated in the embryo, the choice of the promoter is of primary importance. The promoter should not show any activities on its own in electroporated chicken embryo, but should clearly reflect the activity of enhancer inserted in the reporter vector. The tk promoter is proven to satisfy these conditions. *Sox2* promoter can also be used, but tk promoter more sharply responds to *Sox2* enhancers. The hsp68 (heat-shock protein 68) promoter, which is frequently used in transgenic mouse studies, shows too high basal activity in electroporated chicken embryos.

The choice of reporter proteins is another key factor in the analysis of enhancers. Fluorescent proteins are excellent in reporting the enhancer activities in living embryos. Various fluorescent proteins are now available that allow multicolor analysis in live embryos: EGFP for green fluorescence, Venus for yellow fluorescence, mRFP1/mCherry for red fluorescence, and AmCyan1 for cyan fluorescence, with sufficient brightness and relatively fast maturation time. The combination of EGFP and mCherry for two color fluorescence analysis, and that of AmCyan1, Venus and mCherry for three color analysis is recommended for simultaneous detection of multiple enhancer activities. Enhancer reporter vector to express EGFP may be co-electroporated with an mCherry-expressing non-specific vector that ensures successful electroporation of embryo. Co-electroporation of enhancer reporter vectors separately carrying wild type and mutated enhancers and encoding fluorescent proteins of different colors is used for assessing mutational effects. The fluorescence level of reporter proteins can be quantified, e.g. using a software-equipped microscopic CCD camera, or using tissue extracts and a 96-well fluorescence reader. Examples of multicolor analysis are shown in Fig. 7.4.

The size of reporter vector affects the efficiency of embryo electroporation. The entire length of the vector including the inserted enhancer sequence should be less

than 10 kb. Larger sizes of DNA precipitously decrease electroporation efficiency. Using ptkEGFP or related enhancer reporter vectors of 4–5 kb backbone size, insertion of genomic fragments of 4–5 kb is thus recommended.

A variety of electrode shapes and setups have been devised to electroporate embryos of various developmental stages and at specific tissue sites. For the purpose of enhancer analysis, consistent efficiency of electroporation is important. A tip for this is to maintain the inter-electrode current constant, as the electric resistance varies considerably depending on the electrode setup and embryo samples.

Observing the above points, electroporation efficiency is satisfactorily high and reproducible, and enhancer analysis can be accomplished with high accuracy. We were thus able to identify many enhancers regulating *Sox2*, each acting with distinct specificity during neural and sensory development, using stage 4 chicken embryos under modified New's culture.

3.4 Utilization of Identified Enhancers

Once a new enhancer is identified, it can be utilized in various ways. As already discussed, dissecting enhancers to find core sequence and determining regulatory elements comprising the enhancer core helps characterization of interacting transcription factors and of upstream signaling systems.

Another use of the enhancers will be as tools for gene manipulation. The enhancers may be used as drivers of Cre recombinase or vertebrate-adapted Gal4-UAS system. They may also be employed for tissue- and stage-specific gene knockdown using RNAi expression vectors.

3.5 Utilization of Genome Information

The genome sequences of many vertebrate species have recently been determined and are available through websites, e.g. Ensembl (http://www.ensembl.org/). These sequence data are very useful in identifying and analyzing the regulatory regions. Phylogenetically conserved non-coding sequences are candidates of regulatory sequences, and in fact, some, though not all, of the conserved sequence blocks are identified as the enhancers (Bejerano et al., 2004; Woolfe et al., 2005). Thus, a haste approach would be to amplify the conserved sequences using specific primers and PCR and test them for an enhancer activity using the enhancer reporter vector. Chicken-mammal genomic comparison is generally favorable in predicting candidate regulatory regions for their appropriate phylogenic distance (Uchikawa et al., 2003, 2004; Izumi et al., 2007). However, it should be remembered that enhancers are not always conserved, and conserved sequences do not necessarily have regulatory functions.

Various software applications useful for comparative analysis of genomic sequences are available through websites including: Vista (http://genome.lbl.gov/vista/index.shtml), Dcode.org (http://www.dcode.org/), etc. These software

applications illustrate distribution of the conserved sequence blocks, their genomic localization and degree of conservation (Mayor et al., 2000; Loots & Ovcharenko, 2005). However, their performance depends on parameter setting: scanning unit of base sequence length, sequence similarity index, etc. One should optimize the parameters, usually by try and error, for a particular gene locus, and for the variety of animal species of different phylogenetic distance. These software applications using approximation tools for calculation are not perfect in indicating conserved sequences, and the compute-assembled genomic sequences are not error-free. One should be aware of these pitfalls.

We extracted 1 Mb-long genomic sequences surrounding the *Sox2* gene from genomic databases of chicken and mammals. The comparison of chicken and mammalian sequences (mouse or human) indicates that (1) the *Sox2* gene is far from neighboring genes, (2) distribution of highly conserved and less conserved sequences are much more punctuated than the case of comparison of two mammalian species, and (3) distribution of conserved sequences is more compact in the chicken genome, reflecting its one-third size compacted to mammalian genomes.

Comparison of the genomic sequence flanking the *Sox2* gene in a wide range of vertebrate species, mammal, marsupial, chicken, frog and fish, using the Vista program, is schematically shown in Fig. 7.6, and compared with functionally identified enhancers. The sequences having more than 60% identity over the length of 100 bp were scored as conserved sequence blocks in the comparison of chicken sequence with those of mammals or frog (Xenopus), while the threshold values of 60% identity over 50 bp were taken for comparison between chicken and fish sequences. The functionally identified enhancers using chicken embryo electroporation matched a fraction of the conserved sequence blocks.

Comparison of chicken, opossum, mouse and human genomic sequences indicates that mouse sequence uniquely lacked some of the phylogenetically conserved sequence blocks, namely, conserved sequence blocks Nos. 3, 21 and 25. Xenopus genome also possessed the majority of the conserved sequence blocks found in amniotes. In particular, enhancer L active in lens fibers is uniquely conserved between chicken and Xenopus genomes, consistent with the occurrence of *Sox2* expression in ocular lens fibers in these animal species (Kamachi et al., 1998; Schlosser & Ahrens, 2004). The conserved sequence block No. 11, known to be active as an enhancer in ES cells (Tomioka et al., 2002), is conserved only among amniotes. In fish genomes, conservation of the sequence blocks is more limited and appears to depend on the fish species. The sequence blocks are more clearly conserved in zebrafish than in tetraodon. In addition, enhancer N-1 sequence is not found in fish genomes, while other early neural enhancers (N-2, N-3, N-4 and N-5) are conserved throughout the vertebrate species. This genomic feature of fishes reflects the fact that *Sox2* expression is restricted to anterior neural plate in fish, in contrast to pan-neural expression of *Sox2* in higher vertebrates. These observations indicate that variation of the conservation of individual sequence blocks reflect the divergence of regulation of a gene in individual species. Thus, comparative genome studies focusing on regulatory sequences are revealing the evolution of the diversity in the gene regulation.

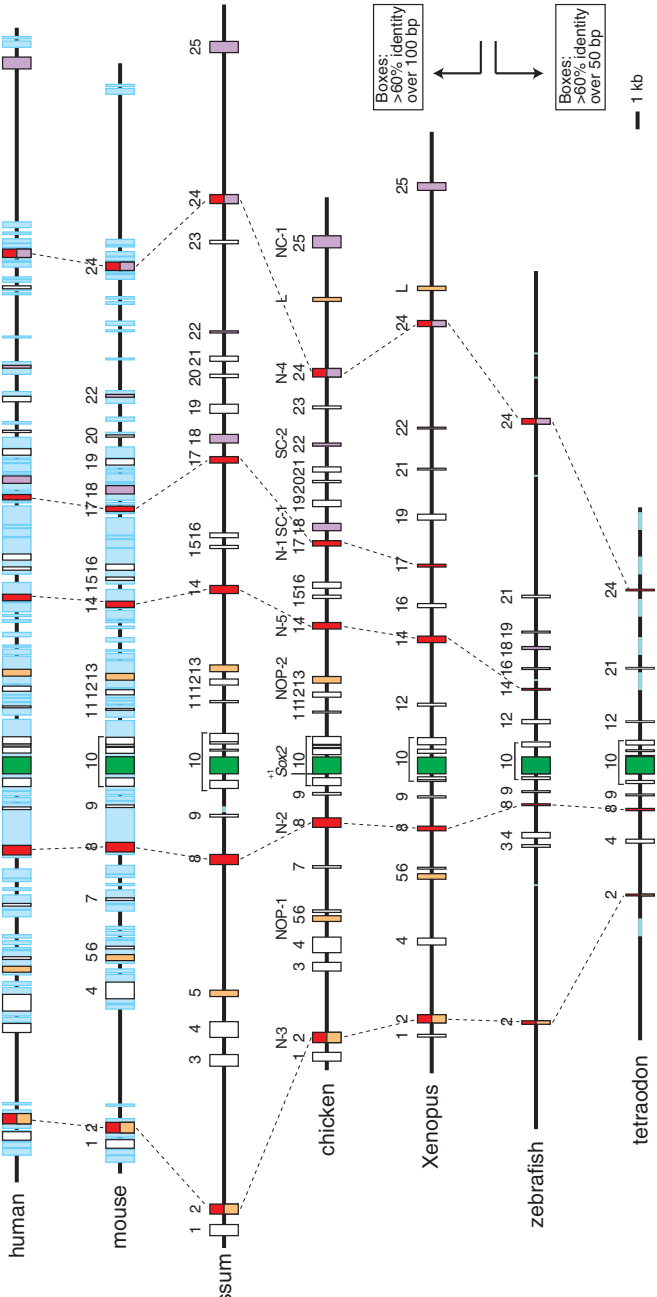

Fig. 7.6 Distribution of conserved sequence blocks and functionally identified enhancers surrounding the *Sox2* gene in various vertebrate genomes. The conserved sequence blocks between chicken and other vertebrates were determined using the Vista program, and are schematically shown as boxes with 60% sequence identity over 100 bp long between chicken and mammals, marsupial or frog, while those with 60% sequence identity over 50 bp long between chicken and fishes. The *Sox2* coding sequence (*single exon*) is shown in green. The colored boxes, except these in blue, indicate their possession of enhancer activities in early/late neural tissues (*red/purple*) and cranial placodes (*orange*). Blue boxes indicate the sequences conserved between human and mouse genomes scored using the same criteria. The conserved sequence blocks are numbered in each species and the abbreviated names of enhancers are indicated on the chicken genome. The nucleotide sequences shown by the blue lines have not determined yet. Modified from Fig. 6 in Uchikawa, M. et al., 2003. Dev. Cell, 4, 509–519, with permission from Elsevier

4 Comments

4.1 Future Perspective

The electroporation technique applied to chicken embryo is very powerful for identification of enhancers and investigation of genetic regulatory mechanisms. The chicken embryo having various advantages in developmental study has gained additional merits by introduction of the electroporation technique. In this chapter, the merit in enhancer analysis was emphasized, but application of the electroporation technique to manipulation of gene expression, based on recombinases, Gal4 type activators, siRNAs and transposon-dependent genomic insertion of vectors holds promise to the future of developmental studies. A limitation of the technique at present is the size limit of DNAs to be electroporated, which is 10kb. Improvement of the gene transfer method allowing efficient introduction of BAC-size vectors to embryonic tissues will further widen the versatility of the technique.

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Chapter 8
Electroporation of Nucleic Acids into Chick Endoderm Both In Vitro and In Ovo

Kimiko Fukuda

1 Introduction

During gastrulation, vertebrate embryos generate three different germ layers, the ectoderm, mesoderm and endoderm. The endoderm layer is situated in the most ventral part of the embryo and differentiates into various tissues including the gut, respiratory and endocrine epithelium. In amniotes, the endoderm spreads out in a sheet-like manner and forms the most ventral layer in the early embryo. Subsequently, the anterior-most endoderm folds ventrally and forms a sack-like structure, the foregut. As the foregut extends posteriorly, the endoderm at the most posterior portion of the embryo forms another sack-like structure, the hindgut, and this grows rostrally. Finally, the foregut and hindgut meet at the level of the small intestine and form a simple tube. After the formation of tube, the endoderm becomes the lining epithelium of the gut and differentiates into various organs according to their position along the anterior–posterior and the dorsal-ventral axes. These include the esophagus, lung, stomach, duodenum, pancreas, liver, small intestine and large intestine. These organs show specific morphologies and express particular factors depending on their function (Wells and Melton, 2000).

It has been well established that the differentiation of the endoderm is controlled by the surrounding tissues, principally the mesodermal mesenchyme (Fukuda and Yasugi, 2005; Kim et al., 1997; Yasugi and Fukuda, 2000). Hence, endodermal differentiation is regarded as a model example of tissue interaction during development. As an example of the types of processes that occur during endodermal differentiation, the presumptive dorsal pancreas precursor cells, which reside at the midline of somite level 4–7 in the endodermal layer of the stage 10 chick embryo (Matsushita, 1999), facilitate dorsal pancreatic morphogenesis. In addition, these cells maintain the expression of early pancreas genes via the activity of activin and bFGF secreted from the notochord at around embryonic stage 12 through the repression of Shh (Hebrok et al., 1998). At a later stage, the pre-pancreas endoderm

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is separated from the notochord by the dorsal aorta. It has also been reported that vascular endothelial growth factor (VEGF) secreted from the dorsal aorta is a key molecule that upregulates insulin expression within the pancreatic endoderm (Lammert et al., 2003). After pancreas bud formation, activin is secreted from the surrounding mesenchyme whereby it induces exocrine cells and prevents an endocrine cell fate arising from the pancreas endoderm (Kumar and Melton, 2003). To further understand the processes of endodermal differentiation, it will be necessary to elucidate the chronological order of the multi-step tissue interactions that take place between endoderm and the surrounding tissues.

The molecular systems underlying endoderm differentiation remain largely unknown. One of the reasons for this is the positioning of the endoderm and endodermal organs. The endoderm is situated at the most ventral position of the embryo, and endodermal organs develop inside the body at later stages. Hence, both molecular and anatomical analysis of these structures without dissection is more difficult in comparison with exposed tissues such as the limb and the neural tube. As a consequence of this, only a limited number of endodermal mutants have been identified when compared with the ectoderm or mesoderm.

Electroporation is a relatively simple and effective transfection method that can deliver nucleic acids into the chick endoderm. Both in ovo and in vitro electroporation methods have now been established in combination with whole embryo culture, New culture, and organ culture. We herein review these methods and some of the results obtained from transfecting the chick endoderm of various stage embryos in this way.

2 Procedure

2.1 In Ovo Electroporation

Chick embryos that had undergone 20–24 h of incubation are exposed by making an opening at the sharp edge of the eggshell. 5 mg/ml DNA with 1% Fast Green in TE is then injected into the subgerminal cavity under the embryo. An anode electrode in the form of a tungsten needle is next inserted below the embryo and a 2 mm^2 platinum plate cathode electrode (Nepa gene, CUY701P2L) is held over the embryo using a micromanipulator. Five 10 V square pulses of 50 ms duration are subsequently applied at 900 ms intervals using a CUY21 electroporator (Bex) (Fig. 8.1a–c). Following electroporation, the eggs are sealed with tape and incubated for a further 24 h.

Chick embryos at between the 18- and 25-somite stage (stage 13–15 on the Hamburger and Hamilton [HH] stage; (Hamburger and Hamilton, 1951)), can be obtained via a 54 h incubation at 38°C. A hole is then made in the shell on the air chamber side and a window is cut into the top of the egg. A 2 mg/mL solution of DNA in 50 μg/mL Nile Blue Sulfate is then injected into the blastocoel. A few drops of PBS are added to the top of the embryo to establish an even electrical

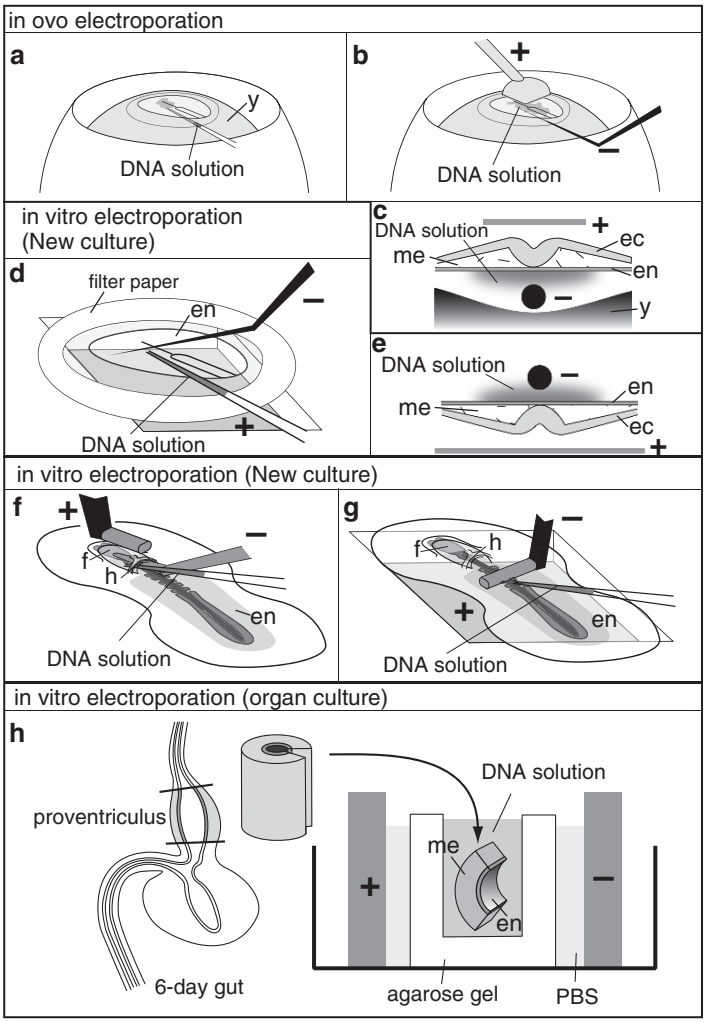

Fig. 8.1 Schematic representation of electroporation methods for transfecting nucleic acids into the chick endoderm. For electroporation in ovo, two steps are involved: injection and electroporation (**a**, **b**). An illustration of the transverse section of the embryo is shown in (**c**). (**d**, **e**) In vitro electroporation using New culture. In the early chick embryo, the endoderm is situated in the most ventral position as a sheet, and electroporation is therefore carried out in a ventral-up position (**d**). (**e**) A schematic illustration of a transverse section of the embryo. (**f**) In the somite stage embryo, the foregut has formed and the DNA solution can be injected into this region. For electroporation into the hindgut, which is still an open sheet-like structure, the apparatus used is similar to that described for the early endoderm (**g**). (**h**) In vitro electroporation combined with organ culture. A well consisting of an agarose gel containing the DNA solution is used to transfect isolated tissue fragments. Note that the gaps between the electrodes are filled with phosphate-buffered saline. +, cathode electrode; –, anode electrode. Abbreviations: ec, ectoderm; me, mesoderm; en, endoderm; f, foregut; h, heart; and y, yolk

current. A negative electrode is next inserted below the embryo parallel to its anterior–posterior axis, and a positive electrode is held by a micromanipulator in a position parallel to and above the embryo but in contact with the PBS solution. Three square pulses of 17 V and of 50 ms duration are next applied to the embryo using a BTX T-820 square wave electroporator. After electroporation, the eggs are resealed with tape and placed at 38°C for 24 to 96 h.

2.2 Electroporation Combined with Modified New Culture

Fertilized hens' eggs are incubated at 38°C for 12–24 h to obtain HH stage 2–5 embryos. These embryos are then explanted in Pannett–Compton saline (Pannett and Compton, 1924) using a modified version of the New culture method (Stern and Ireland, 1981). Embryos with or without ring-shaped filter paper are placed in a ventral-up position on a Petri dish containing a 2 mm^2 platinum plate electrode with a 1 mm deep embankment well. 5 mg/ml DNA in 5% sucrose and 1% Fast Green is injected into each embryo according to the chick endodermal fate map (Kimura et al., 2006). A 2 mm^2 platinum plate can be used as the anode electrode and five 6–8 V square pulses of 50 ms duration are applied at 900 ms intervals using a CUY21 electroporator (Fig. 8.1d, e). Embryos with or without filter paper are then transferred onto a agarose gel with egg white or a vettellin membrane, respectively, and incubated at 38°C for up to 24 h.

To introduce a foreign gene into the ventral foregut chick endoderm at st.10–12, embryos at 2 days of incubation are isolated from eggs using New's methods. Embryos are then placed in the ventral-up position on an agarose gel containing egg white. A 5 mg/ml DNA solution in 1% Fast Green/TE is then injected into the foregut, into which a tungsten needle anode electrode is subsequently inserted. A platinum cathode electrode of 1 mm in length (Nepa gene, CUY613P1) is then held over the heart using a micromanipulator. Five 6–8 V 50 ms square pulses are then applied at 900 ms intervals using a CUY21 electroporator (Fig. 8.1f). After a brief washing of the foregut with Pannett–Compton saline, the embryos are incubated for a further 24 h.

To introduce DNA into the hindgut at st.10–12, chick embryos isolated using the New culture method are placed onto a 2 mm^2 platinum plate anode. After the DNA solution is injected onto the embryo, a 1 mm platinum cathode is used (Nepa gene, CUY613P1) and electroporation is carried out as described for the foregut.

2.3 Electroporation Combined with Organ Culture

Electroporation of plasmid DNA into the proventriculus (PV) epithelium has been previously described (Sakamoto et al., 2000). Briefly, platinum electrodes are fixed onto a glass dish and integrated into a resin chamber (7 mm in height, 8 mm in

width and 5 mm in length; Nepa gene, CUY520P5). A vessel containing a 1.5% agarose gel in Tyrode's solution is then placed into an electrode chamber to which a 14 µl aliquot of plasmid DNA in Tyrode's solution is added. The outside of the gel vessel is then filled with Tyrode's solution. Isolated tissue fragments are dissected and placed into the vessel with their epithelial sides facing the cathode to allow the introduction of DNA into the epithelium. For optimal transfection, 50 ms pulses of 30 V are applied 10 times for a stage 28 PV and 15 times for stage 29 PV over 75 ms intervals using CUY 21 (Fig. 8.1c). The tissues are then immediately washed with Tyrode's solution and cultured for the appropriate time. Explanted PV tissues are then laid onto a Nucleopore filter (0.8 µm) which is placed onto a stainless steel grid which is inserted into one well of a 24-well culture dish (Falcon, 3047). Culturing is then performed at a medium-gas interphase in 5% CO_2 and 95% air at 37°C. The culture medium used is 199 with Earle's salt containing an equal volume of embryo extract prepared from stage 38 embryos (Takiguchi et al., 1988).

3 Application and Results

3.1 In Ovo Electroporation

The first report of a successful DNA transfection into a chick endoderm using in ovo electroporation was described by Grapin-Botton and her colleagues (Grapin-Botton et al., 2001). The chick embryos were electroporated at stage 13–15, at which point there are between 18–25 somites. This previous study of Grapin-Botton focused on pancreatic development, particularly in relation to the function of Pdx-1 which is a transcription factor expressed specifically in the pancreas epithelium in chick. The authors introduced *pdx-1* into the gut epithelium around the pancreas, which includes outside of the normal expression domain for this gene. The chick embryos in these experiments were incubated for either 2 or 3 days, at which point the dorsal and ventral pancreas buds start to form and the pancreas epithelium begins to show blanching morphogenesis, respectively. In subsequent analysis, the ectopic expression of *pdx-1* was detected only in the endodermal epithelium. Cells overexpressing *Pdx-1* began to invaginate into the lumen and form a pancreas bud-like structure during the incubation period. This result suggests that *Pdx-1* plays a vital role in pancreas bud formation. In addition, whereas overexpressed *Pdx-1* was found to induce the expression of *Hlxb-9*, which is normally detected in the dorsal gut tube and later restricted to the beta-cell lineage, these cells never fully differentiate into endocrine or exocrine cells as demonstrated by their inability to express insulin, glucagon or carboxypeptidase A during 48, 72 and 96 h of incubation after electroporation. This strongly suggests that additional transcription factors other than pdx-1 and Hlxb-9 are required for the complete differentiation of endodermal cells into endo- and exocrine cells in the pancreas.

Also in the report of Grapin-Botton et al., there was an examination of the relationship between *pdx-1* and the expression in the gut epithelium of *cdxA* (a small

intestinal epithelium marker), *sox2* (an esophageal and stomach epithelium marker) and *hex-1* (a liver epithelium marker) using in ovo electroporation. At the developmental stage examined for the chick embryo in these analyses, these transcription factors establish graded expression boundaries in the gut. Ectopically expressed *pdx-1* in the esophagus and anterior stomach induced the down-regulation of *sox2*. *Pdx-1* expression in the small intestine and duodenum also suppressed *CdxA* and *hex-1*. These data indicated that *Pdx-1*-expressing cells promote a pancreatic cell fate. To examine the initiation of pancreas regionalization in the chick endoderm, stage 13 is too late as the major regions of the gut epithelium have already regionalized at this point and begun to express specific regional transcription factors, such as Hex-1, Nkx2.1 (a lung epithelium marker), pdx-1, CdxA, CdxB and Sox2.

In their previous study, Dessimos and co-workers have elucidated the role of FGF signaling in endoderm regionalization at earlier stages of embryogenesis in chick (Dessimoz et al., 2006). The authors in this case ectopically expressed FGF4 in gastrula stage embryos using heparin agarose beads. The data presented revealed that the exposure of the chick endoderm to recombinant FGF4 protein results in an anterior shift in the *Pdx1* and *CdxB* expression domains. Moreover, these expression domains remain sensitive to FGF4 levels throughout the early somite stages. Exogenous FGF4 was also found to repress the anterior endoderm markers *hex-1* and *Nkx2.1* and disrupt foregut morphogenesis. In additional experiments in this same report, the ectopic expression of dominant-active (da) *FGFR1* was achieved specifically in the foregut epithelium by electroporation of gastrula stage embryos. The expression of da-*FGFR1* was found to induce the ectopic expression of *Pdx-1* in the foregut endoderm. This finding revealed that FGF signaling controls endodermal patterning directly and not via a secondary induction mechanism from another germ layer. These data therefore show that FGF signaling is critical for the patterning events in the gut tube by promoting a posterior but inhibiting an anterior endoderm cell fate.

3.2 In Vitro Electroporation

Although in ovo electroporation is a powerful tool for elucidating the molecular mechanisms underlying endodermal patterning, the method is hard to apply to studies which needs more restrict and controlled transfection into the endoderm. For example, most anterior organs of the digestive tract, i.e. the esophagus, pharynx, stomach, liver, and pancreas, originate from the foregut. The foregut is also the source of the respiratory organs such as the lung and trachea, and some endocrine organs. Despite the importance of the foregut however, there are few studies to date that have focused on the specification mechanisms that operate inside this region. The presumptive ventral foregut endoderm situates at the anterior-most narrow area. It expands to form the foregut and restricts the fate decisions for each organ during foregut development. For example, the expression of the earliest liver marker gene, *hex-1*, is first detectable at the gastrulation stage in the anterior most endoderm,

a structure which gives rise to whole ventral foregut including the presumptive liver cells. The expression of *hex-1* is then restricted to the posterior-most ventral foregut until stage 10 in chick development (the 10 somite stage). This suggests that regional gain-of function and loss-of function analyses are required to elucidate the mechanisms that regulate fate selection in the foregut endoderm. Experimental designs that combine the precise fate maps of the chicken endoderm (Kimura et al., 2006) and electroporation of in vitro cultures, such as New culture, could more precisely control the area into which DNA or RNA is transfected. Hence, this approach has the potential to accurately delineate the functions of the signaling molecules and transcription factors that are expressed in the foregut (Kimura at al., unpublished data).

In vitro transfection methodologies can be applied to later stage chick embryos in which organogenesis is occurring in the gut tube. We have established an organ culture system in our laboratory for gut tissue fragments of 6 day old chick embryos. At this stage of development, the various regions of the gut tube are distinguishable but still undergoing cyto-differentiation. As an example of this in chick, the stomach is subdivided into the rostral proventriculus (PV, glandular stomach) and the caudal gizzard. Both the PV and gizzard have similar structures in 6 day old embryos, i.e. a simple columnar epithelium surrounded by mesodermal mesenchyme, but the PV epithelium begins to invaginate in a sequential manner into the surrounding mesenchyme to form simple glands after this stage. During this process, homogenous epithelial cells in the PV adopt either a glandular or a luminal fate, which is characterized by the expression of *smad8* (early stage)/ *embryonic chicken pepsinogen gene* (*ECPg*) (later stage), and *chicken spasmolytic polypeptide* (*cSP*), respectively (Yasugi, 2000). It has also been well established that epithelial–mesenchymal interactions are important for the differentiation of the PV epithelium (Fukuda and Yasugi, 2005; Yasugi and Fukuda, 2000). However, the molecular mechanisms that control the onset of binary fate decisions remain unknown.

Notch signaling controls the cell fate decisions that occur during several developmental processes (Campos-Ortega, 1993; Kopan, 2002). We have found in initial studies from our laboratory that the Notch signaling receptors, *Notch1* and *2*, are expressed in the whole PV epithelium during gland formation in chick (Matsuda et al., 2005). Moreover, the expression of a Notch ligand, *Delta1*, was first detected in the uninvaginated epithelium in a speckled manner in 6 day embryos. More Delta1-positive cells were observed in the luminal cells, but not in the glandular cells. To confirm the activation of Notch signaling during gland formation in the PV, we used a Notch reporter construct, pTP-1 venus, which expresses venus (Kohyama et al., 2005) only in cells which delta, serrate and lag2-dependent notch signaling is active. This plasmid was introduced into PV epithelial cells using a combination of in vivo electroporation and organ culture (Sakamoto et al., 2000). The efficiency of transfection was high (above 50%), when 6 day PV tissue fragments were used.

Venus-expressing cells were found in the PV epithelium adjacent to *Delta*-expressing cells. However, although Notch active venus-positive cells could be

detected only in the luminal epithelium after the onset of gland formation, these cells showed no expression of the luminal epithelium maker, cSP. In addition, all Venus-positive cells differentiated into gland cells later. To delineate the function of Notch signaling during gland formation in the PV, we then overexpressed a Notch intracellular domain, CNIC, which activates this pathway (Wakamatsu et al., 1999) into PV epithelium. The resulting Notch signaling-active cells never invaginated into the mesenchyme and never expressed the later gland cell marker, *ECPg*. They also failed to express *cSP* but did express the early gland marker, smad8. From these results, we presented a possible model of fate determination for the endodermal epithelium and also invagination. Our hypothesis is that up to day 6 in the chick embryo, the epithelial cells in the PV are homogenous. At day 6, when some of the cells in the PV epithelium begin to express *Delta1*, Notch signaling becomes active in the neighboring cells. This Notch activation event instructs the endodermal epithelial cells to adopt an immature glandular progenitor fate. The remaining epithelial cells assume a luminal fate under the influence of luminal inducers. Until Notch signaling is subsequently inactivated, the progenitor glandular cells remain immature and express *smad8*. As Notch1 signaling is gradually inactivated, immature glandular cells undergo gland cell differentiation and invaginate into mesenchyme.

At the time of gland formation in the PV, the expression pattern of several transcription factors changes dramatically (Yasugi, 2000). For example, the expression of sox2, which is detectable throughout the uninvaginated epithelium in the 6 day chick embryo, is downregulated only in the gland cells and is thus maintained in the luminal epithelium. In contrast, the transcription factor GATA5 is expressed across the PV epithelium at the onset of gland formation, and after the PV gland has developed is expressed at higher levels in the gland cells compared with the luminal cells. Due to the involvement of these transcription factors in the gland-specific expression of ECPg, the regulatory sequence required for ECPg expression in the PV gland cells was successfully isolated (Fukuda et al., 1995). This 1.1 kb 5'-flaniking region of the *ECPg* gene contains five GATA-binding sites and a Sox-binding site (Watanuki and Yasugi, 2003). Co-transfection of a luciferase reporter construct harboring the *ECPg* regulatory region and a *GATA5*-expression vector into the PV epithelium by in vitro electroporation resulted in increased luciferase activity compared with the control. On the other hand, when this reporter gene was co-transfected with a sox2-expression vector, reporter activity was reduced significantly (Sakamoto et al., 2000). These results suggested that *GATA5* and *Sox2* regulate the expression of *ECPg*, by activation and inhibition, respectively. Further investigations using gel-shift assays, and deletion and mutation analysis (Sakamoto et al., 2000; Watanuki and Yasugi, 2003) of the *ECPg* regulatory region, showed that three GATA-5 binding sites flanking the transcription start site of this gene are critical for its activation. These findings strongly suggested that GATA5 directly binds these three sites and controls the expression of *ECPg*.

Electroporation is a relatively easy and robust way of introducing nucleic acids into chick embryos and has thus become an important technique for analyzing the molecular mechanisms underlying vertebrate organogenesis. Most notably

in the neural tube and brain, analyses of the molecular mechanisms involved in regionalization have been very revealing using this system. In this regard, studies of the endoderm differentiation using electroporation have lagged behind those of the neural system. Recently however, electroporation into endodermal cells has been established both in ovo and in vitro in chick. This has enabled us to control the introduction of DNA and RNA into the endoderm in a spatio-temporal manner. Hence, this methodology will facilitate the elucidation of the mechanisms underlying endodermal differentiation for which complex epithelial–mesenchymal interactions are known to be required.

4 Comments

4.1 In Ovo vs. In Vitro Methods of Electroporation in the Chick Embryo

There are two electroporation techniques that can be used for the introduction of exogenous DNA or RNA into the chick endoderm, i.e. an in ovo and in vitro methods. We outline some of the characteristics of each method below.

In ovo electroporation is relatively easy way to introduce DNA into a chick embryo. No special medium or culture apparatus is required and the handling times are very short. The plasmids introduced by this method are also generally active for a few days. In addition, the electroporated embryo can theoretically be cultured until hatching so that the effects of the introduced gene could be analyzed even at the later developmental stages. Importantly also, the transfected areas such as the rostral, caudal, peripheral and middle regions of the embryo, can be more or less controlled.

Electroporation combined with in vitro culturing needs additional preparation to isolate embryos or tissues prior to transfection. An advantage of this technique is that the embryos are much easier to observe in detail and it is therefore possible to transfect a specific area which gives rise to a limited organ or tissue based upon knowledge of fate maps in chick (Kimura et al., 2006; Matsushita, 1999; Matsushita et al., 2002). Although the embryos or tissues are cultured only for a limited time using in vitro culture system, time course analyses and multiple observations are possible.

4.2 Promoter Specificity

It must always be noted that some promoters function very differently in the endoderm compared with the neural system. For example, the pMT23 vector (Wong et al., 1985) fails to express its gene inserts in the endoderm but does so very efficiently in neural tissues. Thus far, the vectors pCAGGs (Niwa et al., 1991) and

pCAb-IRES-eGFP (McLarren et al., 2003) have been shown to work very well in the chick endoderm. In addition, the thymidine kinase promoter, which is used often to analyze enhancer regions because of its low transcription activity, is very active in endoderm and we therefore do not recommend the use of this promoter for analysis of endoderm gene enhancers.

4.3 siRNA

To better clarify the functions of gene expressed in endoderm, loss-of-function analyses of target genes are essential. Nakamura et al. have previously reported the use of a vector-based small interfering RNA (siRNA) system carrying short hairpin RNA (shRNA) (Nakamura et al., 2004). To date however, the expression of specific genes in the chick endoderm has not been found to be significantly affected by transfection of targeting shRNAs (data not shown). Moreover, we have found that a commercially available mouse RNA polymerase III promoter does not work either in the endoderm. Other methods may thus prove to be useful in this context, such as for example the transfection of chemically synthesized siRNAs (Pekarik et al., 2003; Rao et al., 2004) or the introduction of RCAS retroviral vectors to deliver shRNAs (Das et al., 2006; Harpavat and Cepko, 2006).

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Chapter 9
Electroporation into the Limb: Beyond Misexpression

Takayuki Suzuki and Toshihiko Ogura

1 Introduction

Limb development has been studied for over 100 years by several generations of developmental biologists. The developing limb is one of the best models with which to study pattern formation in vertebrates. We have used chick limb development to answer a simple but basic question, namely, why heterogeneous tissues are formed at correct positions and times from a homogeneous population of cells (Pearse & Tabin, 1998).

Limb development starts as two pairs of tissue bulges in the lateral plate mesoderm (LPM). These are called the forelimb and hindlimb fields (Fig. 9.1). After limb initiation, one can clearly identify three-dimensional axes in the limb buds: the proximal-distal (PD; from shoulder to fingers), dorso-ventral (DV; from back to palm), and antero-posterior (AP; from thumb to little fingers) axes. Morphological changes and differences along these three axes are determined by pattern formation during limb bud stages. Following establishment of these axes, one can visually recognize condensation of cartilages. Muscles, tendons, and neurons migrate and differentiate after cartilage formation. Because the stages and events are easily recognized morphologically and in detail, it is therefore the limb bud is an excellent model with which to study the molecular mechanisms of embryonic patterning and tissue differentiation in vertebrates.

1.1 History of Basic Experimental Strategy

One of the most fundamental discoveries in the study of limb development was based on the pioneering use of tissue transplantation within limb buds. In 1968, John Saunders Jr. discovered cells in ZPA (zone of polarizing activity) located

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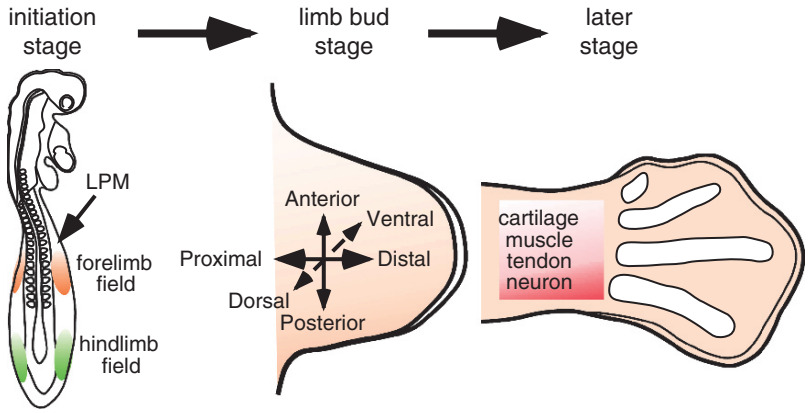

Fig. 9.1 Chick Limb bud development. Forelimb and hindlimb start to develop at the lateral plate mesoderm (LPM). These fields are called the forelimb field and the hindlimb field. After limb initiation, the three axes of the limb bud are specified: antero-posterior axis, proximo-distal axis, and dorso-ventral axis. At later stages, cartilage starts to condense. Subsequently, muscle, tendon, and neuron migrate into the limb and differentiate (*See Color Plates*)

at the posterior edge of the limb bud (Saunders & Gasseling, 1968). When he transplanted ZPA cells to the anterior side of the limb bud, a mirror-image duplication of digits was induced in the resulting limb. In addition, the digits induced at the anterior side had posterior digit identities. This result showed that there must be a gradient of diffusible molecule(s) which determine positional information along the AP axis of the limb bud, with a higher concentration in the posterior region and a lower one in the anterior. In 1993, it was reported that the *Shh* gene is expressed in the ZPA region (Riddle et al., 1993) (Fig. 9.2). SHH is a secreted molecule, and implantation of beads soaked in SHH at the anterior side of the limb bud induced the same mirror-image duplication as that observed for the transplantation of the ZPA cells (López-Martínez et al., 1995). Thus, SHH was proposed to be a morphogen expressed at the posterior side of the limb bud and which specifies positional values along the AP axis.

In addition to Shh, they have been identified, many other secreted molecules with the ability to influence limb pattern were identified. *Fgf8/Fgf4*, expressed in the AER (apical ectodermal edge), and *Fgf10,* expressed in the mesoderm, were both found to be necessary for the outgrowth of the limb bud along the PD axis (Ohuchi et al., 1997; Sun et al., 2002). *Wnt7a* was found to be expressed only in the dorsal ectoderm, and *Wnt7a* knockout mouse showed ventralization of the limb. Thus, it was inferred that *Wnt7a* specifies the dorsal limb bud identity (Parr & McMahon, 1995). As for Shh, these and other signaling molecules can easily be studied by implantation of protein-soaked beads. A second technique easily used for the study of patterning molecules in the limb bud is the expression of transgenes, described below.

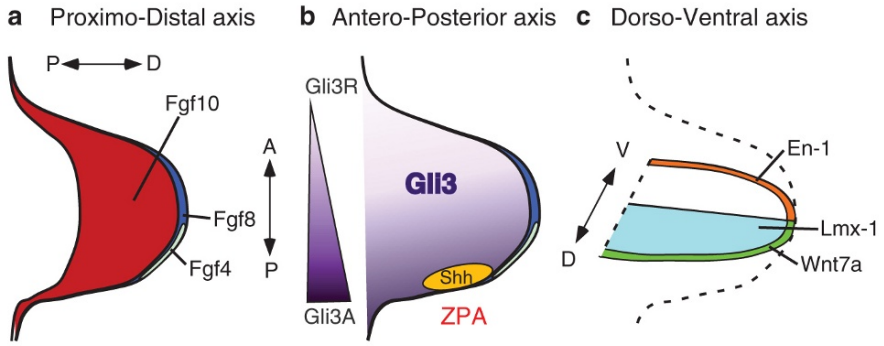

Fig. 9.2 Thee axes in the limb bud. *Fgf8* and *Fgf4* are expressed at the apical ectodermal ridge (AER). In contrast, *Fgf10* is expressed only in the mesoderm. They are each necessary for the other to maintain its expression during limb bud outgrowth along the proximo-distal axis. *Shh* is expressed in the posterior limb bud and establishes antero-posterior limb bud identity through the transcription factor Gli3. SHH signaling inhibits degradation of Gli3, and leads to the formation of full length Gli3, called Gli3A, in the posterior. On the other hand, Gli3 is degraded in the anterior, producing a short form of Gli3, called Gli3R. Thus, a Gli3R–Gli3A gradient exists along the antero-posterior axis on the basis of SHH activity. *Wnt7a* is expressed in the dorsal ectoderm, and *Lmx-1* is expressed only in the dorsal mesoderm. In contrast, *Engrailed-1 (En-1)* is expressed in the ventral ectoderm, where it inhibits *Wnt7a* and *Lmx-1* expression. This combination establishes dorso-ventral polarity (*See Color Plates*)

2 Procedure

Here we detail of protocols to express transgenes into limb by electroporation.

2.1 Preparation of Egg and DNA Solution

Incubate eggs in a humidified incubator (IC800, Yamato) at 39°C until St. 13/14, kept down for easy access. An 18G1/2 needle (NN-1838R, Terumo) or forceps (Dumont No. 5 forceps, 11 cm; No. 500341, World precision instruments) should be used to create a window at the broader edge of the eggshell. ~5 ml of albumin should be gently removed from this hole with a 25 ml syringe (SS-20ESZ, Terumo). Using this procedure, one can procure enough space inside the egg for electroporation.

The pCAGGS (Niwa et al., 1991) or RCASBP (Logan & Tabin, 1998) vector is next diluted with distilled water to a final concentration of 3 to 5 µg/ml and mixed with pCAGGS-EGFP expression vector (0.8 to 1 µg/ml) for electroporation. This concentration of the pCAGGS-EGFP vector is enough to visualize the entire electroporated area (Momose et al., 1999). A cocktail of expression plasmids can then be placed on the cap of a PCR tube (0.2 ml), and mixed with a solution

of 1% Fast Green (diluted in PBS; No. 061–00031, Wako) before electroporation. This electroporation cocktail is then vacuum-extracted by a glass capillary attached to an aspirator tube.

2.2 Injection of DNA Solution into Limb Fields

After opening an eggshell, one can observe the chick embryo under the vitelline membrane. At this moment, a small amount of a Rotring-PBS solution (PBS with 1:40 Rotring; Art-R 591017, Sanford) (approximately 100 to 200 µl) is injected from outside of the sinus terminalis located near the tail bud, using a syringe equipped with a 26G1/2 needle (NN-2613S, Terumo). After bathing the embryo in 1 ml of sterilized PBS, vitelline membrane near either the forelimb or hindlimb field is gently shorn by a sharpened tungsten needle. At St. 14 (the best stage for electroporation into limb buds) 22 pairs of somites are present (Fig. 9.3a). DNA solution is injected into an embryonic space located between the somatic lateral plate mesoderm (LPM) and the splanchnic LPM. An L-shaped platinum cathode is inserted from the hole that was created for injection of the Rotring-PBS solution (Fig. 9.3b), and placed under either the forelimb or hindlimb field (Fig. 9.3c–e).

To inject DNA solution into the forelimb field, the tip of a glass capillary tube is inserted from the anterior side of the forelimb field by pricking the thin embryonic tissue. For injection into the hindlimb field, a glass capillary tip is inserted from the posterior side of the hindlimb field, careful to avoid any damage to the vitelline artery, which typically results in the death of embryo. After insertion of the needle, DNA solution can be injected. After successful injection, one can observe green pigment in the Fast Green-filling forelimb or hindlimb field.

2.3 Electroporation into Limb Fields

We developed three different methods by modifying our original protocol (Ogura, 2002), typically using a platinum anode (Fig. 9.3f).

1. When an anode is placed at the center of either the forelimb or the hindlimb field before electric pulses (8 V, 60 ms pulse-on, 50 ms pulse-off, three repetitions), one can express transgene along the entire limb bud, with strong expression along the middle part of the limb. By positioning an anode at either the anterior side or the posterior side of the limb field, transgene expression becomes restricted to the anterior or posterior portions, respectively.
2. One can expand the domain of transgene expression by moving an anode serially. In this case, the electroporator is set for three pulses (8 V, 60 ms pulse-on, 1 second pulse-off, for three repetitions). For the first pulse, an anode is placed in the anterior limb field. During a 1 s pulse-off pause, this anode is moved to the

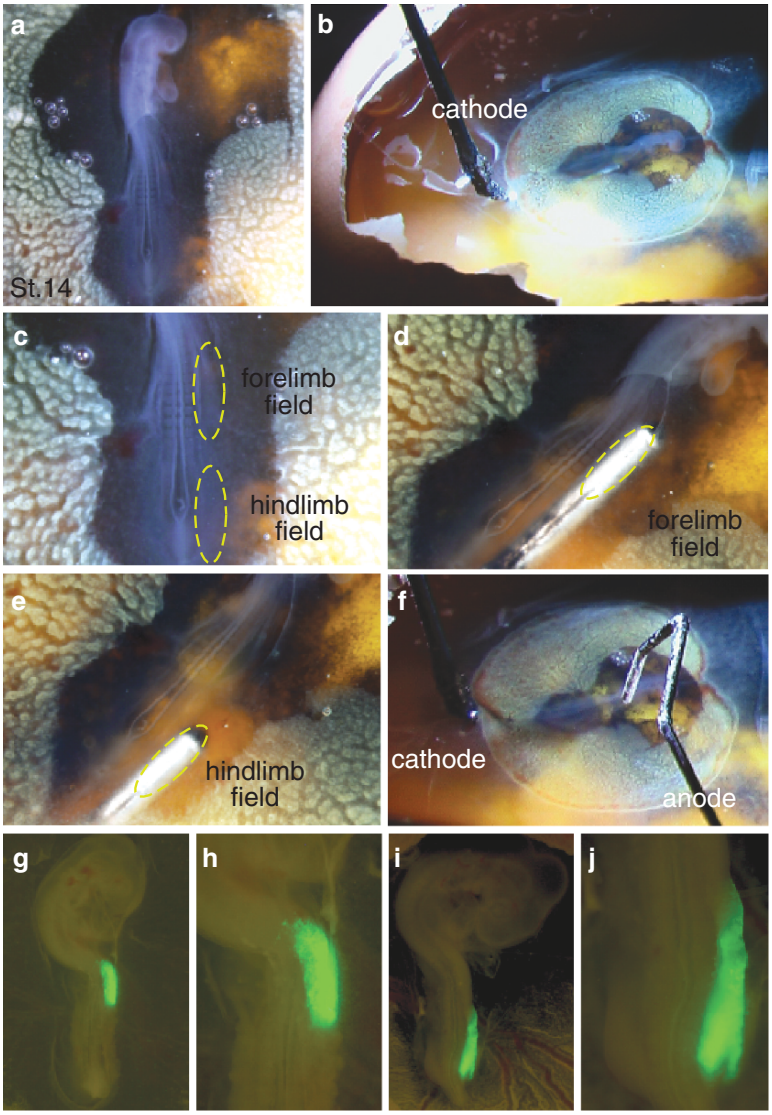

Fig. 9.3 Electroporation into the chick limb field. (**a**) After injection of Rotring-PBS solution, a St. 14 chick embryo is highlighted on the black background. (**b**) A cathode electrode is inserted under the embryo. (**c**) Forelimb and hindlimb fields are shown by a yellow circle. A cathode electrode is placed under the forelimb field (**d**) or hindlimb field (**e**). (**f**) An anode electrode is placed in the forelimb or hindlimb field. (**g–j**) 5 μg/μl of pCAGGS-EGFP was electroporated into the forelimb field (**g, h**) or hindlimb field (**i, j**). EGFP expression is detected at 12 h after electroporation. **h** and **j** show high magnification of **g** and **i**, respectively (*See Color Plates*)

central part of the limb field, and then a second pulse is applied. The anode is moved further to the posterior limb field for the last electric pulse. By applying three electric pulses serially in different parts of the limb field, one can overexpress transgene strongly and uniformly in the whole limb bud.

3. An anode can be placed over the limb field, making a parallel configuration with a cathode for electric pulses (5 V, 60 ms pulse-on, 50 ms pulse-off, three repetitions). In this setting, one can misexpress transgene throughout the entire limb bud. However, lower voltage pulses must be employed, since the electric field formed between two parallel electrodes is wider and a higher voltage in this setting may damage cells.

During electroporation, two electrodes must be kept in solution, not touching the surface of the embryos or the vitelline artery in order to avoid tissue damage. When truncation or shortening of the limb buds is observed, even after electroporation with non-toxic pCAGGS-EGFP (Fig. 9.3g–j), this finding must be replicated in order to confirm the electrodes were free from the embryonic tissues, and not affecting the experimental results.

After electroporation, the cathode is gently withdrawn from the amnion, and 30 µl of a penicillin-streptomycin solution (PBS with 1:100 penicillin-streptomycin; No. 15140–122, Invitrogen) (for 1 L 1xPBS: NaCl 5.8 g, $NaH_2PO_42H_2O$ 0.36 g, $Na_2HPO_412H_2O$ 2.76 g) is used to bathe the embryo. The eggshell window is then sealed firmly with plastic tape. Embryos should be incubated again immediately after sealing.

3 Comments

3.1 Retrovirus and/or Electroporation

In 1992, Morgan et al. showed that misexpression of *Hoxd11* in the chick hindlimb induced posteriorization of digit 1 to digit 2 (Morgan et al., 1992). To overexpress *Hoxd11*, they used the replication-competent retrovirus system (RCASBP). Thus, they introduced a transcription factor into a whole limb bud by retrovirus infection. This experiment made a large impact on chick developmental biology, proving that efficient and widespread misexpression of genes in the limb bud is powerful enough to induce dramatic morphological changes. By using this method, many transcription factors and diffusible proteins were identified as patterning molecules. For example, overexpression of *Lmx1* – a transcription factor normally expressed only in the dorsal mesoderm – was found to result in the dorsalization of muscle and tendon structures in the chick (Riddle et al., 1995). In contrast, misexpression of *Engrailed-1* – normally expressed in the ventral ectoderm – was found to inhibit the expression of *Wnt7a* and *Lmx1* to establish the ventral identity of the limb bud (Logan et al., 1997).

At later stages, *BMPs* (bone morphogenic proteins) *2, 4, 5, 7,* and *GDFs* (growth and differentiation factors) *5* and 6 are expressed around cartilage, and implantation of beads soaked in these proteins promotes differentiation of chondrocytes (King et al., 1996). Similarly, overexpression of a dominant negative BMP receptor 1B by retrovirus infection inhibited its differentiation (Zou et al., 1997). In contrast, misexpression of the transcription factor *Pax3* induced ectopic expression of *MyoD* and subsequent differentiation of muscles at later stages (Bendall et al., 1999).

As evidenced by these results, the retrovirus system is a useful tool for the study of pattern formation and cell differentiation. Although the retrovirus system is powerful in its ability to deliver exogenous DNAs (Morgan & Fekete, 1996), there are some restrictions to its use (Table 9.1). First, the sizes of transgenes need to be less than 2.4 kb in the RCAS system, since longer transgenes inhibit efficient packaging of retrovirus, thereby resulting in a lower titer. Second, it is difficult to obtain high titer viruses when transgenes are toxic to the chick embryonic fibroblasts. Such transgenes include those that inhibit the cell cycle or induce apoptosis.

The biggest disadvantage of this system is the time lag between the injection of virus and the onset of gene expression. After injection, chick cells are infected and a viral genome is integrated into the host genome. Following viral integration, transcription of the integrated transgene initiates, and synthesis of the protein product becomes evident 20 h after injection of the virus solution. Once expression of the viral genome starts, viral particles infect surrounding cells, resulting in spatial expansion of gene expression to the entire limb bud. Although infection is straightforward, expansion throughout the limb bud takes a relatively long time (2 days). Therefore, high virus titers are necessary for robust and extensive expression of transgenes at early stages, such as during initiation of limb outgrowth.

Although retroviral infection is useful, electroporation offers several advantages over the retroviral approach that make it even more powerful and effective (Ogura, 2002). First, there is almost no limit to the size of transgenes, since expression of electroporated transgenes does not require their packaging into viral particles.

Table 9.1 Comparison of electroporation and virus infection

	Electroporation	Virus infection
Size of transgene	No limit	<2.4 kb
Expression level	High	Low
Vector	No limit	RCAS, RCAN
Expression starts from	<3 h	<12 h
Expression area	Whole limb or restricted area	Whole limb
Visualization of experimental area	Yes	Difficult but possible
Damage to tissue	High	Low
Suitable experimental stage	From limb initiation stage	From limb bud stage
Suitable transgene	Transcriptional factor receptors, adaptor protein	Small protein less than 2.4 kb secreted protein

Second, we can use powerful exogenous promoters, such as the CMV or the CMV promoter-based chick beta-actin promoter, to drive expression. Electroporation also allows for a wide range of choice of vector systems. In the limb bud, the pCAGGS vector exhibits a 10 times stronger expression than the RCAS vector. The Xenopus elongation factor promoter exhibits a similar strength in the chick limb bud (Suzuki et al., unpubl. data, 2008). Third, electroporated transgenes are transcribed immediately and expressed as proteins within 3 h. When the RCAS system is used, it takes 20 h for any significant expression of transgenes. In our hands, expression of EGFP at a high level is seen within 6 h after electroporation when the pCAGGS-EGFP expression plasmid is used. This rapid expression of transgenes enables us to study events even at very early stages, such as during the initiation of limb outgrowth. Fourth, in contrast to the wide expansion of transgene expression that occurs with the RCAS system, one can misexpress transgenes in a restricted subdomain of the limb at any position, such as a small portion of the anterior side of the limb. In the case of the RCAS retrovirus system, it is not easy to obtain efficient expression in the limb ectoderm (Suzuki et al., unpubl. data, 2008); the slow proliferation of ectoderm cells makes them relatively resistant to infection and subsequent integration by the viral genome. However, we can overexpress transgenes even in the ectoderm by applying electric pulses after dropping a DNA solution on the surface of the ectoderm (Kida et al., 2004). Finally, one can combine the electroporation and RCAS systems. For example, one can electroporate the RCAS retrovirus vector in the limb. Introduced vector can be integrated in the electroporated cells, resulting in the stable expression of transgenes and production of infective viral particles. When specific pathogen-free eggs are used, the virions produced infect surrounding cells, so that expansion of expression domain can be achieved. In addition, by electroporating a mixture of the RCAS and pCAGGS vectors, one can expect rapid but transient expression from pCAGGS at early stages and stable expression from RCAS at later stages.

By combining the two different methods, with electroporation as a common primary delivery system, one can control expression of transgenes both spatially and temporally. For example, we reported that *Tbx5* and *Tbx4* specify wing/leg identity in the chick limb bud, and that they are also necessary and sufficient for limb initiation (Takeuchi et al., 1999). In the chick embryo, the forelimb bud starts to develop from a restricted part of the LPM at the 15–20 somite level, whereas the hindlimb is formed at the 26–32 somite level at St. 14–16. Before limb bud initiation, both the limb fields and wing/leg identity are specified at the LPM. Therefore, to study limb initiation, we have to introduce a transgene before HH St. 14. Before St. 14, T-box transcription factor *Tbx5* is expressed only in the forelimb field, whereas *Tbx4* is only expressed in the hindlimb field. Takeuchi et al. electroporated RCAS *Tbx5-EGFP* or *Tbx4-EGFP* into future hindlimb or forelimb fields *vice versa*. They must be expressed continuously from initiation stages to later stages, because endogenous Tbx4 and Tbx5 are normally expressed throughout limb bud development. When *Tbx5-EGFP* was electroporated into the prospective hindlimb field, leg morphology completely changed. The scales were transformed to feathers, and the limb had three digits, as seen in the wing. In contrast, misexpression of *Tbx4-EGFP* in the prospective forelimb field induced the opposite transformation

of morphology. Feathers were converted to scales, and, like a leg, the limb had four digits. The phalange structure was also leg-like. The dramatic and revealing quality of these results makes it clear that the electroporation system is an excellent method to study specification of the wing/leg. Furthermore, when *Tbx5-EGFP* or *Tbx4-EGFP* in pCAGGS vector was electroporated at the future frank region, an extra wing or leg was formed, respectively (Takeuchi et al., 2003). Importantly, when *Tbx5* fused with a robust repressor domain from Engrailed (En-Tbx5) was misexpressed at the prospective forelimb field, a wing-less phenotype arose with a loss of a scapula bone (Takeuchi et al., 2003). In this experiment, RCAS En-Tbx5 was electroporated around HH St. 10, 16 h before limb initiation starts. When the prospective limb fields were isolated at HH St. 9 and cultured for 24 h, expression of *Tbx5* and *Tbx4* persisted in the isolated tissues (Saito et al., 2002). This result indicates that the prospective forelimb and hindlimb fields are specified before HH St. 9. Therefore, RCAS En-Tbx5 had to be electroporated as early as possible to inhibit the endogenous Tbx5 function. At the moment, electroporation is the only method that fits the requirements of such experiments.

3.2 Important Reminders About Electroporation

As described above, electroporation is a powerful and excellent method for introducing transgenes into the limb bud and other tissues. But there are several precautions to consider in its use. First, we point out the physical damage induced by electric shocks during electroporation. When the voltage of the applied pulses is high or an electrode is attached to the LPM directly during electric pulses, truncated or malformed limb buds can arise at later stages. Although these artifacts arise only infrequently, it might be difficult to distinguish these artifactual phenotypes from those induced by the transgene. To minimize artifacts, one must be trained enough to obtain normal morphology after electroporation of an EGFP expression vector, which can be used as a negative control and a training plasmid. Second, it is difficult to electroporate an equal amount of DNA in each electroporation experiment. This means that electroporation is only suitable for qualitative analysis, and not quantitative assays.

3.3 New Applications for Electroporation in the Study of Limb Bud Development

Knockout mice are among the most important genetic tools for the study of gene functions, but mice lacking an essential gene may die before the appropriate stage for analysis. In these cases, electroporation provides an alternative method for the study of gene function even in mice.

One possible approach is knocking-down. An RCAS siRNA system was reported to be a useful tool in the study of limb bud development (Kawakami et al., 2003).

In these experiments, the authors inserted a pSuper siRNA promoter and an appropriately designed oligo into the RCAS vector to knock-down *MKP3* and clearly observed a cell death phenotype in the chick limb. If one can construct more effective RNAi vectors, it would be a useful tool even for mice, for which both *in utero* electroporation and *in vitro* embryo culture have recently been established (Tabata & Nakajima, 2001).

Another technique employing the CHAPOL retroviral library was used for analysis of cell lineages in the limb bud (Pearse et al., 2007). The members of this retroviral library each contain an alkaline phosphatase gene, as well as a 24 bp oligonucleotide of random sequence to mark each progenitor cell. After infecting the LPM with these replication incompetent retroviruses, the unique oligonucleotides of individual library members can be used as a tag to identify clonally related cells. Since we can electroporate DNA into a restricted area, it is possible to follow cell fates within a narrow area with this retroviral vector.

In order to be able to detect regional differences in gene activity within the limb bud, we developed an area-specific expression monitoring system. In the RCANBP vector, we inserted an exogenous SMAD1/5/8 responsive promoter to drive expression of the luciferase gene connected downstream. This vector enables us to monitor BMP signaling in the limb bud by measuring the enzymatic activity of luciferase (Suzuki et al., 2008). For normalization, the Renilla luciferase gene driven by the CMV promoter was used. At later stages, when each digit primordium is developing, a dual luciferase assay was performed with small tissue pieces isolated from the limb bud. Differential luciferase activities obtained by this method indicate that each digit primordium has different SMAD1/5/8 activities. This technique can be used for other signaling pathways by changing promoters that drive luciferase or other sensitive reporters.

Another innovative step is the application of electroporation to limb regeneration. It has been shown that nerves are necessary for limb regeneration (Kumar et al., 2007). When salamander limbs are amputated, stumps begin regeneration by forming a blastema. At this time point, regeneration does not occur if the nerves are removed from the limb. Kumar et al. reported that they could re-start limb regeneration even in denervated limbs by electroporating nAG into the blastema of the salamander. This result demonstrates that electroporation is an extraordinarily valuable new tool for the study of limb regeneration.

In the field of limb development, many of the most important genetic and embryological experiments resulted from new technical innovations. As described above, electroporation is one such innovative method. By combining electroporation with other newly developed techniques, new frontiers in modern experimental biology can be opened.

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Chapter 10
Retinal Fiber Tracing by In Ovo Electroporation

Hidekiyo Harada and Harukazu Nakamura

1 Introduction

Axonal tracing techniques are the fundamentals for the investigation of neural circuit formation. In ovo electroporation system allows us to transfect a gene of interest to the desired place in chick embryos (Odani et al., 2008). Recently, Tol2 transposase element, which was originally found in medaka fish (Koga et al., 1996), has been adapted to an in ovo electroporation system (Niwa et al., 1991; Kawakami et al., 1998, 2000, 2004a, 2004b; Kawakami & Noda, 2004; Kawakami, 2005, 2007; Sato et al., 2007). This system assures the integration of the transgene into the genome by electroporation (Niwa et al., 1991; Sato et al., 2007). We applied this system for tracing retinal fibers (Harada et al., 2008). In this chapter, we demonstrate the method of tracing retinal fibers from both small and large groups of the retinal ganglion cell (RGC) with transposon-mediated gene transfer by in ovo electroporation to chick embryos.

2 Principle

The pT2K-CAGGS vector is a CAGGS expression cassette flanked by a Tol2 transposable element that was originally found in medaka fish (Niwa et al., 1991; Koga et al., 1996; Sato et al., 2007). Coelectropoation of pT2K-CAGGS-(cDNA of tracer protein) and pCAGGS-transposase to the optic vesicle results in integration of tracer proteins into the genome. See the chapters of this volume for the detailed principle of in ovo electroporation (Odani et al., Chapter 2, this volume) and of transposon-mediated gene transfer (Sato and Takahashi, Chapter 3, this volume).

We use the enzyme-type tracer and fluorescent protein tracer. The enzyme-type tracer gives good sensitivity, since the signal can be enhanced in the process of

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color development. The fluorescent proteins are easy to observe and to combine with other methods such as immunohistochemistry. Sensitivity of the tracer depends on fluorescence intensity.

Tracer proteins such as GFP may diffuse passively along RGC fibers. At least, a soluble form of EGFP may diffuse from the soma to the terminal of RGC (Fig. 10.1d). Tracer proteins fused with localization signal are also good tools. Tau-LacZ localizes to the microtubule of the axon, which makes it possible to

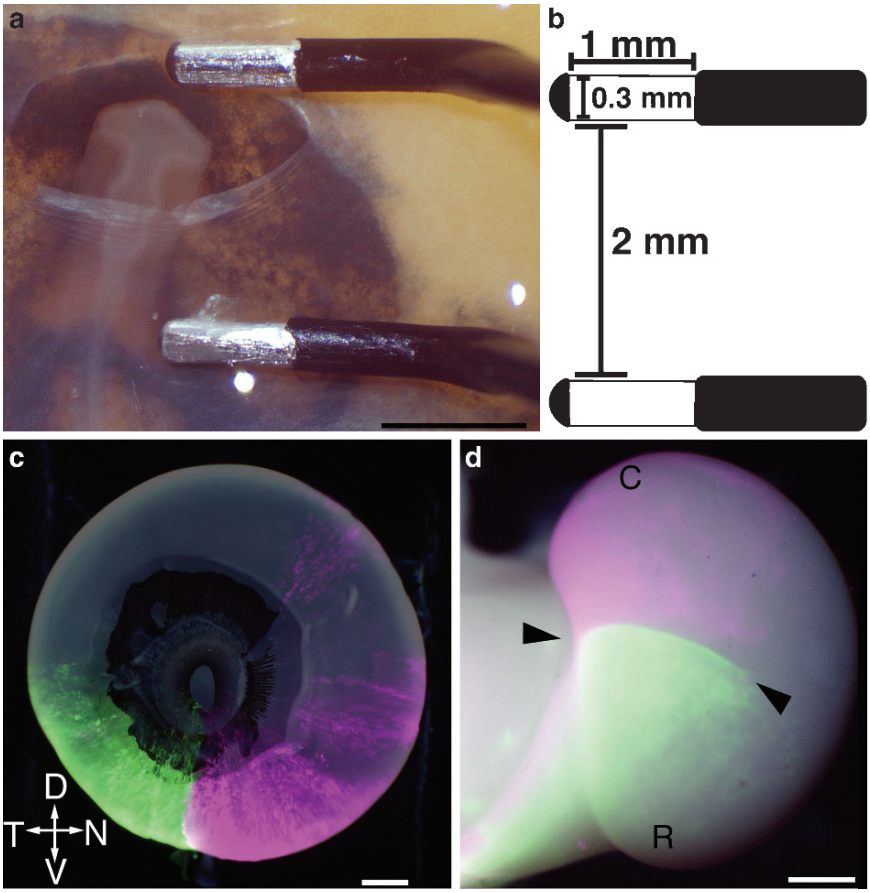

Fig. 10.1 Labeling of large areas of RGC by in ovo electroporation. (**a**) Electrodes on the embryo. One of the electrodes is placed near the anterior side of the optic vesicle and the other is placed just lateral to the otic vesicle. (**b**) Length of the parallel electrodes was 1 mm and the diameter is 0.3 mm. The distance between the electrodes are fixed at 2 mm. (**c**) Retinal whole mount at E15.5. Green fluorescence and red fluorescence were observed after transfection of GFP to the ventrotemporal retina and RFP to the ventronasal retina. (**d**) Tectum whole mount. Sharp boundary of GFP and RFP can be observed between the rostral and caudal tectum (*arrowheads*) (after Harada et al., 2008). D: dorsal; V: ventral; T: temporal; N: nasal; C: caudal; R: rostral; Scale bars: 1 mm (*See Color Plates*)

visualize an axon clearly (Callahan & Thomas, 1994), and GAP-GFP localizes to the plasma membrane, which makes it easy to visualize the cellular outline (Moriyoshi et al., 1996).

3 Procedure

3.1 Preparation of Plasmids

The final concentration pCAGGS-T2TP (transposase) and pT2K-CAGGS vectors that contain EGFP, RFP-monomer-mem (Clontech), or Tau-LacZ (Callahan & Thomas, 1994) is 1 μg/μl dissolved in double distilled water (DDW). Fast green is dissolved at a concentration of 0.025%.

3.2 Chick Embryos

Fertilized chicken embryos are incubated transversely in humid conditions at 38°C for 40–45 hours to reach stage 11 (Hamburger & Hamilton, 1992). A small quantity of albumen is pulled out (1.5–2 ml) from the pointed pole of the egg to aid manipulation. A window is opened on the top shell with surgical scissors.

3.3 Injection of DNA Solution

The vitelline membrane was cut with a microscalpel or sharp tungsten needle. Plasmid solution was injected into the optic vesicle with a micropipette made of glass capillary (GD1: Narishige, Tokyo, Japan). Insertion of a micropipette from the anterior neural pore is the best, but no problems have occurred after penetration of micropipette from the dorsal side of the diencephalon. The plasmid solution filling up the optic vesicle can be checked by fast green.

3.4 Electroporation

3.4.1 Large Group of RGC Labeling

Parallel electrodes (0.3 mm in diameter, 1.0 mm in length, 2.0 mm in distance; Unique Medical Imada) were placed perpendicular to the embryo; one electrode was placed lateral to the otic vesicle and the other anterior to the optic vesicle

(Fig. 10.1a, b). The electrodes were submerged by two or three drops of phosphate-buffered saline (PBS) or Hanks solution. Then, 13 V, 50 ms/s rectangular pulse was charged three times by the electroporator (CUY21, Bex, Tokyo, Japan). For the labeling of retinal fibers with two tracer proteins, a second electroporation was carried out after washing out the plasmid solution by PBS.

3.4.2 Small Group of RGC Labeling

A platinum needle electrode was inserted into the optic vesicle as a cathode and a ball-shaped electrode was placed adjacent to the optic vesicle as an anode. Then, a 7 V, 25 ms/s rectangular pulse was charged twice by the electroporator (Fig. 10.2a).

3.5 Whole Mount LacZ Staining

β-Galactosidase activity on whole-mount embryos was detected as previously described (Suemori et al., 1990; Katahira et al., 2000). For detection of β-galactosidase activity on the whole mount, the retina and the brain were fixed in 1% glutaraldehyde in PBS for 30 min at room temperature and washed three times for 5 min in PBS. Retinal pigment epithelium (RPE) was removed from the retina and the brain was freed from the pia mater before putting into the fixative. Color was developed in the reaction mixture (1 mM $MgCl_2$, 3 mM $K_4[Fe(CN)_6]$, 3 mM $K_3[Fe\ (CN)_6]$, 0.1% Triton X-100, and 1 mM X-gal dissolved in 0.1 M sodium phosphate buffer pH7.5 which contains 10 mM KCl) overnight at 37°C.

4 Application and Results

4.1 Labeling of Large Group of RGC by In Ovo Electroporation

Two weeks after electroporation of pCAGGS-GFP and pCAGGS-RFP, GFP and RFP were observed in the ventronasal and ventrotemporal side of the retina, respectively (Fig.10.1c, see the frontispiece). The GFP/RFP boundary was very sharp on the ventral midline. On the optic tectum, the retinal fiber trajectory was observed clearly in the precise retinotopic manner by green and red fluorescence (Fig. 10.1d, see the frontispiece). The GFP-labeled retinal fibers from the ventrotemporal retina terminated on the dorsorostral side of the optic tectum, and the RFP-labeled retinal fibers from the ventronasal retina terminated on dorsocaudal side of the tectum. A sharp GFP/RFP boundary in the retina was reflected as the rostrocaudal boundary on the tectum.

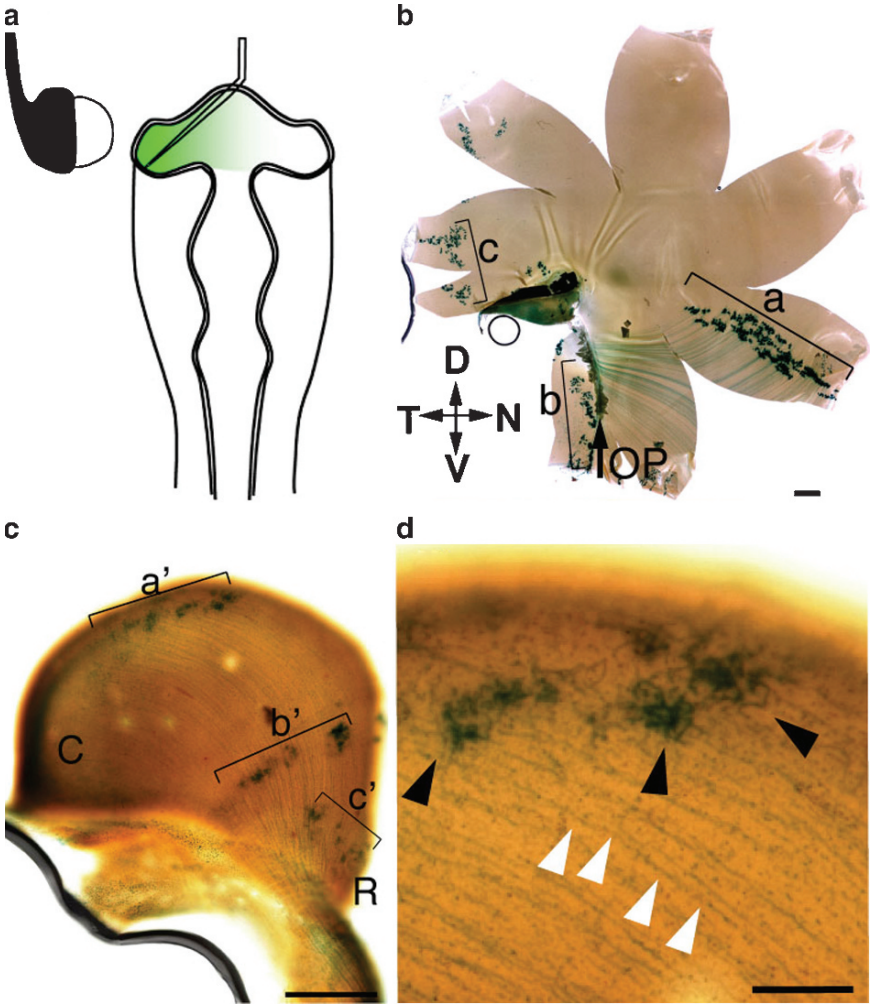

Fig. 10.2 Labeling of local areas of RGC by in ovo electroporation. (**a**) Labeling of a small group of retinal fibers by a needle- and a ball-shaped electrode. A needle electrode (cathode) was inserted in the optic vesicle and a ball-shaped electrode (anode) was put near the optic vesicle. (**b**) Open mount of E13.5 retina. A group of Tau-LacZ-expressing cells and fibers originating from the cells are detected. (**c**) Tectal whole mount. Optic fibers can be traced to the tectum, and TZ is easily recognized because of dense lacZ staining. The a, b, and c on the retina (**b**) may correspond to a., b., and c. on the tectum, respectively. (**d**) Higher-power micrograph around a. of c. Optic fibers and TZ are clearly observed (after Harada et al., 2008). OP: optic pectin; Scale bars: 1 mm (**b**, **c**), 200 μm (**d**)

4.2 Small Group of RGC Fibers

For a small group of RGC labeling, we selected Tau-LacZ as a tracer protein, since Tau is a microtubule-associated protein and localizes to the axon (Callahan & Thomas, 1994). Twelve days after electroporation, a LacZ signal was detected in three small groups in the retina (Fig. 2b, bracket a–c), and terminal zones (TZ) were detected reflecting the retinotopic mapping (Fig. 10.2c, bracket a.–c.). On high-power micrograph, TZ (Fig. 10.2d, white arrowheads) and RGC axons (Fig. 10.2d, black arrowheads) were observed clearly. The size and number of transfection sites in the retina are important for us to infer the TZ of each transfection site. By adjusting the place of the anode (a ball-shaped electrode) and the DNA concentration, we can obtain the desired size and number of transfection in the retina.

5 Troubleshooting

5.1 Fluorescence Photobleaching and Intrinsic Fluorescence Problem

In the process of fixation by 4% paraformaldehyde (PFA) in PBS, GFP and RFP are easily photobleached and intrinsic fluorescence of the embryo is increased. So embryos before fixation are the best in signal-to-background ratio, and it is recommended to take photographs of the optic tectum before fixation. However, fluorescence in the retina is much stronger than in the optic tectum and the retina is much thinner than the optic tectum, so that we do not need to be concerned with the photobleaching problem.

5.2 Selection of Tracer Protein

We must take care of the cytotoxicity of the tracer protein. We have noticed that high-level misexpression of DsRed-monomer-mem, which localizes in the cell membrane because of the N-terminal 20 amino acids of GAP-43, affected survival of nasal RGC after E17.5. On the other hand, GAP-EGFP did not alter proper development. We supposed that membrane-localized RFP may induce cell death, since GAP-DsRed2 and GAP-AsRed, both of which localize in the membrane, also induced cell death. Soluble DsRed2 and AsRed did not induce cell death.

Many tracer proteins have been produced and are being created (Miyawaki, 2005). It is important to select a proper tracer, paying attention to brightness, cytotoxicity, color variation, and modification subcellular localization such as Tau-LacZ.

5.3 Transfection Efficiency and Survival Rate

In case the survival rate is too low, the voltage of electroporation should be checked. We tested the relationship of the voltage at electroporation, transfection efficiency, and survival rate of the embryo. With parallel-type electrodes, effective transfection was obtained by 11 V for the nasal side of the retina, but not for the temporal side. By 13 V, we could obtain optimal transfection for both nasal and temporal RGC. The survival rate was reduced by 15 V. Higher concentration of plasmid solution than 2 μg/μl (1 μg/μl pT2K-CAGGS- /pCAGGS-T2TP) may improve electroporation efficiency even by the low-voltage electroporation.

For transfection of a small group RGC, the distance of the ball-shaped electrode and needle electrode is an essential factor for the area of transfection in RGC. The greater the distance of the ball-shaped electrode from the optic vesicle is, the larger the transfected area in the retina tends to be.

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Chapter 11
Retroviral Vector-Mediated Gene Transfer into the Chick Optic Vesicle by In Ovo Electroporation

Hiraki Sakuta, Ryoko Suzuki, and Masaharu Noda

1 Introduction

The chick embryo offers many advantages for developmental studies over other vertebrate embryos as it allows easy access for in ovo surgical manipulations, such as tissue transplantation and the implantation of cultured cells or chemically treated beads for the local release of humoral factors. In particular, owing to its external position in the embryo, the chick eye is a popular model for studying the patterning mechanism of the central nervous system (CNS). This patterning has a crucial role in shaping functional organization because it is the basis of the specific wiring in the CNS. Genetic analysis is not easy in the chick, as compared with the mouse for which transgene introduction or gene targeting techniques have been well established. However, because methods for the expression of exogenous genes and for gene silencing in the chick embryo have been recently developed, the functional analysis of genes has become possible in combination with classical techniques of developmental biology and neurobiology.

One general method is replication-competent retroviral particle-mediated gene transfer in the chick embryo (Morgan and Fekete, 1996). After the infection of viral particles, the viral RNA is reverse-transcribed and integrated into the host genome. Moreover, because the replication-competent viral vectors harbor all of the genome sequences essential for the production of infectious viral particles, horizontal infection continues to the neighboring cells in the host through reproduction of viral particles. Thus, the stable and efficient expression of a transgene can be achieved by this method. However, the system has several restrictions. For example, only coding sequences of less than 2.5 kb can be packaged into the vector. Also, it takes over 24 h for the proteins encoded in the retroviral vectors to be adequately expressed after the injection of viral particles. Therefore, this method is not suitable for the functional analysis of genes in the early developmental stages.

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The in ovo electroporation of expression plasmids is another way to transfer genes into chick embryos (Muramatsu et al., 1997; Funahashi et al., 1999; Yamagata et al., 1999). This system has the advantage that multiple genes can be simultaneously introduced into embryos with no apparent restriction in the size of the transgenes. However, the expression of transgenes is transient, lasting for only a few days even though the expression is driven by efficient promoters such as pMiwClaI (Yamagata et al., 1999) and pCAGGS (Niwa et al., 1991). This failure to achieve a stable expression is attributable to the inability of plasmids to persist in the embryo: they are not integrated into the host genome, and as a result, become diluted as embryonic cells proliferate.

As a third method to overcome these drawbacks, the in ovo electroporation of retroviral vector DNA was developed. With this method, the rapid, efficient, and sustained expression of a transgene is achieved, because the mRNA is directly transcribed from the introduced retroviral vector DNA shortly after the gene transfer, which is followed by the production of viral particles and continuous widespread infection. Here we describe the retroviral vector-mediated transfer of genes into the chick optic vesicle by in ovo electroporation. These methods enable long-lasting expression, reagent-inducible expression, and silencing of the expression of genes. This article has been partially published elsewhere (Sakuta et al., 2008).

2 Procedure

2.1 Chick Strain

The avian sarcoma-leukosis virus (ASLV) family is classified into five major subgroups, A to E, by the envelope protein (Hughes, 2004): RCAS vectors are derived from Rous sarcoma virus. ASLV-infected chick cells are highly resistant to reinfection by ASLVs of the same subgroup. Chick strains differ in their susceptibility or resistance to different subgroups due to the endogenous proviruses that are closely related to the ASLV. Therefore, it is necessary to use strains susceptible to the subgroup of the retroviral vector: A chick strain susceptible to all five subgroups (C/O) is now available commercially.

2.2 Plasmids

We usually use a replication-competent retroviral vector, RCAS-NS (Fig. 11.2a; Suzuki et al., 2000), which is a modified vector of subgroup B RCASBP (Hughes et al., 1987; Yuasa et al., 1996). The transgene was transferred to RCAS-NS after the coding region was cloned once into the shuttle vector SLAX-NS (Suzuki et al., 2000). SLAX-NS has the 5′-untranslated region of the *src* gene which confers efficient expression on heterologous coding sequences (Morgan and Fekete, 1996). A replication-incompetent retroviral vector, RIAS (Chen et al., 1999), is used for expression of a transgene longer than 2.5 kb (Shintani et al., 2006). RCAN-TRE

(Sakuta et al., 2006), which carries the tetracycline-responsive element (TRE) linked to the minimal cytomegalovirus (CMV) promoter as an internal regulatory element, is used for the doxycycline (DOX)-induced expression of a transgene. RCASDC (Sakuta et al., 2006), which contains a unique restriction site in the U3 region of the 3′ long terminal repeat (LTR), is used for the gene silencing by introducing the short hairpin RNA (shRNA)-expression cassette. Plasmids are prepared using the Qiagen Plasmid Kit (Qiagen, Hilden, Germany).

2.3 Preparation of the Embryos

Fertilized eggs are incubated horizontally at 37.5°C in a humidified incubator until they reach Hamburger Hamilton stage 8–10 (Hamburger and Hamilton, 1951), 26–40 h. After sterilization with 70% ethanol, a small hole is made at the sharp side of the egg with fine forceps, and then 3–5 ml of albumen is removed with an 18-gauge needle attached to a 5-ml syringe. After the hole is sealed with plastic tape, the top of the horizontal shell is cut elliptically with small scissors to open a window over the embryo. If the embryos have not reached stage 8–10, the eggs are further incubated at 37.5°C after the window is sealed with plastic tape.

2.4 In Ovo Electroporation

A pair of platinum electrodes (CUY611P3-1; Unique Medical Imada, Miyagi, Japan) is set with a micromanipulator 4 mm apart. After the window of the shell is opened, the embryo is wetted with about 200 µl of Hanks' saline. The cathode and anode are placed on the vitelline membrane on the left and right sides of the rostral region of the embryo, respectively (Fig. 11.1a, b), and wetted with about 200 µl of Hanks' saline, so that the resistance between electrodes decreases to around 1.0 kΩ. A microglass pipette filled with a DNA solution is inserted into the anterior neural fold at stage 8 (Fig. 11.1a) or the optic vesicle at stage 9–10 (Fig. 11.1b), and the DNA solution is injected by mouth. The microglass pipettes for injection of the DNA solution into the embryo are made from glass capillary tubes 1 mm in diameter (GD-1; Narishige, Tokyo, Japan) using a puller (tip diameter; about 40 µm). About 0.2–0.4 µl of DNA solution in phosphate-buffered saline (PBS) containing 0.05% fast green is injected into individual embryos. The concentration of DNA is 0.1–2.0 µg/µl. After injection of the DNA solution, electric square pulses (20–24 V, 50 ms) are applied by a CUY21EDIT electroporator (BEX, Tokyo, Japan) 3–5 times at an interval of 400 ms. After electroporation, the window is sealed with plastic tape, and the embryos are reincubated at 37.5°C until they reach the desired developmental stage.

Because DNA is negatively charged, it moves from the cathode toward the anode in the electric field, and only the right (anode) side of the embryo is transfected. The left (cathode) side of the embryo serves as a control. Therefore, you can compare the effects of the manipulation in the same embryo.

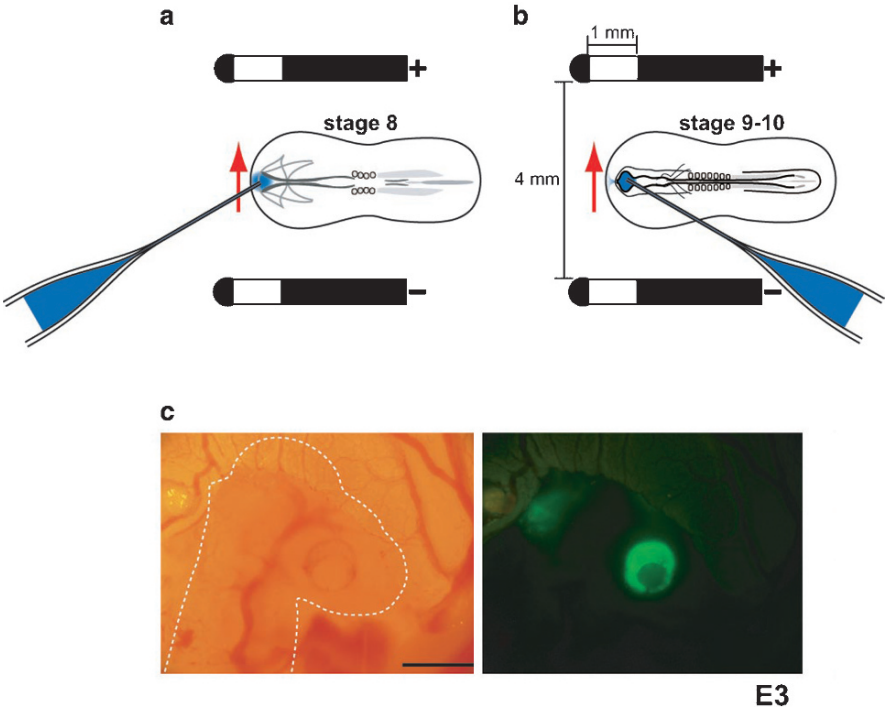

Fig. 11.1 In ovo electroporation into the chick optic vesicle. (**a, b**) Schematic representation of in ovo electroporation. A pair of platinum electrodes (0.5 mm in diameter with an exposed length of 1 mm) is set with a micromanipulator 4 mm apart. The cathode and anode are placed on the vitelline membrane on the left and right sides of the rostral part of the embryos, respectively. To transfect the eye region, the DNA solution is injected into the anterior neural fold at stage 8 (**a**) or the optic vesicle at stage 9–10 (**b**). DNA moves from the cathode toward the anode in the electric field (*arrow*) and the right (*anode*) side of the embryo is selectively transfected. (**c**) Evaluation of transfection efficiency. An E3 embryo electroporated with RCAS/AP carrying the human placental alkaline phosphatase gene and pEGFP-N1 at E1.5 (*left*). The same embryo was observed for EGFP fluorescence, visualizing the right retina expressing the transgenes (*right*). Scale bar: 1 mm. Reproduced from Sakuta et al., 2008

3 Application and Results

3.1 Electroporation of the Replication-Competent Retroviral Vector

3.1.1 Evaluation of Transfection Efficiency

After electroporation, the chick embryo is repositioned to the original right-side up orientation. To monitor the transfection efficiency in ovo under a fluorescence stereoscopic microscope, coelectroporation of an expression plasmid for a marker

protein is useful. For this purpose, we usually use an expression plasmid for the enhanced green fluorescence protein (EGFP), pEGFP-N1 (Clonetech, Palo Alto, CA, USA). We usually check the transfection efficiency at E3 in ovo (Fig. 11.1c) and discard the embryos of low transfection.

3.1.2 Expression of a Transgene After Electroporation

We have electroporated RCAS/EGFP as a model into the anterior neural fold at stage 8. EGFP fluorescence is already detectable in the entire right optic vesicle at 6 h after electroporation (Fig. 11.2b), as early as stage 9, just before the stage when

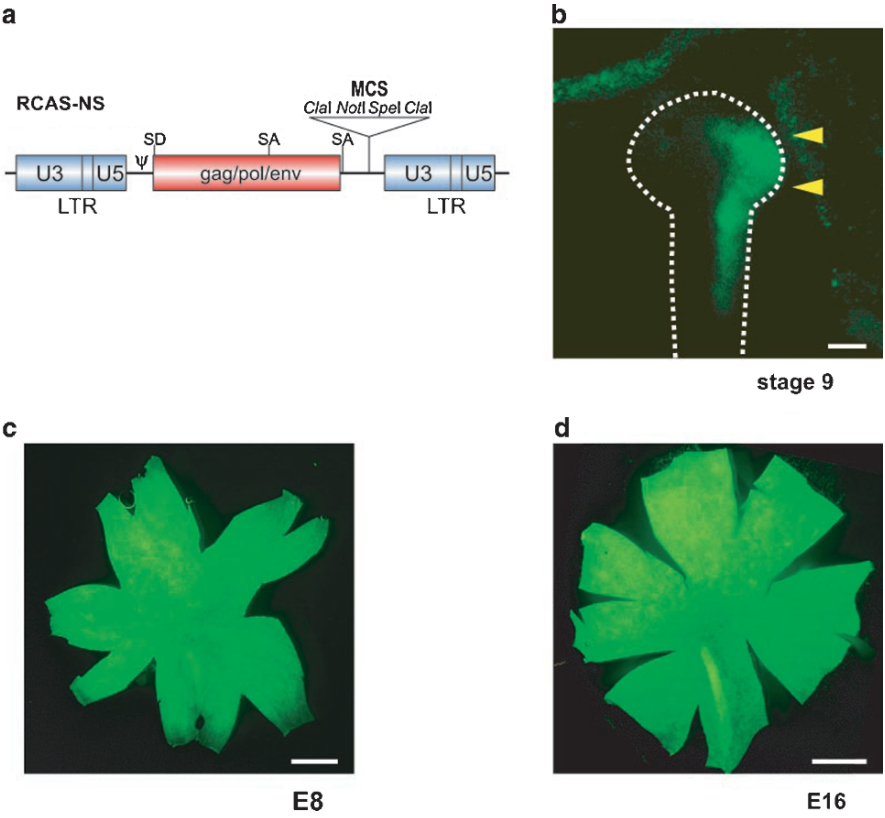

Fig. 11.2 Rapid and sustained expression of EGFP after electroporation. (**a**) Schematic representation of the RCAS-NS vector. LTR, long terminal repeat; SD, splicing donor; SA, splicing acceptor. (**b**) A stage 9 embryo electroporated with RCAS/EGFP at stage 8. EGFP fluorescence was already detected in the entire right optic vesicle (*arrow heads*) at 6 h after electroporation. Anterior is upwards. (**c**, **d**) Flat mount of E8 and E16 retinae transfected with RCAS/EGFP. EGFP fluorescence was continuously detected in the entire retina at E8 (**c**) and E16 (**d**). Temporal is left, and dorsal is upwards. Scale bars: (**b**) 100 µm; (**c**) 2 mm; (**d**) 3 mm. Reproduced from Sakuta et al., 2008

the regional specificity along the anteroposterior axis is reportedly determined (Dütting and Meyer, 1995; Dütting and Thanos, 1995). When a conventional expression vector, such as pMiwClaI and pCAGGS, is electroporated, the expression of the transgene is transient, lasting for merely 2 to 3 days. In contrast, electroporation of the retroviral vector leads to integration of the provirus into the host genome and resultantly a long-lasting expression. As expected, EGFP fluorescence was detected in the entire retina at E8 (Fig. 11.2c), and still at E16 (Fig. 11.2d) when the retinotectal map is fully established (Nakamura and O'Leary, 1989). A higher level of expression of a transgene just after the electroporation can be achieved by coelectroporation of a RCAS-NS vector together with a conventional expression vector carrying the same transgene. The rapid and sustained expression achieved by this method is effective for analyzing the functions of a gene whose expression begins at the early developmental stages (Sakuta et al., 2001; Takahashi et al., 2003).

Because the DNA concentration of a retroviral vector necessary for efficient infection varies depending on the transgene, the optimal concentration must be examined experimentally. The efficiency of retroviral infection can be evaluated by visualizing the expression of the transgene and viral genes. We usually check the efficiency of infection by immunostaining the viral *gag* protein using the monoclonal antibody 3C2 (Potts et al., 1987). When the RCAS vector or its derivative was electroporated under optimal conditions, the whole of the right retina was infected at E8 (Fig. 11.5b).

3.2 Introduction of Modified Retroviral Vectors by In Ovo Electroporation

3.2.1 Introduction of a Long Transgene into the Retina

A replication-competent retroviral vector, RCAS-NS, allows the insertion of a transgene only shorter than 2.5 kb. To circumvent this limitation, we used a replication-incompetent retroviral vector, RIAS (Fig. 11.3a; Chen et al., 1999), which is able to carry up to 4.5 kb of cDNA as a transgene. Because the RIAS vector lacks an *env* gene, only a few retinal cells expressed EGFP at E16 after electroporation of RIAS/EGFP (Fig. 11.3b). When an *env* protein was supplied from RCAS-NS by coelectroporation with the RCAS-NS vector, the infection efficiency of the RIAS vector greatly increased and an expansion of the positive area with EGFP fluorescence was attained (Fig. 11.3c). When we electroporated RIAS/Ptpro(WT) harboring the HA-tagged protein tyrosine phosphatase receptor type O (*Ptpro*, 3.8 kb) gene together with the RCAS-NS vector into the retina, approximately half of the retinal ganglion cells were Ptpro(WT)-positive at E16 (Fig. 11.3d). This efficiency is lower than that obtained with a replication-competent retroviral vector for a short transgene but enough for the functional analysis of a long transgene (Shintani et al., 2006).

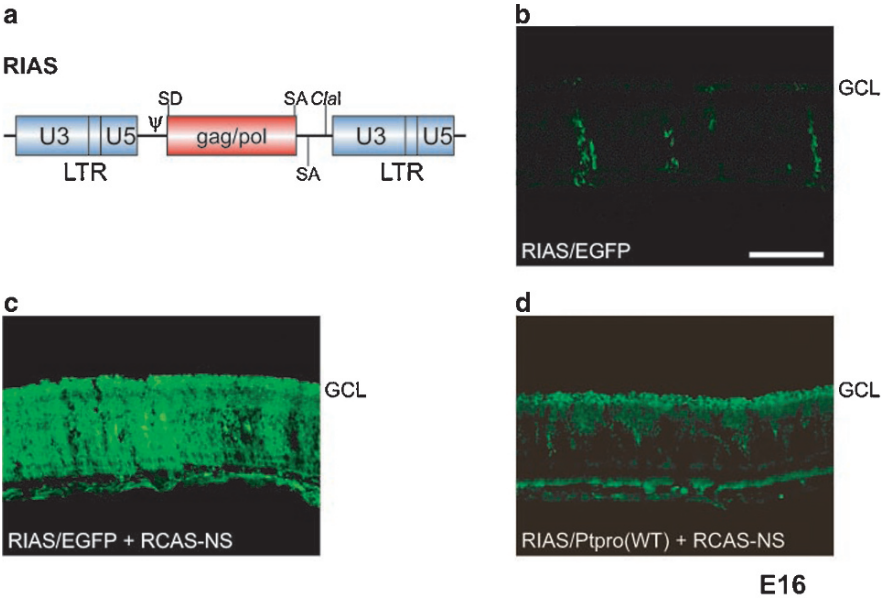

Fig. 11.3 Expression of a long transgene by simultaneous electroporation of the RIAS vector and RCAS-NS vector. (**a**) Schematic representation of the RIAS vector. For the RCAS-NS vector, see Fig. 11.2a. (**b**) When RIAS/EGFP alone was electroporated into the retina at E1.5, only weak expression of EGFP was observed in the ganglion cell layer (GCL) at E16. The retina was immuno-stained with anti-GFP and Alexa488-conjugated secondary antibodies. (**c**) Coelectroporation of RIAS/EGFP with the RCAS-NS vector greatly expanded the transfected area in the retinal GCL. (**d**) Expression of Ptpro(WT) in an E16 chick retina coelectroporated with RIAS/Ptpro(WT) and RCAS-NS. The retina was stained with anti-HA and Alexa 488-conjugated secondary antibodies. Ptpro(WT) was tagged with HA. About 50% of ganglion cells were positive for Ptpro(WT). Scale bar: 100 µm. Modified from Shintani et al., 2006

3.2.2 Reagent-Inducible Gene Expression

It is essential to manipulate the expression of a gene temporally during development. We took advantage of the *tet* regulatory system, in which the expression of a transgene can be activated by tetracycline or an analog thereof such as DOX (Gossen and Bujard, 1992; Gossen et al., 1995). For this purpose, we prepared RCASDC/rtTA and RCAN-TRE vectors (Fig. 11.4a; Sakuta et al., 2006). The TRE-mediated transgene expression depends on the expression level of rtTA (Kistner et al., 1996). The transgene is inserted into the *Cla*I site of RCAN-TRE. We simultaneously introduced RCASDC/rtTA and RCAN-TRE/EGFP (harboring the *EGFP* gene) into the chick retina by in ovo electroporation. DOX was administered twice to the embryos at E5 and E6.5, and the retinae were removed at E8 to confirm that many cells are positive for EGFP fluorescence (Fig. 11.4b). In the control, only a few cells expressing EGFP were observed in the absence of DOX. This reagent-inducible gene

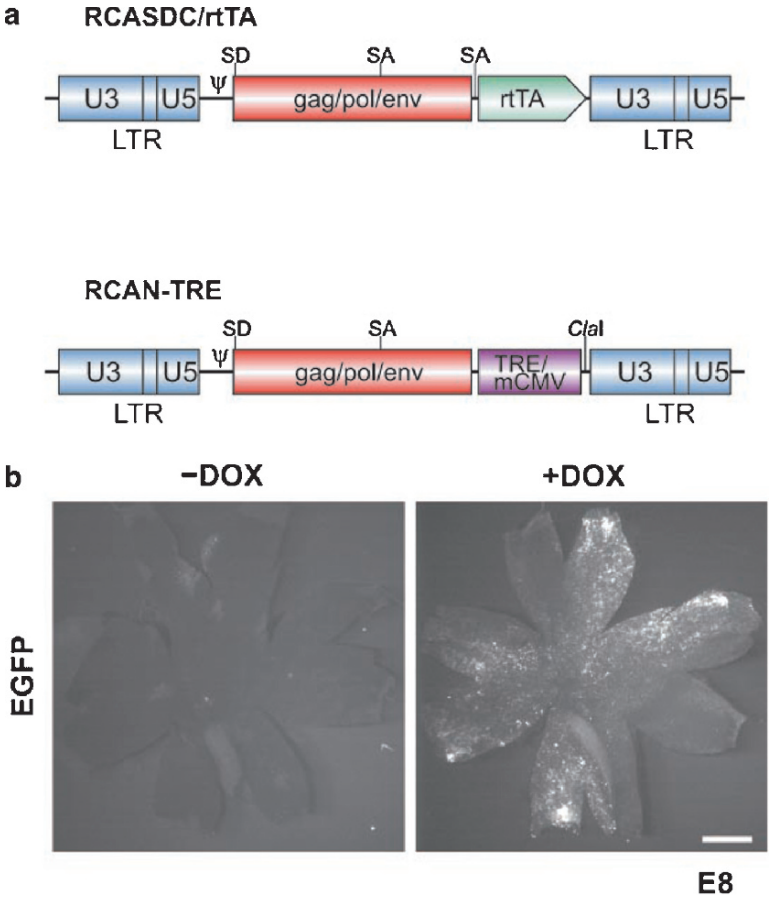

Fig. 11.4 The reagent-inducible gene-expression system for the chick. (**a**) Schematic representation of the RCASDC/rtTA vector and RCAN-TRE vector. rtTA, reverse tetracycline-controlled transactivator; TRE, tetracycline-responsive element; mCMV, minimal CMV promoter. (**b**) EGFP expression in the retinae of E8 embyos with/without DOX-treatment. RCASDC/rtTA and RCAN-TRE/EGFP were simultaneously electroporated at E1.5. EGFP expression is induced only in the retinae treated with DOX. Temporal is left, and dorsal is upwards. Scale bar: 1 mm. Modified from Sakuta et al., 2006

expression system was effective for the functional analysis of bone morphogenetic protein2 (*BMP2*) in the developing retina (Sakuta et al., 2006).

3.2.3 Gene-Specific Knockdown

The loss-of-function analysis of a gene also provides important information for the understanding of its biological roles. RNA interference (RNAi) mediates sequence-specific suppression of gene expression across phyla. It is applicable to

developmental studies by enabling the introduction of a short RNA duplex (small interfering RNA or siRNA) with a sequence homologous to the target gene (Caplen et al., 2001; Elbashir et al., 2001). Moreover, it is known that shRNA, which is transcribed by RNA polymerase III, exerts an RNAi-type effect similar to siRNA (Brummelkamp et al., 2002; Paddison et al., 2002; Yu et al., 2002).

To achieve continuous gene silencing, we generated a RCAS vector with a unique restriction site in the U3 region of the 3′LTR for the insertion of the shRNA-expression cassette (Fig. 11.5a; Sakuta et al., 2006). The provirus generated from the RCASDC vector acquires two copies of the shRNA-expression cassette through the duplication of the LTR at both ends during reverse transcription, and therefore two-fold expression of shRNA is expected in ovo. We show as an example, the introduction of a RCASDC vector carrying the shRNA-expression cassette for *BMP2* by in ovo electroporation. When RCASDC-CU6/BMP2 was electroporated into the right retina, the entire retina was infected at E8, as was evident from the expression of *gag* protein (Fig. 11.5b). Under these conditions, *BMP2* expression was markedly reduced at E8 (Fig. 11.5c). In contrast, *BMP4* expression was not affected at all, at E4 when the expression peaked (Fig. 11.5d). This gene-specific knockdown system was effective for the functional analysis of a target gene (Sakuta et al., 2006; Shintani et al., 2006).

4 Troubleshooting

Several potential problems in in ovo electroporation with retroviral vectors and troubleshooting strategies for them are described below.

4.1 No or Few Transfected Cells

Solutions: A. Increase the volume and/or concentration of DNA to be injected; B. Increase the voltage and/or number of pulses while monitoring cell death; and C. Prepare fresh DNA if impurities or contaminants are suspected.

4.2 Low Survival Rate or Unexpected Morphological Defects After In Ovo Electroporation

Solutions: A. Reduce voltage and/or number of pulses to reduce cell damage; B. Check whether embryos are located in the middle of electrodes, otherwise they are damaged during electroporation; C. Reduce volume and/or concentration of DNA injected if a transgene is suspected to interfere with survival of embryos; and D. Use other chick strains because chick strains differ in their resistance to electroporation.

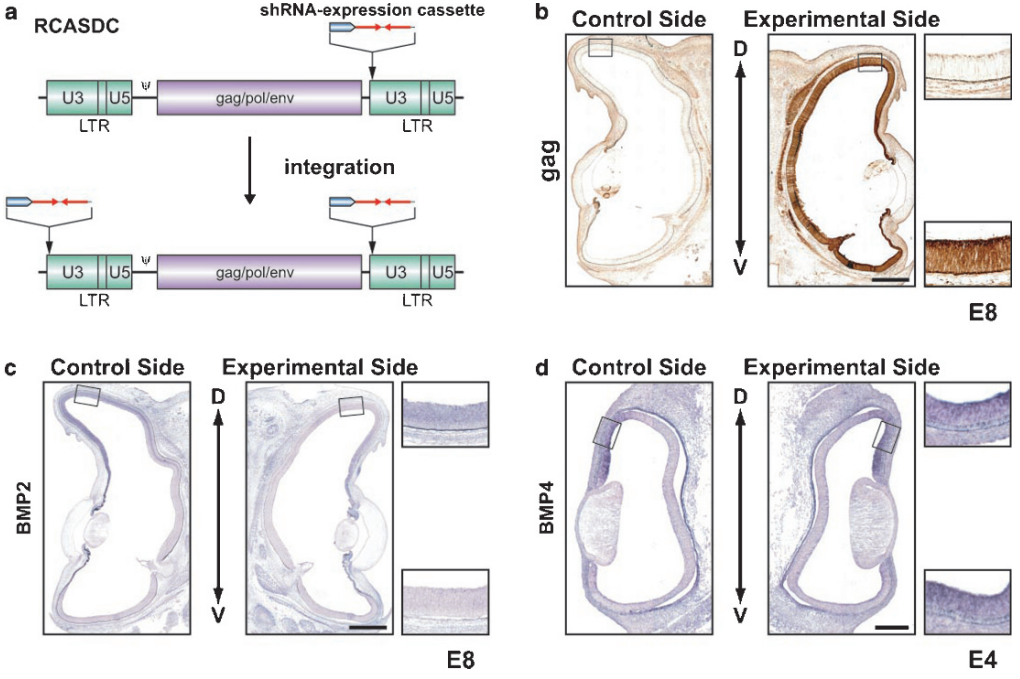

Fig. 11.5 The gene-specific knockdown system for the chick. (**a**) Schematic representation of the RCASDC retroviral vector. The shRNA-expression cassette was inserted into the U3 region of the 3′LTR of RCASDC. The resultant provirus acquires the shRNA-expression cassette sequences at both ends by duplication of the LTR during the reverse transcription, and the viral DNA integrates into the genome of transfected cells. (**b**) Expression of viral *gag* protein in the transfected right eye. RCASDC-CU6/BMP2 was introduced into the embryo at E1.5 by electroporation. Viral *gag* protein was visualized at E8 by immunostaining with the monoclonal antibody 3C2. (**c, d**) Coronal section in situ hybridization of the control retina (*left*) and RCASDC-CU6/BMP2-electroporated retina (*right*). Expression of *BMP2* at E8 was markedly reduced in the retina by electroporation of RCASDC-CU6/BMP2 (**c**). Expression of *BMP4* at E4 was not altered by electroporation of RCASDC-CU6/BMP2 (**d**). The boxed regions in the control retina (*left*) and manipulated retina (*right*) are enlarged on the right-upper and right-lower side, respectively (**c**, **d**). D and V indicate dorsal and ventral, respectively. Scale bars: (**b**, **c**) 1 mm; (**d**) 200 μm. Modified from Sakuta et al., 2006

4.3 High Transfection Efficiency but No or Few Infected Cells

Solutions: A. Increase the concentration of retroviral vectors injected; and B. Test vector constructs for the expression of a transgene in primary cell cultures or cell lines. We usually use chick embryonic fibroblasts (CEFs) for this purpose.

5 Conclusion

We can now achieve the rapid, effective, and constitutive expression of a transgene in the chick embryo using retroviral vector-mediated transfer by electroporation. In addition, the size limit of the replication-competent retroviral vector can be circumvented by using a replication-incompetent vector, and the temporal expression of a transgene can be manipulated by the *tet* regulatory system. Furthermore, electroporation of a retroviral vector carrying a shRNA-expression cassette is an effective way to induce sustained gene silencing in the chick embryo. We hope that these methods help to revive use of the chick as a model animal in developmental biology and neurobiology.

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Chapter 12
Clonal and Widespread Gene Transfer by Proviral Electroporation for Analysis of Brain Laminar Formation

Sayaka Sugiyama and Harukazu Nakamura

1 Introduction

An essential approach to understanding the mechanisms of development is to alter a gene function/expression. In vivo electroporation has been adapted as one such technique (Muramatsu et al., 1997). It is a very useful tool to achieve a gain- and loss-of-function (by using RNAi or morpholinos) of a gene of interest (Funahashi et al., 1999; Fukuchi-Shimogori and Grove, 2001; Kos et al., 2001; Katahira and Nakamura, 2003; Sugiyama and Nakamura, 2003). The technique has allowed the altering of gene expression temporally and spatially. Pulse-labeling technique is an approach to manipulate a specific cell population temporally, depending on its birthday, as this chapter describes. This technique is more advantageous over the BrdU application, as it can reveal cell lineage; it also has the ability to manipulate a gain- and loss-of-function into specific precursor cells (Tabata and Nakajima, 2001; Sugiyama and Nakamura, 2003; Huber et al., 2008).

In spatial terms, widespread gene transfer by electroporation has provided an efficient way to unveil a new gene function on a given tissue (Nakamura and Funahashi, 2001). On the other hand, the spatial precision of gene transfer also has been addressed as each individual cell responds differently to gene expression. Thus, there has been increasing efforts to improve electrodes and create new techniques such as single-cell electroporation (Haas et al., 2001; Kitamura et al., 2008).

Proviral electroporation has been adapted to manipulate clonal and widespread gene transfer. This involves the combination of in ovo electroporation, which is a relatively older technique among the electroporation methods (Muramatsu et al., 1997; Funahashi et al., 1999) and the use of retrovirus to produce long-lasting gene expression (Cepko, 1988; Gray and Sanes, 1991; Itasaki and Nakamura, 1996).

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A combination of these two techniques provides synergistic advantages to introduce a gene of interest only within a single-cell-derived clone in virus-resistant embryos or in cells of a widespread area in virus-sensitive embryos (Sugiyama and Nakamura, 2003). The use of virus-resistant and -sensitive embryos controls the different spatial pattern of gene transfer. A proviral vector is assisted into a virus-resistant cell by electroporation, and its gene transfer is kept clonally due to resistance of the embryo against viral infection. To create a more widespread gene transfer, a proviral vector can be introduced to virus-sensitive embryos and its spread by viral secondary infection is more effective than using electroporation with routine plasmids.

2 Procedure

2.1 Vectors and Morpholino Antisense Oligonucleotide

The lacZ expression vector, pMiwZ (Suemori et al., 1990), was used in pulse-labeling of progenitor cells. The proviral DNA vector, RCAS(BP)B (Hughes et al., 1987), was applied to introduce gene expression into virus-sensitive or -insensitive embryos by electroporation. The RCAS(BP)B-AP vector, into which alkaline phosphatase (AP) had been subcloned, served as a control (Cepko et al., 2000). The entire coding region of *Grg4* with an HA-tag sequence (Sugiyama et al., 2000) was inserted into Cla12 adapter plasmids. Each ClaI fragment of cDNA (~2.4 kb) then was subcloned into the proviral DNA vector RCAS(BP)B. DNA vectors were purified using the plasmid purification kit (QIAGEN) and dissolved in Tris-EDTA (TE) (1–2 µg/µl).

A couple of different sequences for fluorescein-labeled morpholino antisense oligonucleotide were designed by the supplier (Summerton, 1999, Gene Tools). Among them, the effective sequence of morpholino antisense oligonucleotide against *Grg4*, 5´-GCGGATCATCCACGCCGCTTCGGG-3´, was obtained. The supplier recommended oligonucleotide (5´-CCTCTTACCTCAGTTACAATTTATA-3´) as the control. One millimole solution of control or *Grg4* morpholino was used for electroporation.

2.2 Chick Embryos

Virus-sensitive chick eggs (c/o or LM SPF) could be purchased from Nisseiken (Aome, Tokyo, Japan). Conventionally raised fertile chick eggs obtained from a local farm are generally virus-resistant, but they occasionally are virus-sensitive. Stringent checks are required to confirm that the purchased eggs are either virus-resistant or virus-sensitive.

Eggs are incubated at 38°C in a humidified atmosphere. After rinsing the eggshells with 70% ethanol, a small hole is made at the sharp edge of the egg, and 4 ml of albumen is pulled out with 18-gauge needle. This process needs to be finished before the yolk sac plexus is developed inside the eggshell. To access an

embryo, a window is opened on the middle part of the egg at E2 (stage 9–11), E3 (stage 16–18), E5 (stage 27–28), and E6 (stage 29–30). Embryos are staged according to Hamburger and Hamilton (1951).

2.3 Infusion of DNA Vector and In Ovo Electroporation

2.3.1 Pulse-Labeling of Neuroepithelial Cells up to E6 Embryos

Transfection to the neuronal progenitor cells was performed at E3 (stage 16–18), E5 (stage 27–28), or E6 (stage 29–30) with pMiwZ vector (0.5–1 μg/μl). The plasmid solution (1–5 μl) is infused into the aqueductus mesencephali with a micropipette (Fig. 12.1a). Embryos after E5 are freely floating covered by the cholioallantoic membrane. To access the aqueductus mesencephali, the embryo is clamped to the surface of the yolk sac by grasping the amniotic fold with a pair of tweezers. The plasmid solution is injected into the aqueductus mesencephali from a ventral portion of mesencephalon. Adding fast green (final concentration, 0.05%) to plasmid solution is helpful for visualization of injected solution. For electroportaion, the

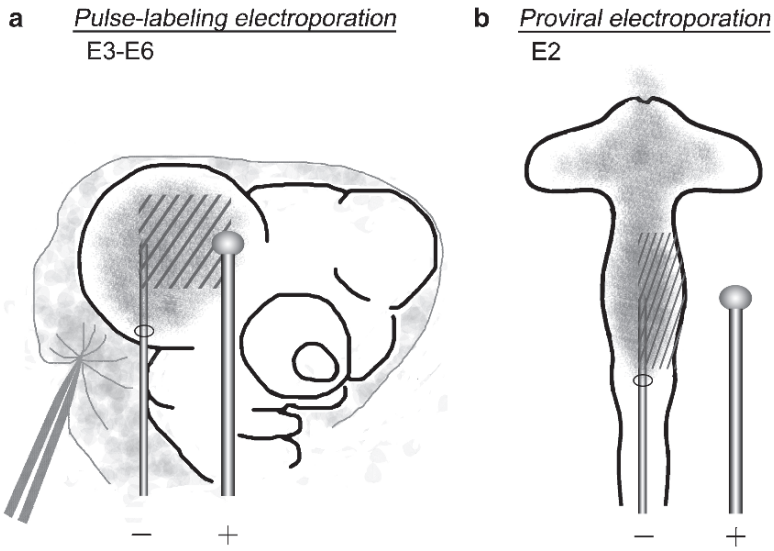

Fig. 12.1 Schema of pulse-labeling or proviral electroporation in ovo. (**a**) Pulse-labeling electroporation up to E6 embryos. Embryos after E5 are covered by the chorioallantoic membrane. To expose the brain, the amniotic fold is pulled up from the embryo with a pair of tweezers and held at brain region. (**b**) Electroporation with proviral vector into a viral-insensitive or -sensitive embryo. The E2 embryos are visualized by injection of India ink just under the yolk sac, and cut at the tip of neural tube to allow fluid of central canal to flow. After microinjection of vector solution (*gray*), a tungsten microelectrode (*cathode*) is inserted into the aqueductus mesencephali and a round platinum electrode (*anode*) is put on the amniotic membrane beside the mesencephalic region. Shaded area is expected to be electroporated

tungsten microelectrode (Nepagene, Ichikawa, Japan; Unique Medical Imada, Natori, Japan) is used as the cathode inserted again into the aqueductus mesencephali, and the round platinum electrode (Nepagene, Ichikawa, Japan; Unique Medical Imada, Natori, Japan) is used as the anode placed just beside the amniotic membrane of the mesencephalic region. A square pulse of 10 V for 25 ms is delivered three times by the electroporator (CUY21EDIT, Nepagene, Japan; Bex, Tokyo, Japan) at intervals of 975 ms. The window on the top of the egg is sealed by Scotch tape and the embryo is placed back into the incubator.

2.3.2 Gene Transfer with Proviral Vector into Viral-Insensitive or Viral-Sensitive Embryos

Expression of the gene of interest is achieved by electroporation with proviral DNA vector, RCAS(BP)B. With virus-insensitive embryos, expression is limited within the descendents of the originally transfected cells. Gene expression, in contrast, spreads from the originally transfected cells to neighboring cells in virus-sensitive embryos by secondary infection.

To visualize the E2 (stage 9–11) embryos India ink is injected just under the yolk sac (Funahashi et al., 1999; Nakamura and Funahashi, 2001). The proviral vector solution (0.1–1 µl) then is infused into the anterior part of the neural tube with a micropipette (Fig. 12.1b). Cutting the tip of the neural tube allows proviral solution to flow into the anterior part of the neural tube. To increase the survival rate of embryos, the tungsten microelectrode (Nepagene, Japan; Unique Medical Imada, Natori, Japan) is used as the cathode and the round platinum electrode (Nepagene, Japan; Unique Medical Imada) as the anode. The tungsten microelectrode is inserted into the anterior neural tube just after infusion of proviral solution, and the platinum electrode is placed beside the anterior neural tube (Momose et al., 1999). A square pulse of 8 V for 25 ms is delivered twice at intervals of 975 ms by an electroporator (CUY21EDIT, Nepagene, Ichikawa, Japan; Bex, Tokyo, Japan).

2.3.3 Loss-of-Function with Morpholino Antisense Oligonucleotide

One millimole solution of control or *Grg4* morpholino (1–5 µl) was injected into the aqueductus mesencephali at E5 (stage 27–28) and electroporation was carried out as described above for E3–6 embryos. Due to its positive polarity, fluorescein-labeled morpholino is introduced on the cathode side.

2.4 Evaluation of Transfection

Electroporated embryos were fixed with 4% paraformaldehyde at E5, E8, or E12. After washing with phosphate-buffered saline (PBS), embryos were dissected in the

mesencephalic region and gradually replaced into PBS containing 30% sucrose for protection of frozen sections.

To evaluate the transfection of RCAS(BP)B virus, immunostaining is performed with anti-gag antibody, AMV-3C2 (monoclonal, Developmental Study Hybridoma Bank, Iowa, USA), which recognizes retrovirus products. The introduced gene expression with RCAS(BP)B is analyzed by in situ hybridization with digoxigenin-labeled RNA probe or immunostaining with anti-HA antibody, 3F10 (monoclonal, Roche), for HA-tagged gene products (Sugiyama and Nakamura, 2003).

Sections of RCAS(BP)B-AP-transfected embryos are processed for AP activity (Halliday and Cepko, 1992). For the AP reaction, endogenous AP is inactivated by heating to 60–65°C for 30 min to overnight. Sections then are placed in AP buffer (100 mM NaCl, 100 mM Tris-HCl pH9.8, 50 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40) containing 0.175 µg/µl X-phosphate (5-Bromo-4-Chloro-3-indolyl-phosphate; Roche), 0.334 µg/µl nitroblue tetrazolium (Roche), until the color development is achieved.

LacZ expression or morpholino distribution in tectal laminae is revealed by the following primary antibodies: anti-β-galactosidase (rabbit polyclonal, ICN), anti-Hu-C/D, 16A11 (monoclonal, molecular probes), and AP-conjugated anti-fluorescein (Roche). The Cy3- or Alexa 488-conjugated goat antibodies are applied as secondary antibodies (molecular probes).

3 Application and Results

3.1 Trace of Pulse-Labeled Neuroepithelial Cells by Electroporation

To pulse-label progenitor cells, we adapted in ovo electroporation with a *lacZ* expression vector, pMiwZ. It is important that only the cells that are dividing (ie, the neuroepithelial) are pulse-labeled. In our system, *lacZ*-positive cells were restricted to the neuroepithelium at 2 h after electroporation (Fig. 12.2b), and were reduced in the neuroepithelium 3 days later (Sugiyama and Nakamura, 2003). Expression of pMiwZ is transient; in rapidly proliferating cells, translation products are diluted initially and peak at around 24 h after electroporation (Funahashi et al., 1999; Momose et al., 1999). In stable postmitotic cells, high levels of plasmid and translation products are still maintained at two weeks after electroporation (Aihara and Miyazaki, 1998; Kishimoto et al., 2002). Consistent with these reports, transfected *lacZ* expression was enough to trace the labeled cells at least for 10 days in the brain laminae (Fig. 12.2). The results indicate the high efficacy of this system to pulse-label progenitor cells.

Thus, the pulse-labeling technique has an advantage to trace a specific cell population back to its progenitor origins in developing tissues. One example of its use is to understand the migration patterns of neuroepithelial cells in laminar

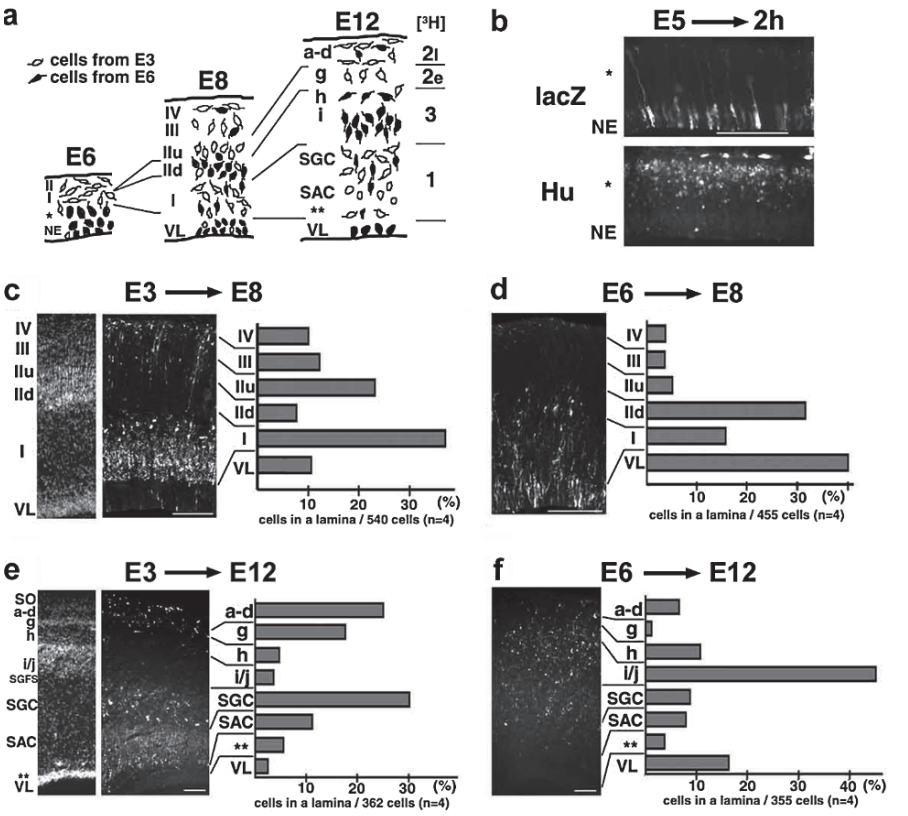

Fig. 12.2 Trace of pulse-labeled cells in laminar formation. (**a**) Tectal laminar formation by early (E3–E5) and late (E6–E8) migratory cells. The laminae of early migratory cells are split into two: upper and deeper laminae, as late migratory cells migrate into middle lamina. (**b**) Two hours after electroporation with pMiwZ vector at E5. Immunohistochemistry with anti-*lacZ* (*upper*) or anti-Hu-C/D (*lower*). The *lacZ* staining was observed in the neuroepithelial layer (NE), but not in the Hu-positive early neuronal layer (*). (**c**, **d**) Distribution of *lacZ*-positive cells at E8. Electroporation with pMiwZ was performed at E3 (**c**) and E6 (**d**). Panels show DAPI staining (**c**, *left*), its *lacZ* counterstain (**c**, *middle*; **d**, *left*), and the number of *lacZ*-positive cells in each lamina (**c**, *right*; **d**, *right*). (**e**, **f**) Distribution of *lacZ*-positive cells at E12. Electroporation with pMiwZ was performed at E3 (**e**) and E6 (**f**). DAPI staining (**e**, *left*), *lacZ* staining (**e**, *middle*; **f**, *left*) and the number of *lacZ*-positive cells in each lamina (**e**, *right*; **f**, *right*). Scale bars: 100 μm. Data adapted from Sugiyama and Nakamura (2003)

formation. Pulse-labeling of tectal neuroepithelial cells at E3 (stage 16–18) or E6 (stage 29–30) revealed that the tectal laminae (La Vail and Cowan, 1971) are formed as a result of early and late migratory waves: the early wave is between E3 and E5 and reaches to the deeper laminae (SGC, SAC, SGP, and SFP) and upper laminae (a–g of SGFS) and the late wave is between E6 and E8 to the middle laminae (h–j of SGFS). The five laminae (IV-I) at E8 were reorganized in an orderly manner into 13 laminae at E12 (Fig. 12.2).

3.2 Lineage Analysis of Clonally Proliferating Cells by Proviral Transfection

To restrict gene transfer within a single-cell-derived clone, electroporation technique was combined with proviral DNA vector, RCAS(BP)B, and performed in virus-resistant embryos. Secondary infection does not occur in virus-resistant embryos, so gene transfer is limited to the descendents of the originally electroporated cells. When the proviral vector RCAS(BP)B-AP was electroporated into virus-resistant embryos at E2 (stage 9–11), a lineage of AP-expressing cells was distributed radially throughout the brain wall in 6 days (Fig. 12.3a). In this system, each clone is sparsely distributed so that one clone is easily distinguished from others. For cell lineage analysis, the proper density of clones might be obtained by modifying the concentration of the proviral vector at electroporation.

One typical example of a cell lineage analysis is given in the clonal gene transfer of *Grg4* transcription inhibitor, which is supposed to lead tectal postmitotic cells to the late migratory fate (Fig. 12.3b) (Sugiyama and Nakamura, 2003; Nakamura and Sugiyama, 2004). When the provirus RCAS(BP)B-*Grg4* was electroporated on virus-resistant embryos at E2 (stage 9–11), the lineages of *Grg4*-expressing cells were selectively concentrated in the deeper lamina II which is composed of late migratory cells.

3.3 Gain-of-Function by Proviral Transfection into Virus-Sensitive Neuroepithelium

Electroporation of proviral vector into virus-sensitive embryos allows widespread expression of introduced gene by secondary infection. Since the retrovirus is integrated into the genome of proliferating cells, the expansion of the retrovirus is very effective with rapidly proliferating cells at an early embryonic age, and robust expression could be detected even 12 days later (Sugiyama and Nakamura, 2003).

After electroporation into virus-sensitive embryos at E2, strong expression of a gene of interest spreads over the entire neuroepithelial cells in 6 days (Fig. 12.4b). The effect of widespread expression is easily analyzed, for instance, by molecular markers, compared with the case of the control provirus, RCAS(BP)B-AP (Fig. 12.4a).

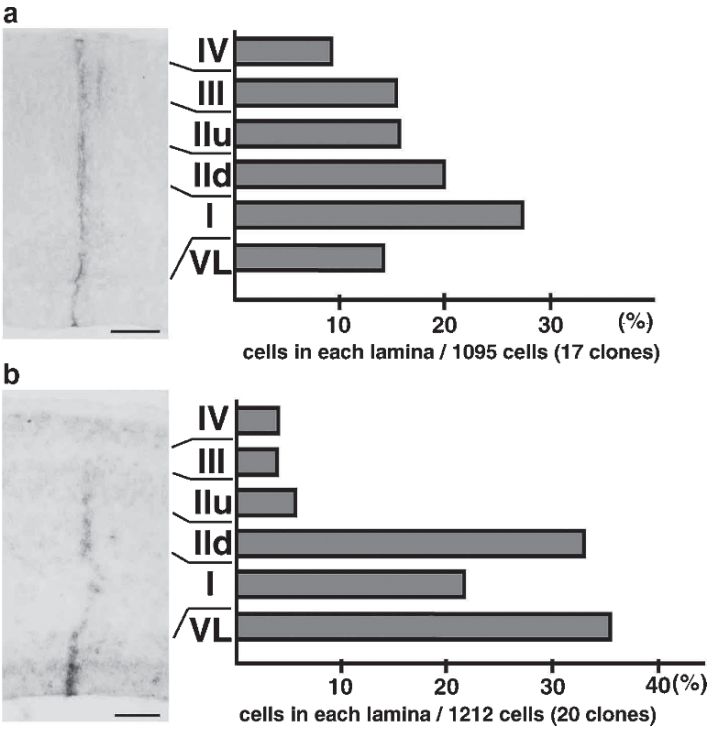

Fig. 12.3 Clonal analysis by proviral electroporation into virus-resistant embryos. (**a**) Electroporation with RCAS-AP in virus-resistant embryos at E2 that were fixed at E8. The AP immunocytochemistry (*left*) and number of AP-positive cells in each lamina (*right*). The AP-positive cells were present throughout all laminae. (**b**) Electroporation with RCAS-*Grg4* on virus-resistant embryos at E2 that were fixed at E8. In situ hybridization for *Grg4* (*left*) and number of *Grg4*-expressing cells in each lamina (*right*). The *Grg4*-expressing cells were found mainly in lamina IId. The labeled cells in lamina I may be those en route to their final destination. Scale bars: 100 μm. Data adapted from Sugiyama and Nakamura (2003)

Widespread expression of RCAS(BP)B-*Grg4* changed the fate of the precursor cells to behave as late migratory cells: the laminae composed of early migratory cells (*Sox2*-positive upper lamina II) were reduced and the lamina composed of late migratory cells (*Sox14*-positive deeper lamina II) was increased (Fig. 12.4b, d).

3.4 Loss-of-Function by Transfection of Morpholino Oligonucleotide

For a loss-of-function of a gene of interest, morpholino antisense oligonucleotide (Heasman et al., 2000) introduced electroporatedly into cells prevented translation. Since fluorescein-coupled morpholino oligonucleotide is positively charged, it is

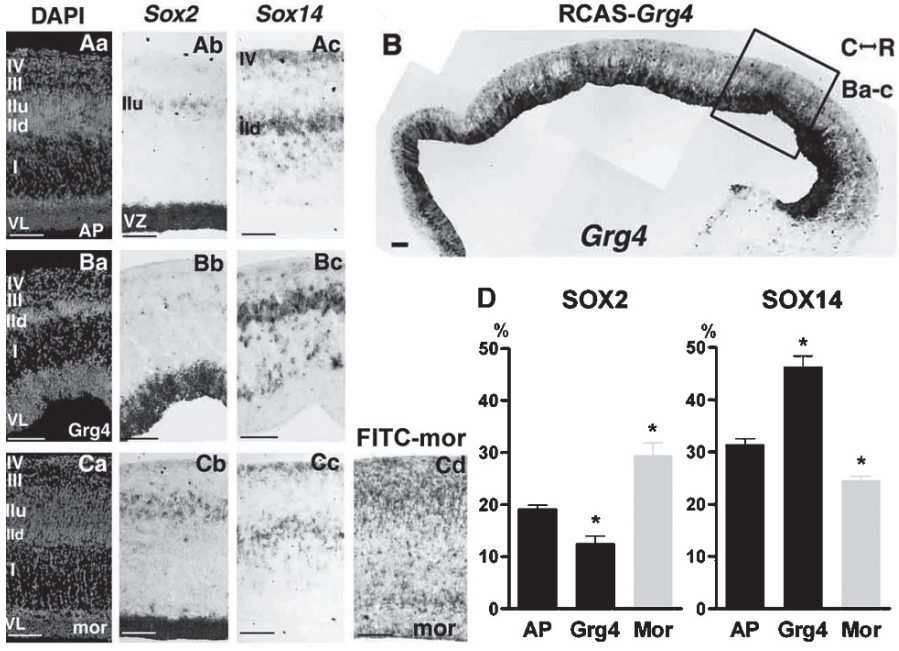

Fig. 12.4 Fate analysis by proviral electroporation into virus-sensitive embryos. (**A**) Control E8 tecta in which RCAS-AP was electroporated at E2. DAPI staining (**a**). The *Sox2* was expressed in lamina IIu and the ventricular layer (VL, **b**). The *Sox14* was expressed in laminae IV and IId (**c**). (**B**) E8 tecta in which RCAS-*Grg4* was electroporated at E2. The *Grg4* expression spread over the entire tectum in virus-sensitive embryos (under low-power magnification). Reduction of lamina I, Iiu, and IV is assessed from the expression of *Sox2* (**b**) and *Sox14* (**c**) counterstained with DAPI (**a**). (**C**) E8 tecta after transfection of morpholino antisense oligonucleotide against *Grg4* at E5. The expression of *Sox2* (**b**) and *Sox14* (**c**) showed that treatment with morpholino oligonucleotide exerts reverse effects: expansion of lamina IIu, reduction of lamina IId. Immunostaining for FITC to show FITC-conjugated morpholino (**d**). (**D**) Relative number of Sox2- and Sox14-positive cells in the different transfection methods. Percentage of *Sox2*- and *Sox14*-positive cells in the lamina II over the total cells of mantle layer was quantified. Asterisks indicate significant differences from the control ($^{*}P < 0.05$, ANOVA). AP, control AP-transfected tecta; Grg4, *Grg4*-transfected tecta; Mor, tecta transfected with morpholino antisense oligonucleotide. Scale bars: 100 μm. Data adapted from Sugiyama and Nakamura (2003)

efficiently introduced by electroporation. It is to be noted that fluorescein-coupled morpholino oligonucleotide moves toward cathode as its charge is opposed to negatively charged DNA. Distribution of the cells containing the fluorescein-coupled morpholino is detectable by immunostaining with antifluorescein andibody (Fig. 12.4c(d)). The *Grg4* antisense morpholino oligonucleotide increased the number of early migratory cells (*Sox2*-positive upper lamina II) and decreased the number of late migratory cells (*Sox14*-positive deeper lamina II) (Fig. 12.4c). Thus, the loss-of-function of *Grg4* successfully exhibits the opposite results from those obtained by the gain-of-function (Fig. 12.4d).

4 Comments

4.1 Perspective

The electroporation technique now provides distinct advantages not only in the understanding of embryogenesis but also the in observation of neuronal morphology and function in well-developed brains (Huber et al., 2008; Nagayama et al., 2007; Umeshima et al., 2007). In recent years, electroporation has been used innovatively in advancing neuroscience. The use of electroporation and voltage-sensitive dye has allowed detection of neuronal responsiveness to a certain stimulus (Nagayama et al., 2007). Light-gated algal channel channelrhodopsin genes have been electroporated by the pulse-labeling technique to give a focal stimulation to a specific neuronal population in awake animals (Huber et al., 2008). If there is a possibility that single-cell electroporation (Haas et al., 2001; Kitamura et al., 2008) could be combined with a unique proviral vector to allow for transsynaptic infection (Wickersham et al., 2007), the understanding of neuronal circuits could advance. Electroporation in combination with novel tools and techniques thus can open many new doors of possibilities in future study.

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Chapter 13
Electroporation into Cultured Mammalian Embryos

Tadashi Nomura, Masanori Takahashi, and Noriko Osumi

1 Introduction

Over the last century, mammalian embryos have been used extensively as a common animal model to investigate fundamental questions in the field of developmental biology. More recently, the establishment of transgenic and gene-targeting systems in laboratory mice has enabled researchers to unveil the genetic mechanisms underlying complex developmental processes (Mak, 2007). However, our understanding of cell–cell interactions and their molecular basis in the early stages of mammalian embryogenesis is still very fragmentary. One of the major problems is the difficulty of precise manipulation and limited accessibility to mammalian embryos via uterus wall. Unfortunately, existing tissue and organotypic culture systems *per se* do not fully recapitulate three-dimensional, dynamic processes of organogenesis observed *in vivo*. Although transgenic animal technology and virus-mediated gene delivery are useful to manipulate gene expression, these techniques take much time and financial costs, which limit their use.

Whole-embryo mammalian culture system was established by New and colleague in the 1970s, and was modified thereafter by several researchers (reviewed by New, 1971, 1978; Sturm and Tam, 1993; Hogan et al., 1994; Eto and Osumi-Yamashita, 1995; Tam, 1998). At first, the whole-embryo culture system was used in the field of teratology, and then applied in a variety of developmental biology fields, because this

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system well maintains the embryonic growth and morphogenesis that are comparable to those *in utero*, and dramatically improves accessibility to the early fetal stages. Furthermore, in combination with gene-delivery techniques such as electroporation, gene expression can be manipulated in a tissue- and region-specific manner (Osumi and Inoue, 2001; Takahashi et al., 2002). Here we introduce this combinatorial procedure applying of the electroporation technique to whole-embryo culture system. We then illustrate the use of this approach in the developmental neurobiology by presenting our findings on molecular mechanisms controlling stem cell maintenance and neuronal migration in the embryonic cortex. Finally, we will discuss future directions and a potential widespread value of this method in developmental biology.

2 Procedure

2.1 Whole Embryo Culture

Currently, the whole-embryo culture system can be applied to a wide range of mammalian embryonic stages from egg cylinder to mid-gestation, for up to two or three days *in vitro* (New et al., 1971, 1976; Cockroft, 1976; Osumi-Yamashita et al., 1994, Osumi-Yamashita 1996; Lee et al., 1995; see Fig. 13.1a). Since rat serum is commonly used as the culture medium, optimal culture condition is archived by using rat embryos (Eto and Takakubo, 1985; Eto and Osumi-Yamashita, 1995). Although the protocol for rat embryos can be applicable to mouse embryos at same developmental stages, embryonic condition is poorer than those of rats, probably due to hetero-specific serum.

Here we briefly introduce the technical aspects of this procedure. First, embryos are isolated from the uterus while maintaining embryonic circulation between the embryo and the placenta. Then, the embryos are cultured in bottles filled with a culture medium containing 100% rat serum, 2 mg/ml glucose and 0.25× antibiotics (25 U/ml penicillin and 25 µg/ml streptomycin). We are using an incubator specially designed for the whole embryo culture, in which 24 bottles can be connected with two rotators (RKI 010-0310, Ikemoto Science, Tokyo, Japan). The bottles are continuously supplied with $O_2/CO_2/N_2$ gas; where the percentage of O_2 and the flow rate are proportionate to the stage of embryogenesis (see also Eto and Osumi-Yamashita, 1995; Osumi and Inoue, 2001 for detailed procedure).

2.2 Electroporation

After few hours of pre-culture in the rotating bottle, the embryo is taken away from the bottle, and transferred into a saline-containing bath-type electrode (CUY-520, Nepa Gene, Tokyo, Japan) filled with Tyrode's balanced salt solution. Then 0.1–0.5 µl of plasmid-containing solution (5 mg/ml) is injected into the brain ventricle (Fig. 13.1b) and square pulses (70 V, 5 Hz, 50 ms, five times) are applied into the

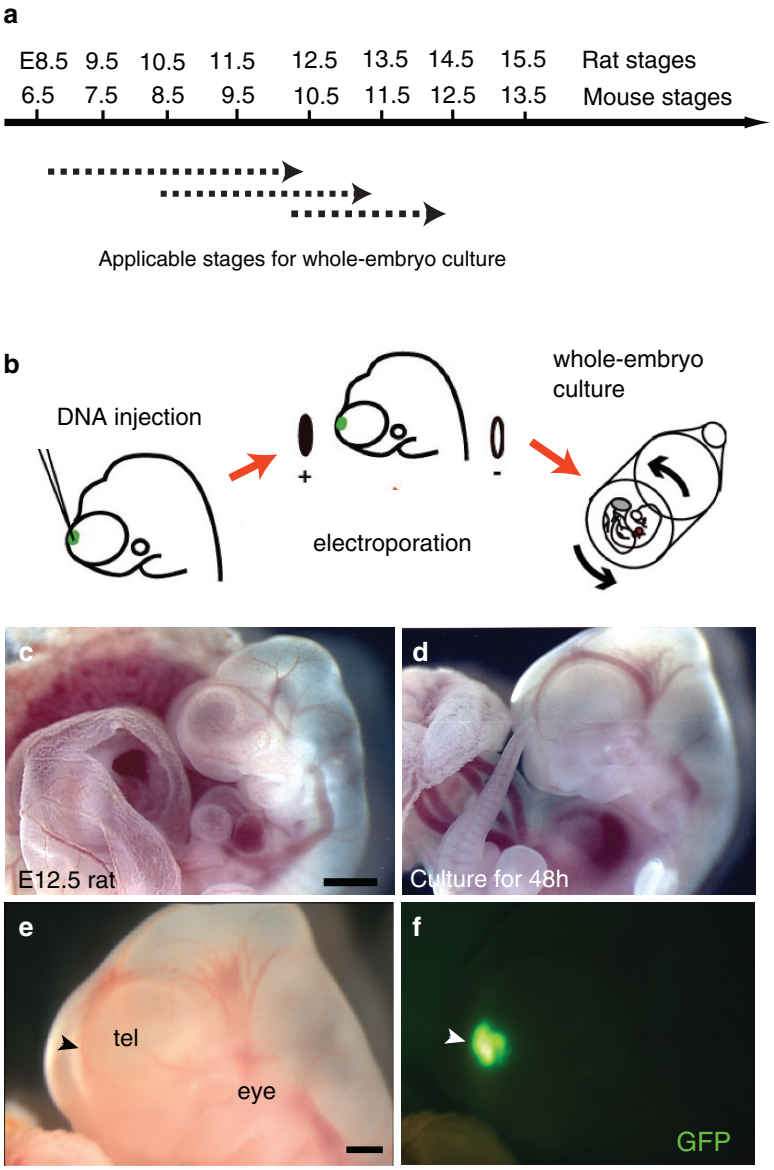

Fig. 13.1 Electroporation followed by whole-embryo culture. (**a**) Embryonic stages that are available for whole-embryo culture. (**b**) Procedure of gene transfer and culture. (**c**, **d**) An embryo (E12.5 rat) at the beginning of culture (**c**) and after 48 h of culture (**d**). (**e**, **f**) An embryo in which GFP-expression vector was electroporated. After 24 h, strong GFP expression was detected at the rostral part of the telencephalon (*black and white arrowheads*). tel: telencephalon. Scale bars: 700 μm in **c**, **e**

embryonic brain using an electroporator (CUY21, Nepa Gene). Alternatively, forceps-type electrodes with different tip diameter and shapes (CUY-650 series) can also be used for focal delivery of exogenous genes (Fig. 13.1e, f). After electroporation, the embryos are transferred back to the culture bottles. The culture medium should be changed after 24 h. The embryo can be cultured in this system for up to 48–72 h depending on initial embryonic stages (Fig. 13.1a, c, d).

3 Application and Results

A combination of the whole embryo culture and electroporation technique is particularly useful for cell-lineage analysis, and gain- and loss-of-function studies of specific genes during embryogenesis. For example, we have applied this system to various types of experiments such as fate-mapping of the developing forebrain (Inoue et al., 2000), analysis of the compartmentalization of the telencephalon (Inoue et al., 2001), neuronal specification in the developing hindbrain (Takahashi and Osumi, 2002), proliferation of neuroepithelial cells (Arai et al., 2005), and neuronal migration in the developing olfactory bulb and olfactory cortex (Nomura and Osumi, 2004; Nomura et al., 2006). We will here describe two of our previously published studies on the role of Pax6 and its downstream target gene in cortical neural stem cells and neuronal migration (Arai et al., 2005; Nomura et al., 2006), preceded by a brief review of previous research.

3.1 The Role of Pax6 and Fabp7 in Embryonic Cortical Progenitor Cells

The mammalian cortex is an intricate structure in which a billion neurons and glial cells are precisely distributed in an elaborate and highly ordered neuronal circuits (Nieuwenhuys, 1994). All cortical neurons are generated during embryogenesis, and they are derived from the neural progenitor cells located in the ventricular and subventricular zone (Caviness and Takahashi, 1995; Takahashi et al., 1995). These progenitor cells proliferate to increase their own numbers, and concomitantly differentiate into several types of neurons in spatially and temporally regulated manners (Fig. 13.2a). This highly organized process is precisely regulated during corticogenesis, although the underlying molecular mechanisms are largely unknown (McConnell, 1995; Caviness et al., 2000; Miller and Gauthier, 2007; Molyneaux et al., 2007).

Over the last decade, we have focused our interest on the role of *Pax6* gene in the developing central nervous system. *Pax6* encodes a transcription factor that contains two DNA-binding domains, a paired-box and a homeobox, and regulates a variety of events during neural development (Walther and Gruss, 1991; also reviewed in Callaerts et al., 1997; Osumi, 2001; Simpson and Price, 2002). Previous studies have shown that Pax6 is a prerequisite for neuronal differentiation, via the regulation of the expression of *Neurogenin2* (*Ngn2*), which strongly promotes neuronal

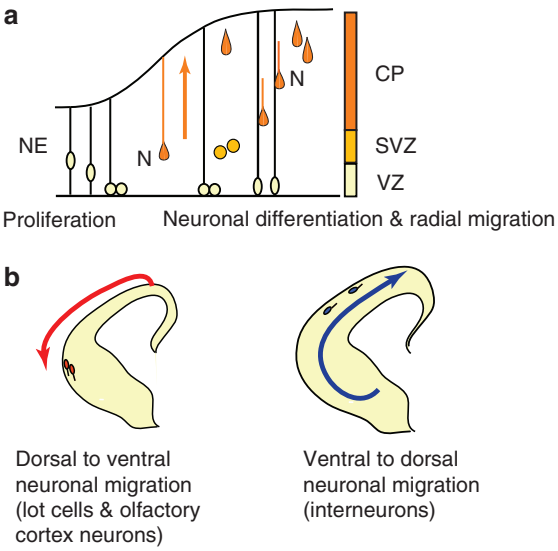

Fig. 13.2 Stage-specific events in the developing cortex. (**a**) Neurogenesis and radial neuronal migration. In the early stages of mammalian cortex, neuroepithelial cells (NE) proliferate in ventricular zone (VZ) and subventricular zone (SVZ). As development proceeds, neuroepithelial cells produce neurons (N), which leave the VZ and migrate into cortical plate (CP). (**b**) Tangential neuronal migration. Left: dorsal to ventral migration of neurons in the early stages of cortex (Tomioka et al., 2000; Nomura et al., 2006). Right: ventral to dorsal migration of cortical interneurons (Marin and Rubenstein, 2003)

differentiation (Scardigli et al., 2003). On the opposite, other studies have revealed that Pax6 is required for the proliferation and maintenance of neural stem cells (Warren et al., 1999; Estivill-Torrus et al., 2001; Maekawa et al., 2005). These contradictory reports suggest that Pax6 plays a dual role during neuronal development, regulating both proliferation and neuronal differentiation of neural stem cells, potentially by regulating expression of distinct target genes.

We have previously identified a gene encoding fatty acid binding protein 7 (Fabp7/BLBP), as a candidate target gene of Pax6 in the developing rat brain (Arai et al., 2005). *Fabp7* is expressed in Pax6-positive cortical neuroepithelial cells, and its expression is markedly reduced in the *Pax6* mutant cortex (Arai et al., 2005). To test whether Pax6 could also activate *Fabp7* expression in the developing cortex, we took advantage of the electroporation and whole-embryo culture technique, where Pax6-expression vector was introduced into E11.5 rat cortex. 24 h after whole-embryo culture, a strong induction of *Fabp7* expression was detected within the cortical region where *Pax6* was overexpressed. Moreover, a robust expression of *Fabp7* was also induced by overexpression of *Pax6* in the *Pax6* mutant telencephalon (our unpublished data). These data demonstrate that Pax6 has the potential to activate *Fabp7* expression during early cortical development (Arai et al., 2005).

Recently, RNA interference technique (RNAi), which efficiently suppresses the expression of endogenous genes, has been applied to post-implantation embryos

(Calegari et al., 2002, 2004). To elucidate the role of *fabp7* in the developing cortex, we designed several types of *Fabp7*-specific siRNA, expressed using *pSUPER* basic vector (oligoengine, Seattle, WA). These vectors were introduced into the developing cortical primordium by electroporation. To study the effect of *Fabp7*-siRNA on the proliferation of neuroepithelial cells, BrdU (5-bromo-2′-deoxyuridine) that get incorporated into the DNA during S-phase was added to the culture medium, following electroporation. In comparison to control vector, electroporation of *Fabp7-siRNA* remarkably reduced the number of BrdU-incorporated cells in the developing cortex. Furthermore, we noticed that *Fabp7-siRNA* electroporation induced ectopic localization of Tuj1-positive neurons in the cortical ventricular zone (Fig. 13.3a, b). These results suggest that the reduction of *Fabp7* expression promotes premature cell cycle exit and neurogenesis, and that *Fabp7* is required to keep the neuroepithelial cells in an immature state in developing cerebral cortex (Arai et al., 2005).

During embryogenesis, different transcription factors control the expression of specific target genes in a spatially and temporally restricted manner. We showed that the electroporation-based gene delivery in whole-embryo culture is a convenient and useful *in vivo* assay system allowing such studies on stage and tissue-specific transcriptional regulation. Specifically, we demonstrated that, although Pax6 activates the expression of *Fabp7,* no strong induction of *Ngn2* expression was detected in the cerebral cortex, which has been shown to be activated by Pax6 in the developing spinal cord (Scardigli et al., 2003). These data highly suggest that differential control of downstream target genes is the central mechanism underlying the dual role of Pax6 for the maintenance and differentiation of neuroepithelial cells. Electroporation in the whole-embryo culture system can also be used for promoter/enhancer analysis, and at present we are investigating the promoter region of *Fabp7* using this technique (Numayama-Tsuruta and Osumi, in preparation). Complementary approaches such as ChIP on chip or *in silico* analysis, to target promoter/enhancer regions will further extend the analysis of downstream target genes.

3.2 Studies on Neuronal Migration During Early Cortical Development

During cortical development, differentiating neurons leave the proliferative zone and migrate into the developing cortical plate and form various cortical regions according to their genetically defined characteristics (Kriegstein and Noctor, 2004). Two distinct modes of neuronal migration, radial and tangential migration, have been reported in the developing cortex (Marin and Rubenstein, 2003). Radial migration of excitatory projection neurons is an essential step for the generation of cortical laminated structure (Fig. 13.2a). In contrast, cortical interneurons undertake tangential migration from the subcortical (striatal) regions to reach the cortex, and integrate into the cortical neuronal circuits (Fig. 13.2b). Furthermore, recent studies have indicated that Cajal-Retzius cells, which play an essential role for the establishment of the cortical laminar structure, exhibit tangential migration to spread

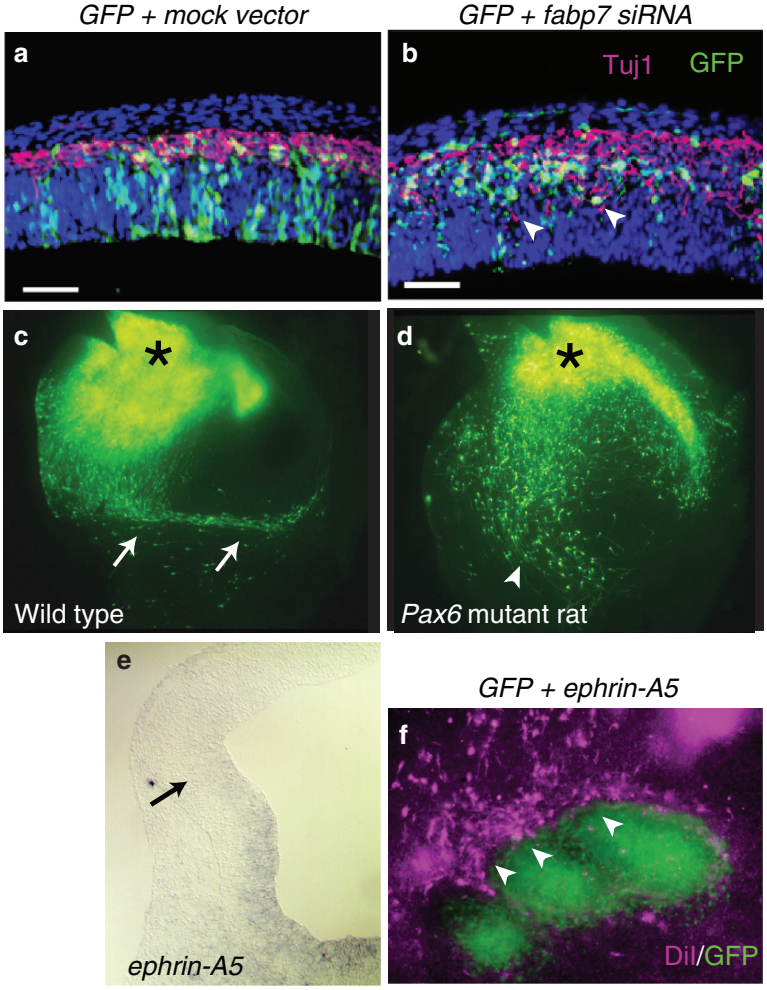

Fig. 13.3 Applications of electroporation in whole-embryo culture. (**a**, **b**) A loss-of-function study of *fabp7* by electroporation of siRNA-expression vector. Compared to control (**a**), introduction of *fabp7*-specifc siRNA induced ectopic localization of TuJ1-positive neurons (*white arrowheads* in **b**; Arai et al., 2005). (**c**, **d**) Dorsal to ventral migration of olfactory cortex neurons. In wild type, neurons born at the dorsal part of the cortex (*an asterisk* in **c**) migrate ventrally and aligned at the future olfactory cortex (*arrows* in **c**), whereas in the *Pax6* mutant rat, the neurons invade into the ventral part of the cortex (*an arrowhead* in **d**; Nomura et al., 2006, 2007). (**e**) Expression of *ephrin-A5* in the wild type telencephalon. *Ephrin-A5* is expressed at the ventral part of the telencephalon, and its expression border coincides with the future olfactory cortex (*a black arrow*). (**f**) Electroporation of *ephrin-A5*-expression vector into the lateral cortex limits migration of the DiI-labeled olfactory cortex neurons (*white arrowheads* in **f**; Nomura et al., 2006)

over the entire surface of the cortex during early embryonic stages (Takiguchi-Hayashi et al., 2004; Bielle et al., 2005; Yoshida et al., 2006; Garcia-Moreno et al., 2007a). We have also reported that additional migration mode that occurs in the early developing cortex, which migrate from the dorsal to the ventral part of the telencephalon, and settled at the future olfactory cortex (Fig. 13.2b; Tomioka et al., 2000; Nomura et al., 2006). This neuronal subtype is a heterogeneous population that can be distinguished by several markers (Sato et al., 1998; Kawasaki et al., 2006; Nomura et al., 2006; Garcia-Moreno et al., 2007b). Functionally, some of these neurons have been suggested to play a crucial role for guiding olfactory axons (Sato et al., 1998).

We have also shown that Pax6 regulates migration patterns of a population of the olfactory cortex neurons (Nomura et al., 2006). These neurons are born at the dorsal part of the embryonic cortex at E11.5 (in rats; corresponding to E9.5 in mice), and migrate ventrally toward the cortical-striatal boundary (CSB), which corresponds to the future olfactory cortex. After arriving at the CSB, they migrate caudally, and aligned at the CSB (Fig. 13.3c). These neurons contribute to the most superficial layer of the mature olfactory cortex. However, in the *Pax6*-deficient mutant, these neurons migrate ventrally ignoring the CSB, and further migrate into the ventral part of the telencephalon (Fig. 13.3d). As a consequence, these neurons ectopically accumulated in the ventral part of the *Pax6* mutant telencephalon. This phenotype suggests that Pax6 regulates the expression of guidance molecule(s) that are responsible for stop and/or alignment of the olfactory cortical neurons at the CSB.

To identify the molecule(s) regulating the migration into the olfactory cortex, we took advantage of our newly established long-term culture system that combined the whole-embryo culture with telencephalic organotypic culture (Tomioka et al., 2000; Nomura and Osumi, 2004; Nomura et al. 2006; Nomura and Osumi, 2007). After labeling the olfactory cortex neurons by electroporation of GFP-expressing plasmid, the embryos were cultured in the whole-embryo culture for 24 h, and the cortical hemisphere were dissected out and placed on a collagen-coated semi-permeable membrane (Transwell-COL, Coster 3,492, Corning, USA) and further cultured in a medium (DMEM/F12, 10% FCS and antibiotics) containing several antagonists against cell surface/secreted molecules. By using this system, we identified ephrin-A5, a member of GPI-anchored cell surface proteins, as a candidate regulator of the migration pattern of the olfactory cortex neurons. *Ephrin-A5* is expressed in the ventral part of the wild-type telencephalon (Fig. 13.3e), whereas its expression is remarkably decreased in the *Pax6* mutant telencephalon. Furthermore, electroporation of *Pax6*-expression vector into the mutant telencephalon induced *ephrin-A5* expression. These results provide strong evidence that *Pax6* is a necessary and sufficient factor to activate the expression of *ephrin-A5* in the developing cortex.

To further study the role of *ephrin-A5* on neuronal migration, we misexpressed *ephrin-A5* in early stages of the embryonic cortex. After labeling the olfactory cortical neurons by microinjection of DiI-containing solution (D-282, Molecular Probes), *ephrin-A5* expression vector (*pCAX-ephrin-A5*) was electroporated into the lateral part of the cortex, corresponding to the migratory pathway of these neurons. The embryos were cultured using the whole-embryo culture system for 48 h, and

then the migration patterns of the labeled neurons were analyzed. We found that exogenous *ephrin-A5* limits the migration of olfactory cortex neurons: the neurons migrated ventrally but avoided entering the *ephrin-A5*-positive area, and aligned at the border of the *ephrin-A5* expression region (Fig. 13.3f). These results indicate that ephrin-A5 has the potential to limit the migration of the olfactory cortical neurons, and reduced expression of this gene in the *Pax6* mutant causes abnormal invasion of the neurons into the ventral part of the telencephalon. A similar defect in the olfactory neuron migration was detected in the *ephrin-A5*-deficient mice, further supporting above conclusion (Nomura et al., 2006).

Several different factors have been recently implicated in the guidance of migrating neurons during early stages of cortical development. For example, CXCL12, a member of the CXC subfamily of chemokines, has been shown to play an essential role in the migration of Cajal-Retzius cells (Borrell and Marin, 2006). Furthermore, netrin-1 regulates dorsal to ventral migration of the lot-cells, which are an important neuronal subtype for guiding the lateral olfactory tract (Kawasaki et al., 2006). However, the factors involved in early cortical neuronal migration identified so far are quite few in comparison to those known to regulate the migration of cortical interneurons, which occurs at later developmental stages. This is largely due to difficulties in manipulating gene expression in the early developing cortex, which greatly limits functional analysis of specific molecules on the neuronal migration. As described in detail above, the electroporation and whole-embryo culture system overcomes these technical problems, and allows for the analysis of genes regulating early cortical neuronal migration.

4 Comments

4.1 Further Applications and Future Directions

The method of electroporation in whole-embryo culture is a powerful tool to analyze molecular mechanisms underlying early stages of mammalian development. Indeed, the technique has already been applied to a variety of studies that examined developmental processes such as gastrulation (Davidson et al., 2003; Ybot-Gonzalez et al., 2007), pancreatic development (Pierreux et al., 2005), hematopoietic development (Giroux et al., 2007) and brain development (Itasaki et al., 1999; Inoue and Krumlauf, 2001). Electroporation techniques will open further experimental possibilities for manipulating gene expression. For example, introduction of dominant-negative constructs or siRNAs to specific gene will enable to test effects of the silencing gene in small number of cells. Moreover, recent advances of inducible site-specific recombination system (using the Cre-ER/loxP and Flp/FRT systems) also allow ones to manipulate gene expression in stage- and tissue-specific manner (Matsuda and Cepko, 2007; Barnabe-Heider et al., 2008). An application of this system in the whole-embryo culture system extends opportunities to control gene expression in a quick and efficient manner.

One restriction of the current whole-embryo culture system is the limitation in culture periods; it is impossible to culture embryos more than E12.5 in mouse embryos and E14.5 in rat embryos. This is largely due to the failure to establish a nutrient exchange between the embryo and the placenta. Despite several attempts to improve culture conditions, there has been no report to date of a successful development of a functional placenta *in vitro*. Although this prevents the analysis of more advanced developmental events occurring at late-gestation stages, combinatory approaches such as organotypic culture or *in utero* manipulation might override this hurdle and the results (Saito and Nakatsuji, 2001; Yozu et al., 2005; Nomura and Osumi, 2007; see Fig. 13.1a).

Following completion of human and other mammalian genome projects, an enormous amount of information regarding our genetic make up has been revealed. Surprisingly, the estimated number of protein-coding genes in the human genome is much smaller than expected, which raises the question about how a limited number of genes can create our complicated body structures. In addition, we have still limited knowledge about dynamic cellular events during embryogenesis. The introduction of gene-delivery techniques in combination with a culture system broaden the possibilities for experimental strategies focused on cell and developmental biology that will be essential for the research in the post-genomic generation.

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Chapter 14
In Utero Electroporation: Assay System for Migration of Cerebral Cortical Neurons

Hidenori Tabata and Kazunori Nakajima

1 Introduction

During the development of the cerebral cortex, the majority of cortical neurons are generated in the ventricular zone (VZ) facing the lateral ventricle and then migrate toward the pial surface to form the highly organized 6-layered cerebral cortex. Detailed profiles of these processes and their molecular mechanisms had been largely unknown because of the absence of an efficient assay system. The *in vivo* electroporation system was initially devised for use within chick embryos (Funahashi et al., 1999; Itasaki et al., 1999; Momose et al., 1999; Muramatsu et al., 1997), and we and other groups have used that system as a basis for developing an *in utero* electroporation system, which allows plasmid DNA to be introduced into cortical progenitor cells in developing mouse embryos in the uterus (Fukuchi-Shimogori and Grove, 2001; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001; Takahashi et al., 2002). *In utero* electroporation of other sites in the brain, including the hippocampus (Navarro-Quiroga et al., 2007), cerebral basal ganglia (Borrell et al., 2005; Nakahira et al., 2006), cortical hem (Takiguchi-Hayashi et al., 2004), and dorsal thalamus (Bonnin et al., 2007), has recently been reported. Introducing green fluorescent protein (GFP) enables the entire processes of migration and layer formation to be visualized (Ajioka and Nakajima, 2005; Sasaki et al., 2008; Tabata and Nakajima, 2002, 2003), and the role of any gene involved in these processes can be easily assessed by overexpressing the proteins or their mutants (Ohshima et al., 2007), or by knocking down the genes by the RNA interference technique (Bai et al., 2003). Furthermore, the Tet-On/Off system and/or other plasmid-vector-based technologies will expand the potential of the analyses. In this section we review the principles and methods of gene transfer into the cortical wall of mouse embryos by means of the *in utero* electroporation system.

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2 Materials

2.1 Instruments

- Electroporator: CUY21E or CUY21SC (Nepa gene, Chiba, Japan)
- Tweezers-type electrode: CUY650P3 for embryonic day (E) 12.5, or CUY650P5 for E13 or older (Nepa gene)
- Aspirator tube assembly (Drummond)
- 1-mm diameter glass capillary tubes (GD-1; Narishige, Tokyo, Japan)
- Optical fiber light
- Sterile gauze: 7.5 × 7.5 cm
- Surgical instruments: fine forceps × 2, surgery scissors × 2, ring forceps, needle holder
- Surgical tape
- Nylon suture
- Silk suture

2.2 Reagents

- HEPES-buffered saline (HBS): Dilute 2 × concentrated HBS (51558, Sigma) with autoclaved water at 1/2
- 0.1% FastGreen solution: Dissolve the powder (F7258, Sigma) with autoclaved water
- Autoclaved phosphate-buffered saline (PBS)
- Diluted Nembutal solution: Dilute Nembutal solution (5% Pentobarbital sodium salt solution) with autoclaved water at 1/10

2.3 Preparation of Plasmid DNA (See Also Comments 1 and Fig.14.3b)

Plasmid solution prepared by conventional methods, such as with the Qiagen kit, can be used for *in utero* electroporation. If precipitates form in the plasmid solution, further purification with phenol/chloroform followed by chloroform extraction is required, because such precipitates may cause hydrocephalus. Plasmids are dissolved with HBS. The location of the DNA solution is monitored by adding 0.1% Fast Green solution at 1/10 volume to the aliquot of DNA solution to be injected.

2.4 Preparation of Micropipettes

The micropipettes for injection are made with a puller (PC-10, Narishige, Tokyo, Japan) from 1-mm diameter glass capillary tubes (GD-1; Narishige). The tip of the

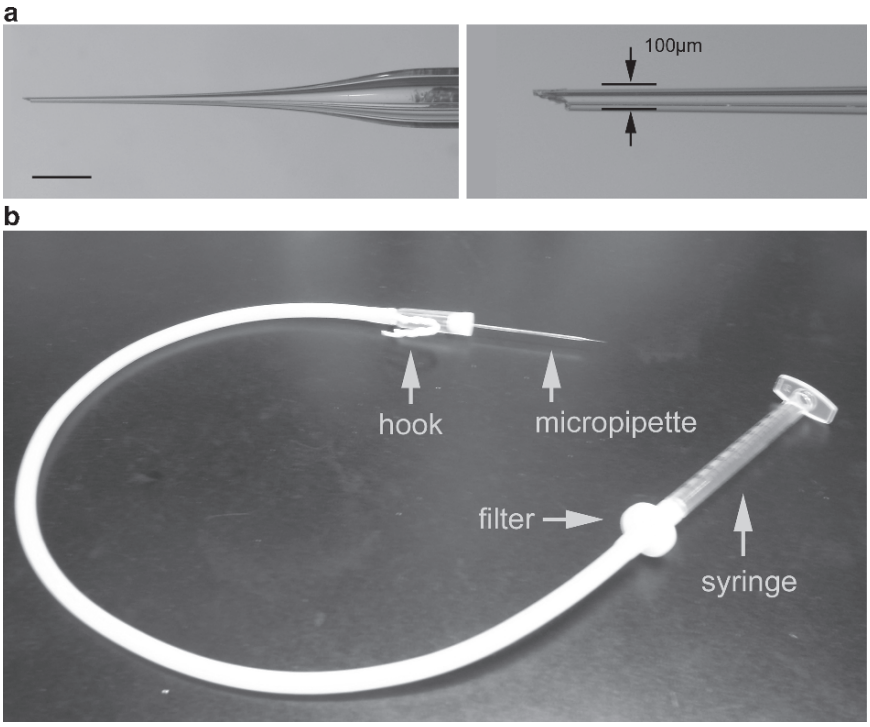

Fig. 14.1 Preparation of the aspirator tube assembly. (**a**) Shape of micropipette. A high-power view of the tip is shown in the right panel. The tip of the needle has been cut obliquely. (**b**) The aspirator tube assembly. The mouthpiece of the original product has been replaced with a 0.22 μm filter and 1 ml syringe, as a new mouthpiece. Scale bar in (**a**), 1 mm

micropipettes is cut obliquely with a fine forceps under a dissecting microscope (Fig. 14.1a). The micropipettes are attached to the aspirator tube assembly (Drummond) (Fig. 14.1b).

3 Procedure

1. A pregnant mouse is intraperitoneally injected with a 12 μl/g body weight dose of 1/10 diluted Nembutal solution (see Comments 2).
2. Ten minutes later the mouse is placed on its back on a dissecting board, and its limbs are held in place with a surgical tape.
3. After disinfecting the abdominal skin with 70% ethanol, an approximately 2 cm midline skin incision is made extending anteriorly from the level of the midpoint between the most posterior two pairs of nipples using one set of forceps and scissors (Fig. 14.2a).
4. A 2 cm midline incision is then made in the abdominal wall along the linea alba using another set of forceps and scissors. A piece of sterile gauze with a hole

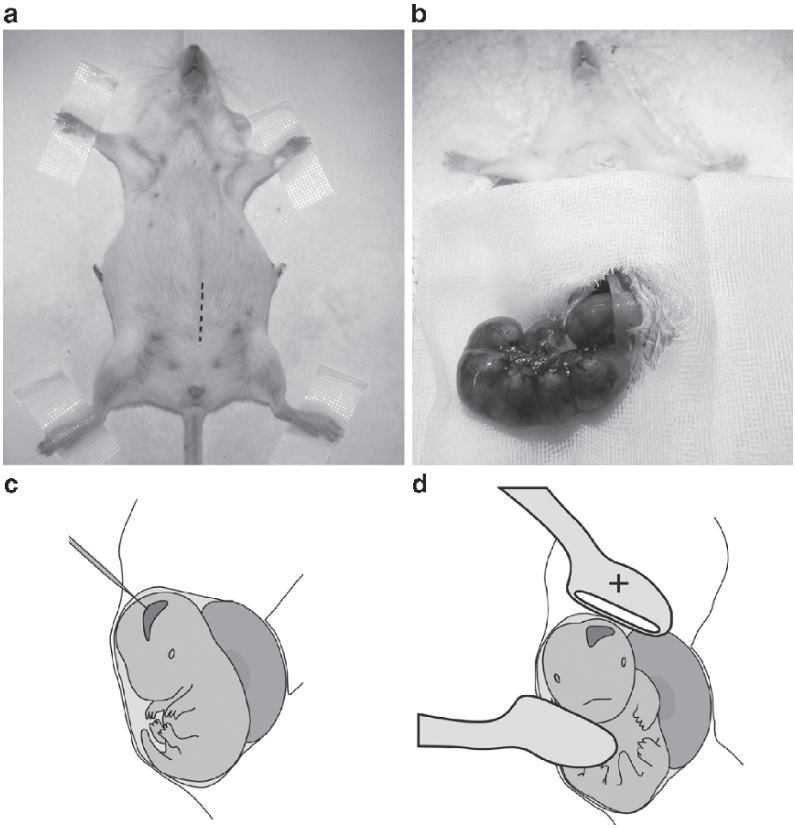

Fig. 14.2 *In utero* electroporation procedure. (**a**) Anesthetized pregnant mouse placed on a dissecting board. The broken line indicates the site of the incision. (**b**) One uterine horn is drawn out through a hole in a piece of sterile gauze. (**c**) Plasmid DNA is injected into one or both lateral ventricles through the uterine wall. (**d**) Electronic pulses are applied from outside the uterine wall

cut in the center is placed over the incision, and one uterine horn is drawn out through the hole in the gauze (Fig. 14.2b).

5. After observing the orientation of the embryos through the wall of the uterine horn, a micropipette is inserted into the lateral ventricle, and 1~2 μl of plasmid solution is injected by expiratory pressure using the aspirator tube assembly (see Comments 3–6, Fig. 14.2c).
6. After soaking the uterine horn with PBS, the head of embryo is pinched with a tweezers-type electrode (CUY650P3 or CUY650P5), and electronic pulses are applied with a CUY 21E electroporator (see Comments 7, Fig. 14.2d). The electroporation conditions are shown in Table 14.1. The voltage should be adjusted so that the actual current would become 40–60 mA.
7. After the procedure is completed on the first uterine horn, it is replaced in the abdominal cavity, and the other uterine horn is exposed and subjected to the same procedure.

Table 14.1 The electroporation conditions

Age	Electrode diameter	Voltage	Pulse-on	Pulse-off	Number of pulses
E12.5	3 mm	33 V	30 ms	970 ms	4
E13 ~	5 mm	30~35 V	50 ms	950 ms	4

Pulse-on; the duration of square pulse.
Pulse-off; the interval between pulses.

8. When the procedures on the second uterine horn have been completed, the abdominal cavity is filled with PBS.
9. The abdominal wall and skin are closed with nylon sutures and silk sutures, respectively.
10. Since the body temperature of the mouse decreases as a result of the exposure of uterus and the effect of anesthetization, the mouse should be warmed with hands or on a plate warmer (see Comments 8).
11. After surgery, the mice should give birth to the electroporated pups normally. If CAG-promoter-driven expression vector of enhanced GFP (CAG-EGFP) (Niwa et al., 1991) has been injected, the GFP fluorescence would be visible through the skin under a fluorescence stereomicroscope on postnatal day 1.

4 Application and Results

4.1 Optimization of Electroporation Conditions

Several conditions must be optimized to make *in utero* electroporation more efficient.

4.1.1 Number of Pulses

A 4 μg/μl dose of CMV promoter-driven *luciferase*-expression vector (CMV-luc) was injected into the lateral ventricle at E14.5, and one, two, or four electronic pulses were applied. The transfected brains were sampled 24 h later, and the efficiency of gene-transfer was evaluated on the basis of luciferase activity (Fig. 14.3a). Application of one pulse was insufficient to achieve significant gene transfer. When more than one pulse was applied, the efficiency of gene-transfer increased almost linearly with the number of pulses. The electric pulses create tiny pores in the plasma membrane, and move DNA toward the anode in the electric field. One pulse might be enough to create the pores, not to drive the DNA toward the anode.

4.1.2 Plasmid Concentration

CMV-luc was injected in dose of 0.5, 2, or 5 μg/μl at E15.5 and four pulses were applied. The brains were sampled 24 h later, and the luciferase activity of each

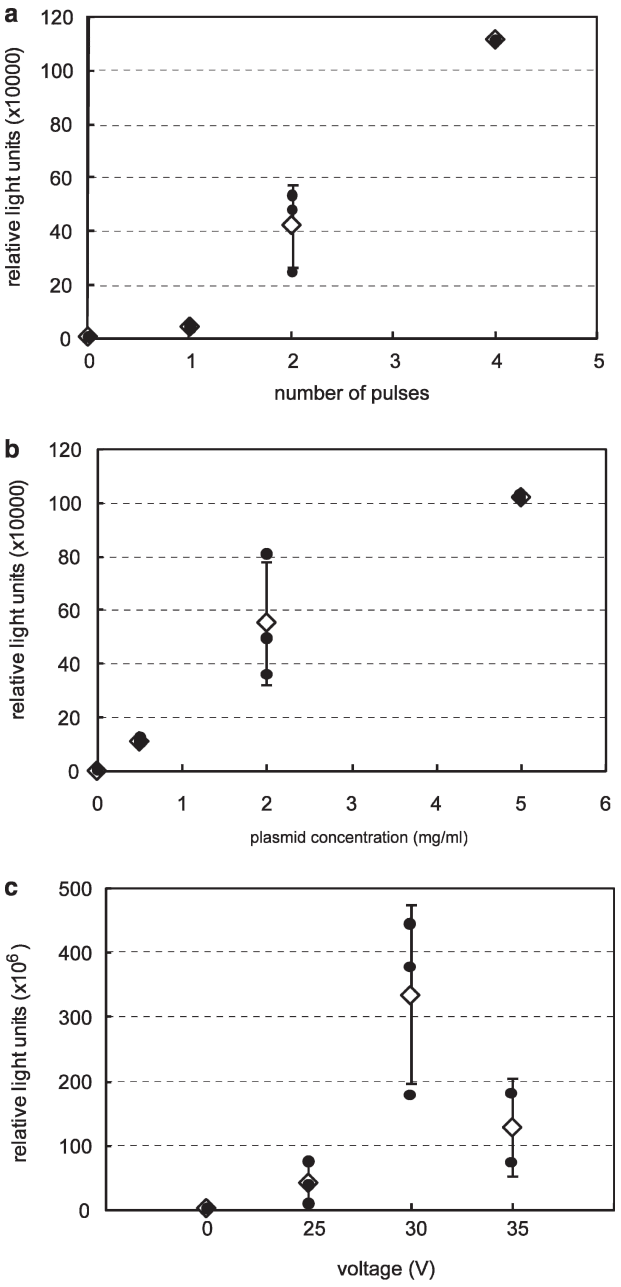

Fig. 14.3 Optimization of various factors for more efficient *in utero* electroporation. Effect of number of pulses (**a**), plasmid concentration (**b**), and voltage (**c**) on transfection efficiency. (**a**) A 4 μg/μl dose of CMV-luc was injected into the lateral ventricle at embryonic day 14.5 (E14.5), and different numbers of 30 V pulses were applied. The transfected brains were sampled 24 h later, and luciferase activity was measured. (**b**) CMV-luc plasmid prepared in various concentrations was injected at E15.5 and four 30 V pulses were applied. and transfected brains were sampled 24 h later. (**c**) 4 μg/μl dose of CMV-luc was injected at E15 and electric pulses of various voltages were applied four times. The brains were sampled at E18. The duration of the square pulses applied in all experiments was 50 ms, and the interval between pulses was 950 ms

sample was analyzed (Fig. 14.3b). The efficiency of gene-transfer increased almost linearly with the concentration of plasmid DNA injected.

4.1.3 Voltage

CMV-Luc was injected in dose of 4 µg/µl at E15 and four pulses were applied. The brains were sampled at E18, and the luciferase activity of each sample was analyzed (Fig. 14.3c). Luciferase activity was detected when a voltage of around 30 V was applied, and lower voltages did not bring about effective gene transfer. This finding seems to indicate that a high level of energy is needed to produce the pores in the plasma membrane. When a voltage of 35 V was applied, the expression of the plasmid that was introduced tended to reduce. It may have resulted from cell death.

4.2 Tracing the Lineage of GFP-Positive Cells

CAG-EGFP (5 µg/µl) (Niwa et al., 1991) was injected into both hemispheres at E12.5. After fixing the embryos at E14.5, the brains were dissected out and examined for GFP fluorescence under a fluorescence stereomicroscope (Fig. 14.4a). Fluorescence was observed in the lateral region of the hemisphere onto which the anode had been placed and in the medial region of the opposite hemisphere. GFP-positive cells were observed in the VZ 12 h after electroporation. Accumulation of GFP-positive cells in the subventricular zone (SVZ) and scattered distribution of GFP-positive cells in the intermediate zone (IZ) were observed 1.5 days after

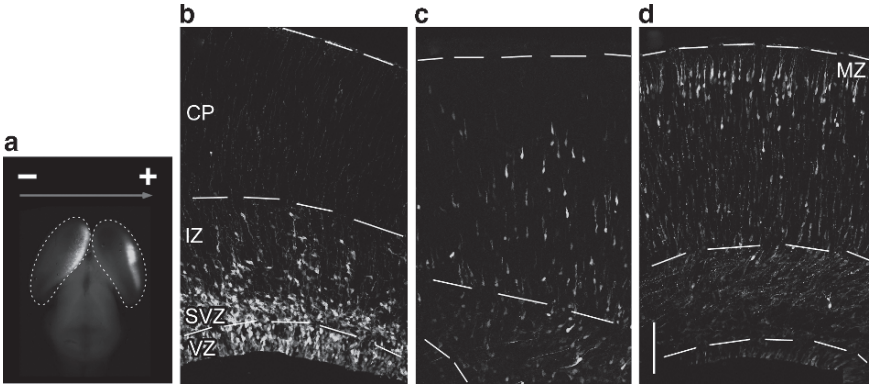

Fig. 14.4 GFP expression after electroporation. E12.5 mouse embryos were subjected to *in utero* electroporation and fixed 2 days later. The brains were removed and examined under a fluorescence stereomicroscope (**a**). Changes in the position and morphology of GFP-positive cells after *in utero* electroporation (**b–d**). E14.5 (**b,c**) or E15 (**d**) mouse embryos were transfected with CAG-EGFP and then fixed 1.5 days (**b**), 3 days (**c**), and 6 days (**d**) after electroporation. Abbreviations: VZ; ventricular zone, SVZ; subventricular zone, IZ; intermediate zone, CP; cortical plate, MZ; marginal zone. Scale bar, 50 µm

electroporation (Fig. 14.4b). By 2.5 days, the number of GFP-positive cells in the IZ had increased, but only a few GFP-positive cells were found within the cortical plate (CP) at that time. By 3 days, many GFP-positive cells assuming bipolar morphology with their leading process pointing toward the pial surface were found in the CP (Fig. 14.4c). At 6 days after electroporation, the major population of GFP-positive cells was observed beneath the marginal zone (MZ) (Fig. 14.4d), and the cells were extending primitive dendrites into the MZ.

5 Comments

1. The efficiency of electroporation is highly dependent on the DNA concentration. The higher the concentration of DNA used, the higher the level of expression became (Fig. 14.3b). When CAG-EGFP is used, a concentration of 1 μg/μl is sufficient to visualize the migrating neurons. Overexpression of unstable or large proteins requires higher concentrations, such as 5 or 10 μg/μl. When a mixture of two different plasmids is injected, all of the transfected cells should contain both plasmids.
2. Sometimes the anesthesia is inadequate to carry out surgery at the dose of Nembutal described in Procedure Step1. In this case, half the original dose should be applied intraperitoneally.
3. Do not grasp the uterus too tightly. Hook the uterus with ring forceps first, and then draw the uterus out with the fingers.
4. Fast Green enables the distribution of the DNA solution in the lateral ventricle to be seen through the uterine wall.
5. Pushing the back of the head of the embryos so that the head is pressed against the uterine wall facilitates the injection process. The micropipette should be inserted with momentum but should not deeply.
6. We avoid injecting the embryos closest to the vaginal duct, because of the possibility of inducing abortion if they are damaged.
7. The plasmid can be introduced into a larger region of the cortex by placing the cathode on the chin of the embryo and the anode at the center of the injected hemisphere.
8. A long operation time decreases the embryo survival rate. The abdominal cavity should be open for no more than 30 min.

6 Troubleshooting

Gene transfer by electroporation has the major advantages of being a simple procedure and achieving a high level of expression of the gene introduced. However, the risk of damages to the brain may be higher than with other gene transfer systems. The troubleshooting was listed below (Table 14.2).

Table 14.2 Troubleshooting

Problem	Cause	Solution
Dam dies	Operation time too long	Not every embryo needs to be injected. Select embryos to inject and record their location
	Excessive bleeding due to abortion	See "high abortion rate" below
High abortion rate	Operation time too long	See above
	Severe embryo injury	Handle embryos gently. We recommend getting used to handling embryos by starting with E14 or E15 embryos, the ages at which they are easiest to inject
	Disruption of blood flow to the uterus	Make larger skin and abdominal wall incisions. Make sure blood flow is not obstructed by twisting of blood vessels during the operation
	Delayed recovery from the anesthesia	Keep mouse warm during and after the operation
Hydrocephalus	Embryo injury	See solution to "high abortion rate"
	Poor DNA quality	Remove all precipitates by centrifugation or by phenol/chloroform treatment
	Voltage too high	Determine appropriate voltage
	Volume of DNA injected too large	Do not inject more than 2 μl of DNA into one lateral ventricle of an E15 embryo
	Tip of the micropipette blunt	Cut tip obliquely

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Chapter 15
Practical Application of Microelectroporation into Developing Mouse Brain

Tomomi Shimogori and Masaharu Ogawa

1 Introduction

One key approach toward understanding the genetic mechanisms underlying embryonic development involves the overexpression or misexpression of target genes in specific regions and at specific time points. The mouse gene-knockout system has been used extensively for loss-of-function studies due to the availability of a large number of mutant lines and the technical advantages of this system. In contrast, gain-of-function analyses have been performed through the production of knock-in and transgenic animals and with the use of various viruses (Cornetta 2006; Jakobsson et al., 2003; Hashimoto and Mikoshiba, 2004). However, it is not always possible to express or suppress genes in a spatially and temporally restricted manner, and the generation of genetically modified mice and recombinant viruses is time consuming and labor intensive. With the aim of solving these problems, many attempts have been made to apply the electroporation technique in research on developmental biology. Due to the accessibility of the avian embryo, it has been used as a classic model system for the study of developmental events in vertebrates. A novel technique for successful gene delivery into chick embryos has been established; this technique is known as in ovo electroporation and appears to be an excellent method, permitting quick and direct examination of the function of the delivered genes (Muramatsu et al., 1997; Itasaki et al., 1999; Momose et al., 1999; Nakamura et al., 2000; Yasuda et al., 2000). It seems that this technique can be adapted to the mouse embryo and would permit more rapid functional analysis of genes than is achieved by the generation of knockout or transgenic mouse lines. However, the inaccessibility of embryos in the mammalian uterus renders *in utero* manipulations targeting precise regions difficult or impossible at most stages of development. Efforts have been undertaken by various researchers to establish an *in utero* electroporation system, and there have been several reports of systems that enable successful gene delivery into mouse embryos, with time- and region-specific expression (Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001; Borrell et al., 2005).

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The establishment of paddle-type electrodes has made possible the delivery of current from outside the uterine wall, targeting a relatively specific region. This technique is useful for gene function analysis over relatively large regions. Moreover, further refinement of electrodes and application of current have made this approach useful for examination of smaller regions. In addition fine-wire electrodes (microelectrodes) that can be inserted into the uterus, at a point close to the target region, have been developed (Fukuchi-Shimogori and Grove, 2001). With this method, more precise targeting is possible and the area of the transfection region can be controlled. Further, this method permits the use of different electrode orientations and thus enables gene transfer along different axes (Fukuchi-Shimogori and Grove, 2003). Furthermore, the introduction of new light sources and a novel method for holding the uterus in place has improved the visibility of embryos through the uterus, thus permitting gene transfer in mice as early as on embryonic day (E) 9.5 (Shimogori et al., 2004). Thus, many aspects of neural differentiation and patterning can be investigated in a time-dependent manner. Further, the co-electroporation of fluorescent reporter genes and the target genes allows the visualization of successfully transfected embryos under a fluorescence microscope. Although *in utero* electroporation involves transient gene delivery, marker genes such as fluorescent reporter genes (e.g., enhanced green fluorescent protein [EGFP] and enhanced yellow fluorescent protein [EYFP]) or alkaline phosphatase can be co-transfected with the target genes and can function as transient lineage tracers in fate-mapping and migration experiments. In addition, the Cre recombinase-loxP system can be used in combination with electroporation for permanent gene delivery (Tsien et al., 1996). For instance, a Cre construct can be introduced into the R26R reporter mouse strain (Soriano, 1999) for permanent cell lineage analysis. Furthermore, the loss of gene function can be analyzed by introducing dominant-negative molecules, antisense morpholinos, and small interfering (si)RNA constructs into the embryos. Such experiments performed using a combination of wild-type (WT) or mutant embryos can directly reveal various phenotypes and patterns of cell behavior. In this review, we describe methods of microelectroporation and discuss some of the results obtained using this unique technique.

2 Principle

2.1 Theory of Electroporation

The electrically mediated transfer of DNA into tissues involves the formation of transient pores in the cell membrane, allowing the introduction of large molecules such as DNA into the cytoplasm (Neumann et al., 1982). Since DNA is negatively charged, the electroporation field causes the DNA to move across the membrane from the negative side to the positive side. The pores formed last longer when a high voltage and a long pulse duration are applied. However, these conditions

can induce irreversible damage to the cell membrane, resulting in cell death. For higher efficiency and less tissue damage, the application of a low voltage with the minimum possible pulse duration, using of a square-wave electric pulse generator, is recommended.

3 Procedure

3.1 Surgery

Anesthesia is induced with sodium pentobarbital (50 µg/g body weight, intraperitoneally) in a pregnant mouse (E9.5–E15.5), and the abdominal hair is shaved off using a razor blade and 50% EtOH (Shimogori and Ogawa, 2008). An abdominal incision is then created using a pair of fine scissors (RS-5865, Roboz, Gaithersburg, MD), and cotton gauze moistened with phosphate-buffered saline (PBS) is placed around the wound. The uterine horns are then lifted and placed on the cotton gauze. The uterus is kept moist with PBS throughout the procedure. For visualization of the embryos, the experimenter holds a flexible optic fiber cable (Leica, Germany) between the index and middle fingers and places it under the uterine horn. No microscope or magnifier is required for the visualization. The tip of the fiber cable and the uterus are moistened with warm (37°C) PBS. The uterus is then positioned between the optic-fiber light source and the thumb of the experimenter and gently squeezed to push the embryos closer to the uterine wall. After the embryos have been positioned, 1 µl of plasmid DNA solution (1 µg/µl), mixed with the non-toxic dye fast green (Sigma, St Louis, MO), is injected into the ventricle via a pulled-glass capillary, using a micromanipulator (KDS310, KD Scientific, Holliston, MA). This process facilitates the discrimination of the brain from the outside of the uterus. Subsequently, a fine tungsten electrode (negative electrode) and a platinum electrode (positive electrode) are inserted into the uterus, on either side of the target region. Further, three square-wave current pulses (7–10 V, 100 ms) in a series are delivered three times at 1-s intervals, using a pulse generator (model 2100, AM Systems, Carlsborg, WA). However, the injected DNA solution does not easily diffuse away from the point of injection because the neural cavity is closed, electroporation immediately after injection of the DNA solution is recommended. The uterine horn is then brought back to its original position with some moisture (approximately 500 µl of PBS). The incision in the uterine wall is closed using sutures, and the skin incision is closed with a 9-mm autoclip (RS-9260, Roboz). Note that a shorter surgical time (less than 30 min per mouse) will yield better embryonic viability. The animal is kept warm by placing a heating pad under it until it recovers from anesthesia (approximately 2 h). The viability of the embryos and the transfection efficiency vary depending on the age of the embryo and the target region of electroporation. In the case of electroporation performed on E11.5 in this study, 60% of the embryos survived, and successful electroporation was achieved

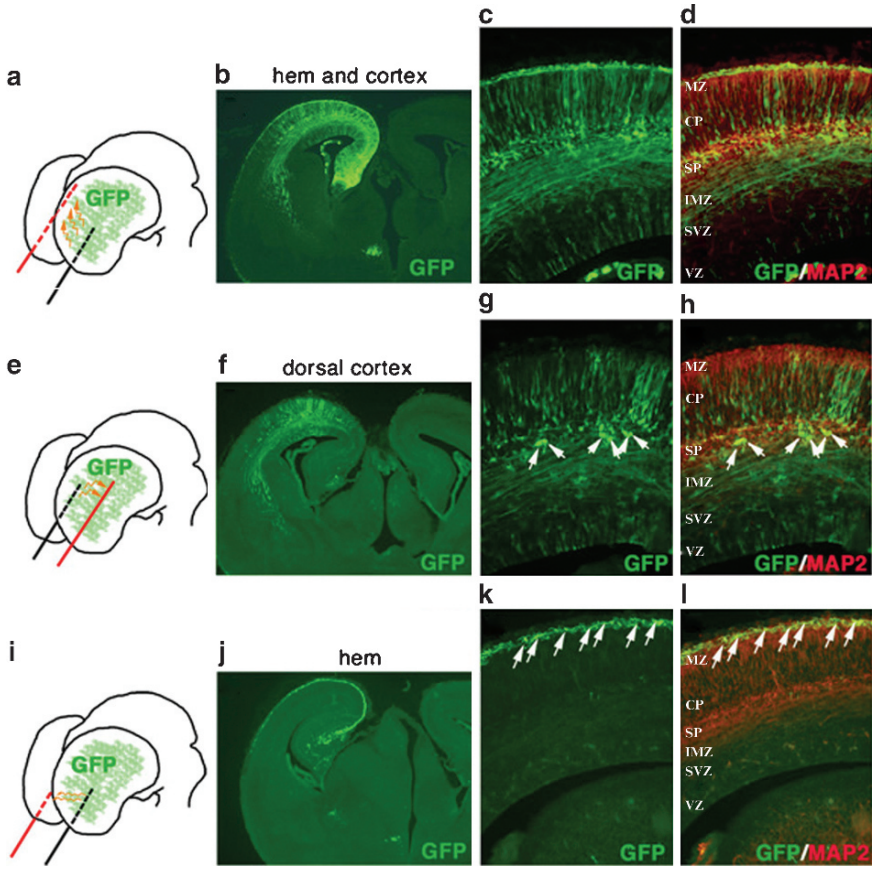

Fig. 15.1 Electroporation targeting the developing telencephalon. Electroporation targeting a large area in the telencephalon was performed on E11.5; the embryos were harvested on E16.5 (**a**) and subjected to immunohistochemical staining with anti-GFP and anti-MAP2 antibodies (**b**–**d**, **f**–**h**, **j**–**l**). Electroporation specifically targeting the dorsal telencephalon was performed on E11.5 (**e**), and the embryos were harvested on E16.5 (**f**–**h**). Electroporation targeting the hem in the medial wall of the telencephalon was performed on E11.5 (**i**), and the brains were excised on E16.5 (**j**–**l**). Arrows indicate subplate neurons which are strongly labeled with MAP2 (**g**, **h**), and Cajal-Retzius cells localized in MZ (**k**, **l**) (*See Color Plates*)

for 50% of the surviving embryos. The conditions used for each electroporation step should be optimized depending on the stage of embryonic development and the target region of electroporation.

3.2 Electrodes

The shape of the electrodes used is critical for the success of targeted electroporation. One end of each electrode wire (positive electrode, 125-mm bare tungsten

wire; negative electrode, 125-mm bare platinum wire; AM Systems, Carlsborg, WA) is coiled over gold-plated pins (model 5482, World Precision Instruments, Sarasota, FL) and fixed with parafilm. The entire wire is then inserted into the stem of a cotton swab and wrapped with parafilm at both ends. The tip of the wire is evenly sharpened with sandpaper to achieve an external diameter of 20– 30 µm in the tip region and of 60 µm in the portion beyond 1 mm from the tip. Then, a thin coat of nail polish is applied over the wire for insulation. After the nail polish has dried, it is removed from the tip by using an acetone-soaked cotton swab. In order to prevent damage to the uterus, the portion of the wire within 1 mm of the tip should not be more than 70 µm in diameter. Next, a glass capillary tube (No. 50611, Stoelting Wood Dale, IL) is pulled using a micropipette puller, and its tip is pinched off with a pair of forceps. The external diameter of the tip is 20–30 µm, and that in the portion beyond 1 mm from the tip is 60 µm. The capillary is connected to the micromanipulator, which is filled with mineral oil, and the DNA solution is sucked directly from an Eppendorf tube prior to the surgery.

3.3 In Situ *Hybridization and Immunohistochemistry*

In this study, harvested mouse embryos were immersed in 4% paraformaldehyde in phosphate-buffered saline and processed for single- or two-color non-radioactive whole-mount *in situ* hybridization (ISH) (Grove et al., 1998). From some of the embryos, the brain was dissected, fixed, cryoprotected, sectioned into 40-mm semicoronal sections using a sledge microtome (Leica, Germany), and processed for ISH according to a previously described method (Grove et al., 1998). In this method, the chromagens nitroblue tetrazolium (350 mg/ml; Nacalai, Japan) and tetranitroblue tetrazolium (350 mg/ml; Research Organics, Cleveland, OH) are used.

For immunohistochemistry (IHC), the embryos were fixed by perfusion, cryoprotected, embedded in OCT compound (Miles), and frozen on dry ice. Further, tissue sections were cut on a cryostat (10 µm) and mounted on glass slides. For double immunolabeling, a rabbit polyclonal antibody against microtubule-associated protein 2 (MAP2; diluted 1:1,000; kindly provided by M. Niinobe, Osaka University, Osaka, Japan), a rabbit anti-Tbr1 antibody (1:4,000; Chemicon) or a rabbit anti-nestin antibody (1:1,000; kindly provided by Y. Tomooka, Tokyo University of Science, Tokyo, Japan) was used, along with a rat anti-GFP monoclonal antibody (1:100; Nacalai, Kyoto, Japan). The staining was visualized using Cy3-conjugated goat anti-rabbit IgG (1:1,000; Chemicon) or FITC-conjugated donkey anti-rat IgG (1:1,000; Jackson Immunoresearch, PA).

3.4 Construct

A target siRNA construct was generated in the piGENE mU6 Puro vector (IGENE, Japan). The ApoER2 target sequence is GCAGCAGACTAATATCTAA.

4 Application and Results

4.1 Targeted Electroporation 1: Dorsal and Medial Telencephalon

For electroporation targeting a specific region in the telencephalon, the configuration of the two electrodes is altered. For example, for electroporation of a large area in the cortex, the insulation is removed from the tip of the electrode. This is done by inserting the wire deep into an acetone-soaked cotton swab to remove the nail polish. In contrast, for electroporation of a smaller area, the wire electrodes are prepared such that a small portion of their tips is exposed. Selection of the correct size of electrode and adequate arrangement of electrodes are key importance for successful electroporation. Here, we describe some of the results that were obtained using different types of electrodes placed in various positions. To observe the pattern of cortical neuron migration, we introduced an EGFP expression vector driven by the human EF1 promoter into the telencephalon of embryos on E11.5. The embryos were subsequently fixed and analyzed by IHC on E16.5. Since the expression vectors were injected into the lateral ventricle of the embryos, transfection occurred only at the cortical ventricular zone (VZ) (Tabata and Nakajima, 2001), and this made it possible to trace neuronal migration from the VZ. During electroporation targeting a large area of the medial telencephalon, the plasmid DNA is transfected into most of the medial structures of the "cortical hem" and also into the dorsal cortex. For this process, electrodes having a large portion of their tips exposed (approximately 300 μm of the tip of each electrode) are used, and inserted into each lateral ventricle (Fig. 15.1a). In the case of electroporation targeting the cortical hem and cortex of the embryos, a large number of GFP-expressing cells were observed in the intermediate zone (IMZ), subplate (SP), cortical plate (CP) and marginal zone (MZ) 5 days after electroporation. These cells were visualized by performing IHC for MAP2 (Fig. 15.1b, c, d). In the case of electroporation performed specifically for either the dorsal cortex or the cortical hem, highly insulated electrodes (with only approximately 100 μm of the tip exposed) are used, positioned in different configurations. To target the dorsal cortex, the negative electrode is inserted on the side of the telencephalic ventricle where the DNA solution is injected, and the positive electrode is positioned outside the brain (Fig. 15.1e). Following electroporation targeting the dorsal cortex, GFP-expressing cells were observed in the IMZ, SP, and CP of the cortex but not in the MZ (Fig. 15.1f, g, h, arrows). In contrast, electroporation targeting the cortical hem is performed by inserting one electrode into each lateral ventricle (Fig. 15.1i). In embryos subjected to this procedure, GFP-expressing cells were observed in the MZ (Fig. 15.1j, k, l, arrows). Thus, differences in the distribution of GFP-expressing cells in various regions following electroporation can provide information regarding the origin of individual cells. These findings are consistent with those of cell lineage analysis previously performed using specific knock-in or transgenic mice (Yoshida et al., 2006; Zhao et al., 2006).

4.2 Targeted Electroporation 2: Posterior Telencephalon

In the developing and mature central nervous system (CNS), newly generated postmitotic cells are known to take various migration routes to reach their final destination (Lois et al., 1996; Anderson et al., 1997). Recent findings suggest that one of the nuclei in the amygdala complex, namely, the nucleus of the lateral olfactory tract (nLOT), is generated in the posterior telencephalic neuroepithelium and migrates anteriorly (Remedios et al., 2007). Considering those of a previous study (Remedios et al., 2007), we performed targeted electroporation on E11.5 to label the posterior telencephalic neuroepithelial cells and followed their migration route (Fig. 15.2). For electroporation targeting the posterior telencephalon, the negative electrode is inserted into the lateral ventricular hemisphere into which the DNA is injected, while the positive electrode is positioned laterally/ventrally on the outside of the brain (Fig. 15.2a). The brains are harvested from the embryos on E16.5 and processed for IHC for GFP and Tbr1 (nLOT marker) (Remedios et al., 2004) in order to determine whether the electroporation had appropriately targeted the nLOT. In the brain of embryos that were subjected to electroporation targeting the posterior telencephalic neuroepithelium, cells exhibiting strong GFP expression signals were found in the ventral telencephalon (Fig. 15.2b). Furthermore, a cluster of GFP-positive cells was detected in the region anterior to the electroporation site (Fig. 15.2b, arrow). The detailed structure of these cells was visible in sagittal brain sections, and IHC for GFP clearly revealed the arc-shape migration route of the cells (Fig. 15.2c, arrows). Further, we performed IHC for Tbr1, using the same sections (Fig. 15.2d), and found that the expression signals overlapped accurately; this indicated that the electroporation with GFP had successfully targeted the nLOT progenitor cells (Fig. 15.2e). Furthermore, it has been reported that in reelin-deficient mutant mice, i.e., *rl/rl* mice, the nLOT is present in an abnormal position; this suggests that reelin is involved in nLOT development (Remedios et al., 2007). Hence targeted electroporation into posterior telencephalon was repeated using *rl/rl* mutant mice, and GFP-positive cells were noted to be localized at abnormal positions (Fig. 15.2g, arrow) that were identical to the positions at which Tbr1-positive cells were observed (Fig. 15.2h and i, arrows). Observation under a higher magnification revealed in detail the abnormalities in the nLOT cells in the brain of the *rl/rl* mutants when compared with the WT mice (Fig. 15.2f and j, arrowheads). The nLOT cells in the WT embryos were oriented in same direction as the leading process (Fig. 15.2f, arrowheads), whereas the orientations of the cells and the leading process were unorganized in the *rl/rl* mutant brain (Fig. 15.2j, arrowheads). To visualize the relationship between the migration route of the cells and the radial glia, we stained the WT mouse brain sections, which had been subjected to electroporation and GFP immunostaining, with an antibody against nestin (marker for the radial glia; Fig. 15.2k–n). Nestin staining revealed complex fiber extension in the ventral telencephalon, and the merged images of nestin staining and GFP staining revealed two steps in the route of nLOT migration along the radial glial fiber (Fig. 15.2m and n, arrows).

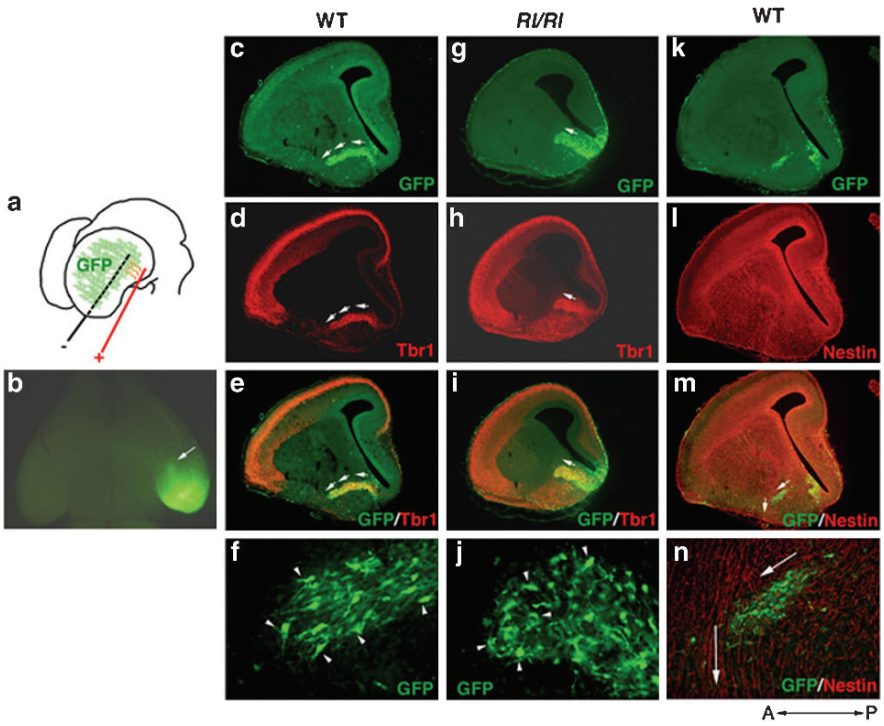

Fig. 15.2 Electroporation targeting the posterior telencephalon. On E11.5, the negative electrode was inserted into the lateral ventricle where the GFP-plasmid solution was injected, while the positive electrode was placed outside the brain (**a**). The brain was harvested on E16.5, and observation in the ventral view revealed strong GFP expression signals (**b**). The anteriorly migrating cell cluster could also be detected (**b**, *arrow*). Sagittal sections (anterior to the left) of WT (**c**–**f** and **k**–**n**) and *rl/rl* mutant mouse brains (**g**–**j**) were obtained on E16.5 and processed for IHC for GFP (**c**, **g**, **k**, **f**, **j**), Tbr1 (**d**, **h**), and Nestin (**l**). Merged images of GFP and Tbr1 staining (**e**, **i**) and GFP and nestin staining (**m**, **n**) are shown

4.3 Targeted Electroporation 3: Diencephalon

In addition to cell differentiation and migration, appropriate axonal projection into their target area is an important event occurring during the development of the CNS. To trace axonal projections, tracer substances such as plant lectin, wheat germ agglutinin (WGA), and the tau-lacZ reporter are selectively delivered to specific types of neurons. GFP and YFP, when delivered to axons, permit the detection of specific neuronal projections. To demonstrate this, a plasmid expressing EYFP under the control of the chicken β-actin (CAG) promoter was electroporated into the embryonic diencephalon on E11, and the embryo was harvested on postnatal day (P)18. DNA solution was injected into the 3rd ventricle of the diencephalon; the negative electrode was inserted into the neural tube, while the positive electrode was placed on the side of the brain (Fig. 15.3a). ISH performed on E12.5

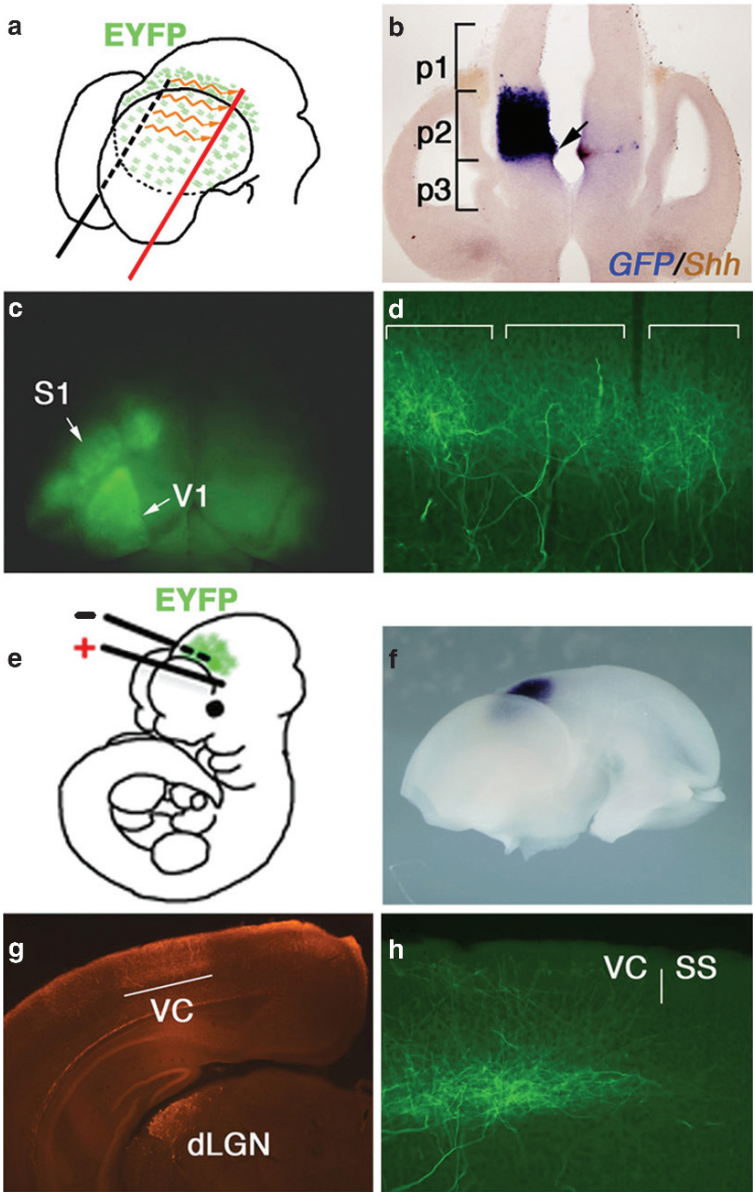

Fig. 15.3 Localization of EYFP in the thalamocortical (TC) axons. Plasmids carrying the EYFP is electroporated into the embryonic diencephalon on E11 (**a**), and the embryos were harvested on E12.5 (**b**) or P18 (**c**, **d**). ISH for *GFP* enabled visualization of electroporation that specifically targeted p2 in the developing diencephalon (**b**, *blue stain*). The ZLI position (border of p2 and p3) is reflected in *Shh* expression (**b**, *brown stain*, *arrow*). Maps of cortical areas such as S1 (primary somatosensory area) and V1 (primary visual area) are illuminated by thalamocortical axons from the thalamus (**c**). For nucleus-specific gene transfer, electroporation targeting the diencephalon was performed on E10.5 (**e**), and the embryos were harvested on E11.5 (**f**) or P18 (**g**, **h**). ISH (**f**) and IHC (**g**, **h**) were performed for GFP. (**g**) On P18, not only restricted electroporation into the dorsal lateral geniculate (dLGN) but also GFP expression in the visual cortex (VC) were observed. (**h**) Observation under a higher magnification revealed the detailed features of the axon shape and arborization in the VC but not in the somatosensory cortex (SS)

revealed restricted gene delivery into the developing thalamus (Fig. 15.3b). The analyses clearly demonstrated that the transfected cells were only expressed in the p2 (presumptive thalamus), which lies next to the zona limitans intrathalamica (ZLI), as visualized by *Shh* ISH (Fig. 15.3b, arrow). The brains were harvested on P18 and observed under a fluorescence microscope to examine YFP expression. Surprisingly, however, the electroporation is restricted to the diencephalon, the cortical area map (primary somatosensory, S1 and primary visual cortex, V1) is clearly distinguished by fluorescence from YFP (Fig. 15.3c). This demonstrates transfection into the functional nuclei of the thalamus, via diffusion of the fluorescence protein into the thalamocortical axons, which project into and illuminate their target areas in the cortex. It is easy to visualize axon terminals in layer four of the cortex in brain sections and to identify individual barrels in the S1 barrel field cortex (Fig. 15.3d) (Erzurumlu and Kind, 2001). Electroporation into the diencephalon, when performed on E10.5 (Fig. 15.3e and f), results in specific transfection in the dorsal lateral geniculate nucleus (dLGN) since this nucleus is formed at an earlier stage compared to the other nuclei (Altman and Bayer, 1988). Immunostaining for YFP, performed using an anti-GFP antibody, revealed strong expression signals in the dLGN and primary visual cortex (Fig. 15.3g). Immunohistochemical labeling also revealed that YFP-labeled fiber arbors terminated at the border between the visual and somatosensory cortices (Fig. 15.3h). Thus, this method permits detailed examination of the visual cortex.

5 Comments

5.1 Advanced Application

5.1.1 Permanent Transfection

In utero electroporation involves transient time- and region-specific gene delivery, which enables the functional analysis of genes at specific time points during development. However, although this technique offers the advantage of transient expression, it is not suitable for the long-term expression of transfected genes. The use of the Cre recombinase-loxP system in combination with electroporation enables permanent gene delivery (Tsien et al., 1996). For instance, a Cre construct can be introduced into the R26R reporter mouse strain (Soriano, 1999) for permanent cell lineage analysis. To confirm this, we co-electropored Cre and EGFP construct into the diencephalon of an R26R mouse in a spatially-restricted manner (Fig. 15.4a, b, e, f). Electroporation targeting the p3 region of the diencephalon (presumptive prethalamus area) is performed by inserting the negative electrode into the 3rd ventricle and the positive electrode outside the brain (Fig. 15.4a). To achieve region-specific electroporation, both electrodes are positioned close to the diencephalic floor plate (Fig. 15.4b). The electroporated region is permanently marked by the expression of

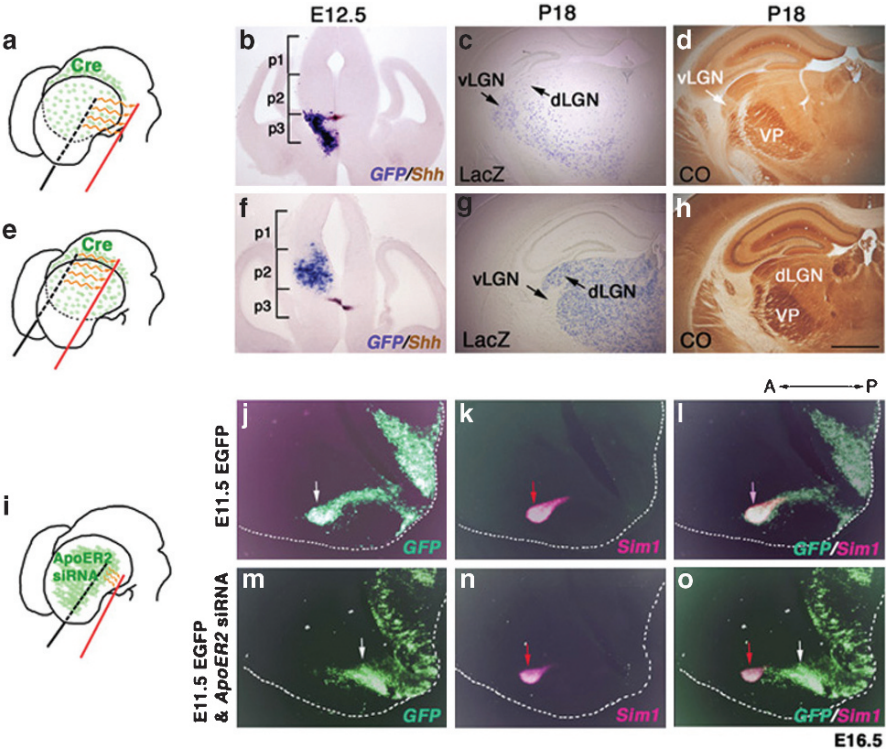

Fig. 15.4 Electroporation of Cre into R26R mice for cell lineage analysis and the use of siRNA for silencing specific mRNA expression. The Cre-expressing plasmid and EYFP were specifically transferred into the p3 or p2 region of the developing diencephalon in the R26R reporter mouse strain on E11 (**a**, **e**), and the mouse brains were harvested on E12.5 (**b**, **f**) or P18 (**c**, **d**, **g**, **h**). ISH for *GFP* electroporation initially targeted p3 (**b**, *blue staining*) or p2 (**f**, *blue staining*). On P18, the expression of the transfected genes was lost in many of the cells. In contrast, X-gal histochemistry permanently stains transfected cells (**c**, **g**). Adjacent sections were subjected to histochemistry for cytochrome oxidase (CO) to observe the nuclei in the thalamus, e.g., sensory nuclei such as the ventroposterior (VP) nucleus, dLGN, and vLGN (**d**, **h**). Further, following p3-targeted electroporation, most of the cells in the vLGN and cells in specific areas of the thalamus were labeled (**c**). However, following p2-targeted electroporation, lacZ expression exclusive to the vLGN but everywhere in the thalamus was observed (**g**). Introduction of the siRNA into specific cell populations demonstrated impaired cell migration. Electroporation targeting the telencephalon was performed on E11.5 (**i**), and the embryos were harvested on E16.5. The brains were sectioned sagittally and anterior to the left. ISH for GFP revealed specific nLOT cells and their migration route (**j**, *white arrow*). False-color overlay of GFP and Sim1 revealed that the anterior-most migratory cell population exhibited positive *Sim1* expression (**k**, *red arrow*; **l**, *pink arrow*). In contrast, in the brains that were electroporated with the ApoER2-siRNA construct, abnormal nLOT migration was observed, which resembled that observed in the *rl/rl* mutant brain (**m**, *white arrow*). Furthermore, cells that were not targeted during electroporation with the ApoER2-siRNA construct exhibited normal migration and Sim1 expression (**n**, **o**, *red arrow*)

β-galactosidase, as visualized by X-gal histochemistry. It demonstrated successful transfection to prethalamic region of the thalamus at P18 such as ventral lateral geniculate (vLGN) which the structure is revealed by cytochrome oxidase activity (Fig. 15.4c and d). In the case of electroporation performed with the electrodes positioned closer to the roof plate of the diencephalon (Fig. 15.4e), most of the trangenes are deposited in p2 (Fig. 15.4f). This method of restricted electroporation reveals that most of the thalamic cells are generated in the p2 region (Fig. 15.4g and h).

5.1.2 Gene Silencing with siRNA

Gene expression alteration is widely used in attempts to unravel various molecular mechanisms. Currently, the introduction of small interfering RNA (siRNA) into different types of cells is gaining popularity as a major strategy for gene alteration (Fire et al., 1998; Young-Pearse et al., 2007). To determine whether this technology can be used in electroporation, we generated an siRNA construct for one of the receptors functioning in the reelin pathway, namely, ApoER2 (Bar et al., 2000). Electroporation targeting the caudal telencephalic neuroepithelium was performed as described above, and the ApoER2-siRNA construct was injected along with the EGFP construct (Fig. 15.4i). In the brain of the control embryos, which had been electroporated with EGFP alone, ISH revealed that the GFP-positive cells migrated anteriorly along a distinct arc-shape migratory route (Fig. 15.4j, white arrow). The anterior-most cell population exhibited positive *Sim1* expression (Single-minded, nLOT marker) (Fig. 15.4k, red arrow) that overlapped with GFP expression (Fig. 15.4l, pink arrow). In contrast, in the brain of the embryos that had been electroporated with the ApoER2-siRNA construct, GFP-positive cells exhibiting an abnormal migratory route were observed; these cells stopped migrating at a more dorsal position (Fig. 15.4m, white arrow). Surprisingly, in these embryo brains, *Sim1*-positive cells were detected in the normal position (Fig. 15.4n, red arrow). However, Sim1 expression did not overlap with GFP expression in any of these cells (Fig. 15.4o, red and white arrows). These results suggest that the cells, which did not receive the ApoER2-siRNA construct, continued migrating along the normal route until they reach an appropriate anterior/ventral position (Fig. 15.4n, red arrow). However, further investigations are required to elucidate the mechanism underlying the siRNA-mediated arrest of cell migration. Taken together, our results reveal that employing siRNA construct to interfere signaling pathway can be used for further understanding of the developmental mechanism.

5.2 Future Applications

All experiments described in this review were performed using plasmid DNA. However, electroporation can be used to introduce dyes, chemical reagents, antibodies, and any other charged macromolecule into specific groups of cells.

The most commonly used promoters for forced gene expression are the CMV, human EF1, and CAG promoters. Although these promoters are preferentially expressed in certain cells following transfection, they are ubiquitously transfected within a few days after electroporation (Tabata and Nakajima, 2001). To accurately examine the expression of these promoters within a particular region, a plasmid carrying the promoter regions of genes expressed in that region can be constructed.

It should be noted that although electroporation can be performed at any stage during embryonic development, it should preferably be performed during the stage at which the target cells are generated. In cases where the target gene is known to have functions in postmitotic neurons and could alter cell differentiation, it is necessary to delay the expression of the gene product. This is possible through the combined use of tamoxifen-inducible Cre-ER(T) recombinases (Feil et al., 1996) and reporter mice (Indra et al., 1999). The induction of such a delay will make it possible to control ectopic gene expression following electroporation.

The removal of target cells is one of the best methods for determining the effects of cells on surrounding tissues. The expression of a cellular toxin gene, namely, diphtheria toxin subunit A (DTA), which effectively kills cells, is a powerful method for the ablation of specific tissues (Maxwell et al., 1986).

Recently, synaptic transmission blockade via the expression of the tetanus toxin light chain (TeNT-LC) has been used to silence neuronal activity (Wang et al., 2007). The electroporation of a TeNT-LC-expressing plasmid along with fluorescent marker proteins, which stain axonal projections as well as dendrites, enables the behavioral and morphological analysis of neurons, while simultaneously suppressing the neuronal activity.

6 Troubleshooting

Preservation of the embryonic viability is the most critical criterion for obtaining efficient results. To prevent embryonic death during the electroporation procedure, the following factors should be considered.

All embryos die within 24 h after electroporation. Further, the uterine horn is extremely delicate: applying excessive pressure to the uterine wall or damaging it with the capillary or electrodes could cause embryonic death within 24 h. Therefore, the uterus should be handled very gently, and the appropriate capillary and electrode diameters should be carefully selected. The use of a capillary or electrode with a large diameter could result in leakage of the amniotic fluid from the uterus. Further, it is useful to perform electroporation for only one of the uterine horns and to assess whether handling during the procedure is sufficiently gentle by comparing this uterine horn with the control one.

Since embryos that have been subjected to electroporation often die when compared with normal control embryos, it is possible that the use of an excessively high voltage during electroporation causes severe cell death. Therefore, it is necessary to attempt the procedure under various conditions of voltage.

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Chapter 16
Single Cell Electroporation Method for Mammalian CNS Neurons in Organotypic Slice Cultures

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1 Introduction

Axon tracing is an essential technique to study the projection pattern of neurons in the CNS. Horse radish peroxidase and lectins have contributed to revealing many neural connection patterns in the CNS (Itaya and van Hoesen, 1982; Fabian and Coulter, 1985; Yoshihara, 2002). Moreover, a tracing method with fluorescent dye has enabled the observation of growing axons in living conditions, and demonstrated a lot of developmental aspects in axon growth and guidance (Harris et al., 1987; O'Rourke and Fraser, 1990; Kaethner and Stuermer, 1992; Halloran and Kalil, 1994; Yamamoto et al., 1997). More recently, genetically encoded fluorescent proteins can be used as a powerful tool to observe various biological events. Several gene transfer techniques such as microinjection, biolistic gene gun, viral infection, lipofection and transgenic technology have been developed (Feng et al., 2000; Ehrengruber et al., 2001; O'Brien et al., 2001; Ma et al., 2002; Sahly et al., 2003). In particular, the electroporation technique was proved as a valuable tool, since it can be applied to a wide range of tissues and cell types with little toxicity and can be performed with relative technical easiness. Most methods, including a standard electroporation technique, are suitable for gene transfer to a large number of cells. However, this is not ideal for axonal tracing, because observation of individual axons is occasionally required. To overcome this problem, we have developed an electroporation method using glass micropipettes containing plasmid solutions and small current injection. Here we introduce the method in detail and exemplified results with some example applications and discuss its usefulness.

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2 Materials

2.1 Animals

- Embryonic day (E) 15 or postnatal day (P) 0 to 2, Sprague-Dawley rat (CLEA JAPAN, Tokyo, Japan)

2.2 Chemicals and Reagents

- Culture medium: A 1:1 mixture of Dulbecco's modified essential medium and Ham's F12 (DMEM/F12; GIBCO, 12500-062) with several supplements containing insulin and transferrin
- Rat tail Collagen
- Hanks balanced salt solution (HBSS)
- Seventy percent ethanol
- Plasmid containing the coding region of enhanced yellow fluorescent protein

2.3 Culture Equipment

- Incubator (37°C, 5% CO_2, humidified)
- Culture membrane (Millicell-CM PICMORG50; Millipore, Tokyo, Japan)
- Thirty-five millimeter Petri dish

2.4 Electroporation Equipment

- Stimulator (Master 8, A.M.P.I, Jerusalem, Israel)
- Biphasic isolator (BSI-2, BAK ELECTRONICS, MD, USA)
- Amplifier (Model 1800, A-M Systems, WA, USA)
- Oscilloscope (VC-6723, Hitachi, Tokyo, Japan)
- Micromanipulator (MO-10, Narishige, Tokyo, Japan)
- Stereomicroscope (SZ40, Olympus, Tokyo, Japan)
- Universal stand (SZ-STU2, Olympus)
- Light illumination system (LG-PS2, LG-DI, HLL301, Olympus)
- Electrode puller (PC-10, Narishige, Tokyo, Japan)
- Glass capillary: OD 1 or 1.2 mm (G-1 or G-1.2, Narishige)
- Silver wire: 0.2–0.5 mm (Nilaco, Tokyo, Japan)
- One hundred ohm resistor
- One milliliter syringe

3 Procedure

3.1 Culture Procedure

The method of slice cultures was described previously (Yamamoto et al., 1989; Yamamoto et al., 1992). In brief, the dorsal thalamic region was dissected from E15 rat embryos, and cortical slices were dissected from the visual and somatosensory cortices of postnatal day 0 or P2 rat. These were cultured on collagen-coated membranes in serum-free, hormone-supplemented medium on culture membranes, and maintained at 37°C in an environment of humidified 95% air and 5% CO_2.

3.2 Preparation of Plasmid Solution

A plasmid containing the coding region of enhanced yellow fluorescent protein (pCAGGS: EYFP, a generous gift from Dr. Hatanaka) (Niwa et al., 1991) was used for electroporation. The plasmid was isolated (QIAGEN Plasmid Maxi Kit, QIAGEN, Tokyo, Japan), and resuspended at concentrations of 1.0–1.5 mg/mL in HBSS.

3.3 Electroporation Set-Up

Glass capillaries were pulled by a micropipette puller. Pipettes (electrode pipette and ejection pipette) with different tip diameters were prepared. The tips were broken off and fire-polished (electrode pipette tip diameter 30–50 µm for cortical neurons; 100–200 µm for thalamic neurons; 50 µm for ejection pipette).

An electroporation stage was set up under a stereomicroscope. The equipment used is shown in Fig. 16.1. A stimulator was connected to a biphasic isolator. For delivery of negatively charged DNA, the electrode pipette was connected to the negative terminal (anode) of the isolator. The current through the pipette was passed across a resistor in series and amplified by a factor of 100 using an amplifier. The amplifier was connected to an oscilloscope to monitor the current injection. To eject plasmid-containing solution to cultured slices, the ejection pipette was connected to a 1 mL syringe with a plastic tube.

3.4 Electroporation Procedure

1. Clean the equipment including the stereomicroscope and silver wire with 70% ethanol.
2. Fill the electrode and ejection pipettes with plasmid-containing solution (5 µL) by back-filling.

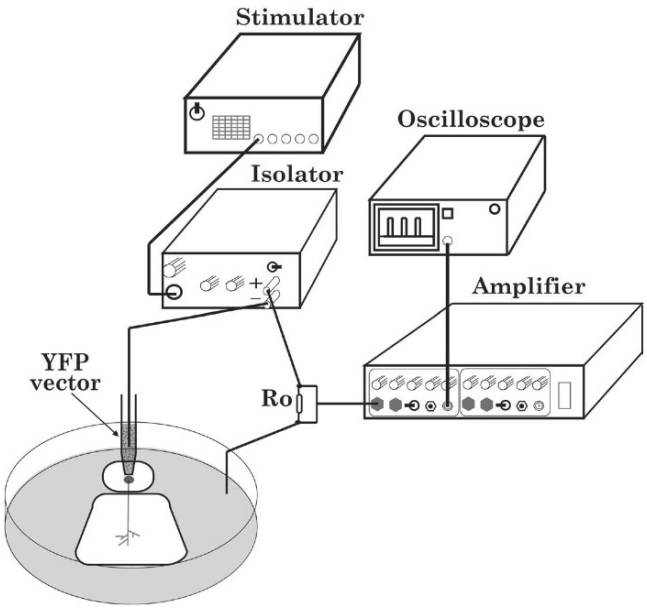

Fig. 16.1 Schematic diagram of the electroporation setup. Wiring is depicted by black lines. The electrode pipette including the plasmid solution is placed into the cultured explant. The shape and amplitude of the current can be monitored by measuring the voltage drop across a resistor (Ro) with an oscilloscope

3. Insert a silver wire inside the electrode pipette.
4. Position the tips of both pipettes with the manipulators.
5. Place the dish culturing slices on the electroporation stage.
6. Insert another silver wire (for a ground electrode) into the culture medium.
7. Position the ejection pipette in thalamic explants or cortical slices under the microscope.
8. Eject the plasmid-containing solution (0.1 to 0.5 μL).
9. Place the tip of the electrode pipette on the region immediately after the ejection.
10. Deliver electrical pulses (3 to 5 trains of 200 square pulses of 1 ms duration at 200 Hz, and of 100 to 150 μA amplitude for cortical neurons, 500 to 700 μA for thalamic neurons).
11. Monitor the amplitude of pulses on the oscilloscope.
12. Retract the pipettes.
13. Put back the slice into the incubator immediately.
14. For additional slices, repeat the procedure from step 5.

4 Application and Results

4.1 Cortical Cell Axons Labeled by the Single Cell Electroporation Method

Axonal morphology of cortical neurons was examined in slice cultures. After 1 to 6 days in culture, single or a few cells in the upper layers were transfected with the *eyfp* plasmid by the electroporation method. Brightly labeled cells appeared within 24 h after transfection. The EYFP labeling further demonstrated that cortical neurons developed in morphology even after a few weeks in culture (Fig. 16.2). Indeed, radially and horizontally extending axons were clearly labeled together with pyramidal shaped soma and dendrites. The primary axons run radially towards the white matter, while collaterals elongated in the upper and deep layers. This axonal projection pattern resembles that found in vivo. Occasionally glial cells were also transfected, indicating no apparent restrictions on the types of cells which were susceptible to electroporation.

4.2 Time-Lapse Imaging

Combination of electroporation with culture technique provides a straightforward method for time-lapse imaging of labeled cells. Repeated confocal imaging

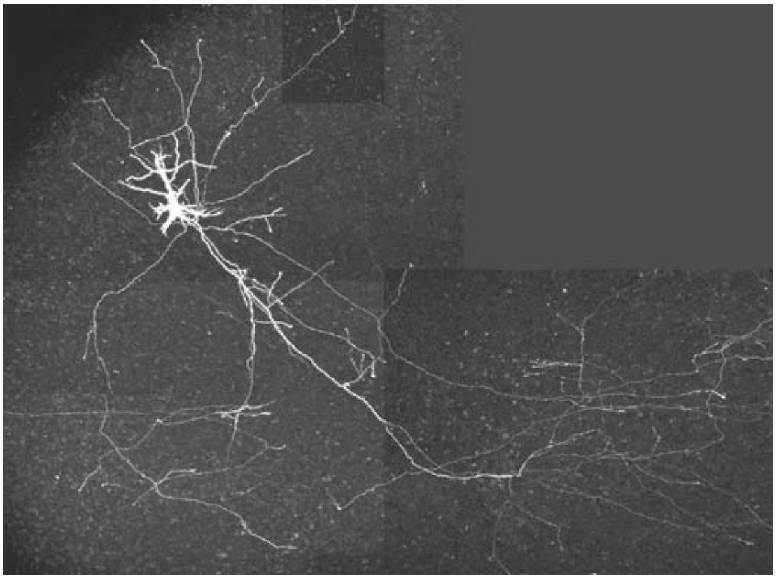

Fig. 16.2 An example of a cortical neuron labeled with EYFP by electroporation. Complete cellular morphology is brightly labeled. Scale bar, 200 µm

allowed us to monitor changes of neuronal morphology in detail. We studied branch formation of thalamocortical (TC) axons in cocultures of the thalamus and cortex. As shown in Fig. 16.3, TC axon arbors became larger and more complex over 6 days. The average number of branch points and the total axon length increased gradually during culture. Analysis of individual axonal branches revealed that more than 80% of branches showed changes in length and that TC axon branching patterns were generated dynamically by ongoing branch addition and elimination with a preference toward branch accumulation in the upper layers.

4.3 Application to Acute Slices

We also tried to observe axonal projection pattern in acute cortical slices. Upper layer cells were transfected with the EYFP vector in cortical slices immediately after dissection. The slices were then cultured for 24 h, which allow the protein to be synthesized and diffuse into processes. The EYFP-labeling in acute cortical slices also allowed the tracing of axonal morphology when single cells were tranfected (Fig. 16.4).

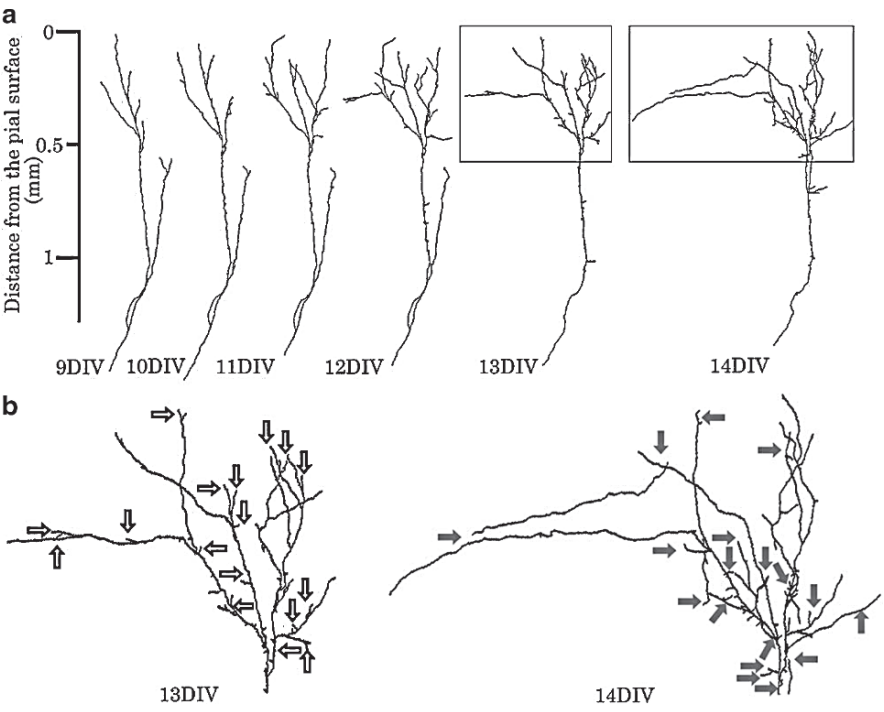

Fig. 16.3 Time-lapse imaging of TC axon branching. (**a**) Tracing of the terminal axon arbor. This axon was followed daily over 6 days (9 to 14 DIV). (**b**) Higher magnification of two boxed regions in (**a**) shows examples of added branches (*shaded arrows*) and lost branches (*open arrows*). From Uesaka and others (2007) with permission from the *Journal of Neuroscience*

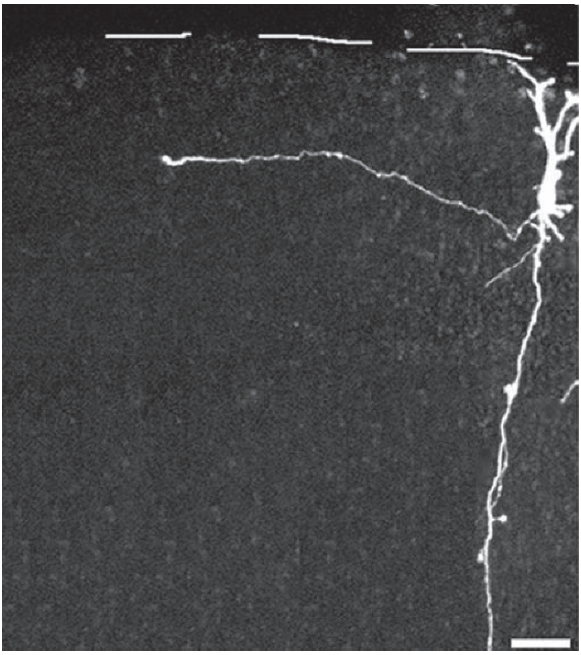

Fig. 16.4 Fluorescence photomicrograph of a P7 rat cortical slice showing a YFP-labeled cell 24 h after transfection. From Uesaka and others (2005) with permission from the *Journal of Neuroscience*. Scale bar, 100 μm

5 Comments

5.1 Overall Assessment of the Technique

We developed an electroporation method that is suitable for axonal tracing. This method provides a large advantage of labeling clearly visible single or a few cells, with the soma, dendrites and axons. Electroporated neurons expressing the fluorescent protein had typical axon morphologies similar to those in vivo, indicating that electroporation does not harm the normal development. Although a method in which plasmid vectors are introduced with patch electrodes is also available to transfer genes into single cells (Haas et al., 2001), the present method is more simple and convenient for the investigator that are not used to electrophysiology. Another advantage is the long-term imaging of neural morphology including axons and dendrites. Time-lapse imaging showed that labeled axons could be followed over several days. In addition, we could observe clear axon morphology even 3 weeks after transfection. Thus, the present electroporation method could contribute to imaging of neural morphology.

5.2 Limitations

There are some limitations to the present method. The electroporation efficacy was extremely low after 1 week *in vitro*. Similarly, we could not transfer genes into acute slices dissected from rats older than postnatal day 7. This might be attributed to the changes of properties of postmitotic neurons. Another limitation is the difficulty in controling the number of labeled cells, because it depends on the volume of ejection plasmid, the shape of the electrode tips and the depth of the electrode in the slices. However, with carefully varying these parameters an acceptable success rate should be attained for repeated experiments.

5.3 Applications of the Technique

The present method can be applied to fundamentally all CNS cells, because the slice culture technique is available for various brain areas such as hippocampus and cerebellum. In addition, this method could also be used to transfer various genes other than the fluorescent proteins. Indeed, we have performed cotransfection of the genes encoding fluorescent proteins with those that regulate cytoskeleton proteins (Ohnami et al., 2008). Thus, this electroporation technique provides a useful method for the study of gene function in the CNS.

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Chapter 17
Temporal and Tissue-Specific Control of Gene Expression in the Peri-Implantation Mouse Embryo Through Electroporation of dsRNA

Miguel L. Soares and Maria-Elena Torres-Padilla

1 Introduction

The delivery of nucleic acids into embryos – either DNA molecules for transient expression or double-stranded RNA for gene silencing by RNA interference (RNAi) – remains a challenging aspect of functional studies on live organisms. Electroporation has long been a standard method for the active transfer of the negatively charged nucleic acids into mammalian cells (Andreason and Evans, 1988). This technique employs electric pulses to create transient pores in the cytoplasmic membrane through which the nucleic acids are actively delivered. It was not until the conditions for culture of whole embryos became consistent, however, that it has been applied successfully for transfection of mouse concepti.

Nucleic acids delivery by electroporation has been achieved at various stages of mouse embryonic development. Conditions for successful electroporation in pre-implantation stages have been established and optimized allowing whole-embryo targeting of zygotes, morulae and blastocysts (Grabarek et al., 2002; Soares et al., 2005). Post-implantation embryos have undergone the procedure from as early as embryonic day (E) 5.25 up to E13.0 (Calegari et al., 2002; Mellitzer et al., 2002; Holm et al., 2007; Soares et al., 2008). This temporal specificity is particularly useful when studying signaling pathways at work within relatively small time windows in development. In early post-implantation development, for instance, just before gastrulation, signaling events shaping the establishment of the body axes of the mouse conceptus have been studied by temporal-specific electroporation of dsRNA (Soares et al., 2008).

Whereas in pre-implantation stages the whole embryo (up to ~64 cells) is targeted by the nucleic acids delivered by electroporation, post-implantation stage embryos can be regionally targeted in a tissue-specific manner. The larger size

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of the embryo and the availability of a cavity to which the nucleic acids can be previously injected make electroporation particularly effective in directing these molecules to specific tissues/regions of the conceptus. This directed electroporation is achieved by orienting the electric field to the region to be transfected. The unidirectional migration of nucleic acids is invaluable for the study of localized gene expression, whereby a given area will be under the influence of the effects induced by the electroporated molecules and adjacent non-targeted areas may serve as internal negative controls.

Since the demonstration of RNAi as an effective tool to induce sequence-specific knockdown of gene expression in mammalian cells (Elbashir et al., 2001), electroporation has been progressively adopted as a means to introduce dsRNA molecules into tissues, organs and whole embryos. Short 21-nucleotide dsRNA molecules (siRNAs) as well as longer dsRNA molecules of several hundred nucleotides can be easily delivered into cells by electroporation, thus inducing temporal and region-specific RNAi in cultured embryos. Despite well documented non-specific effects of long dsRNA in most mammalian cells, which trigger an interferon response leading to a generalized block of translation and the onset of apoptosis (Lee et al., 1994; Stark et al., 1998), dsRNA of up to 650 base pairs does not elicit the same effects in pre-implantation and early post-implantation mouse embryos (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000; Soares et al., 2005, 2008). Here, we focus on the methodology for electroporation of peri-implantation embryos with long dsRNA as a means to dissect the role of components of key signaling pathways in early mouse embryogenesis by RNAi.

2 Procedure

2.1 Culture and Electroporation of Pre-implantation Mouse Blastocysts

Culture conditions that allow normal embryonic development of pre-implantation mouse embryos *in vitro* are well established (Lawitts and Biggers, 1991, 1993; Erbach et al., 1994; Summers et al., 1995). Experimental intact blastocysts collected directly from the uteri of three and a half day pregnant mice or grown *in vitro* from zygote or morulae stages are cultured in KSOM medium supplemented with 4 mg/ml BSA, at 37°C under a 5% CO_2 atmosphere. Conditions for electroporation of blastocysts differ from those for electroporation of oocytes, zygotes or cleavage-stage embryos. The same experimental equipment and general settings can be used for different stages, but the specific morphology and cellular organization of 32 to 64-cell blastocysts require the use of particular electroporation parameters altogether.

Grabarek and colleagues (2002) laid the ground for efficient electroporation of early pre-implantation mouse embryos and oocytes for delivery of dsRNA. By following the incorporation of rhodamine-labeled dextran, a non-toxic 3 kDa

molecule that is easily visualized under fluorescence microscopy, they tested the impact of different parameters such as electric field intensity, duration and numbers of pulses, and buffer composition on uptake efficiency and embryo survival, both in microslide and flat electrode chambers. The initial experiments were carried out with embryos devoid of *zona pellucida*, a layer of extracellular glycoproteins important for the development of the embryos *in vitro* and *in vivo* following uterine transfer. The optimal parameters for *zona*-free maturing oocytes and zygotes consisted in delivering two series of three pulses of 1 ms duration with 10 V intensity, in hepes-buffered saline (HBS). Whereas the efficiency of uptake (measured by the number of rhodamine-positive cells) and the development potential (based on oocyte maturation to metaphase II and zygote survival to morulae/blastocyst stages) were comparable with the use of either type of electroporation chamber, the survival rate of the electroporated embryos was higher with the latter. Cell survival following electroporation was increased further (up to 100%) by the weakening of the *zona pellucida* with acid Tyrode's solution (Nicolson et al., 1975), rather than its complete removal. However, the electrical parameters had to be increased to three series of four pulses of 1–2 ms duration with 20 V intensity for comparable levels of dextran incorporation. Under these conditions, no dextran uptake was observed in intact *zona* oocytes or embryos, suggesting that such an intact layer may also be impenetrable by other macromolecules with the parameters employed.

Successful delivery of dsRNA into zona-weakened zygotes and 4-cell stage embryos using the optimized conditions followed. Transgenic embryos expressing GFP from the ubiquitous elongation factor-1α (EF-1α) promoter were electroporated with GFP long dsRNA (600 bp) at a concentration of 20 μg/ml in HBS and cultured for up to 4 days to morulae/blastocyst stages. Downregulation of GFP was evident by the absence of fluorescence in up to 95% of these embryos, without any significant effect on developmental potential (Grabarek et al., 2002).

These experiments clearly show that electroporation is an effective tool to deliver macromolecules, in general, and nucleic acids, in particular, to early pre-implantation mouse embryos and oocytes *in vivo*. But the efficiency of incorporation at these stages, both quantitatively and qualitatively, is not entirely unexpected. Embryos with up to four blastomeres have their cells highly accessible as the exposed cell surface area is extensive. In contrast, at later stages, from the 16-cell stage onwards, some cells become fully internalized and therefore less accessible. Furthermore, when targeting blastocysts for functional studies, which will require embryo transfer to the reproductive tract of pseudo-pregnant mothers for implantation, an intact *zona pellucida* is desirable. These circumstances make the electroporation of morulae and blastocysts experimentally more demanding.

Using a similar approach, we were able to determine the most efficient conditions for electroporation of late pre-implantation embryos. Following the incorporation of rhodamine-dextran under identical experimental settings, it became clear that an increase in both electric field intensity and concentration of dextran were necessary for the successful targeting of all the cells of the electroporated embryos. Interestingly, adjustments of electrical parameters alone (electric intensity up to 28–30 V) resulted in successful delivery of rhodamine-dextran into intact *zona*

morulae and blastocysts without compromising their viability (unpublished results). This represents a major advantage for subsequent development *in utero* upon embryo transfer to pseudo-pregnant mice, if so required by experimental design.

The optimized parameters were then applied to the delivery of short and long dsRNAs into morulae and blastocysts by electroporation. Intact 16-cell stage embryos and 32- to 64-cell blastocysts were electroporated with increasing concentrations of FAM-labeled ubiquitin-like protein protease 1 (ULP1) siRNA and Alexa Fluor 488-labeled red fluorescent protein (RFP) long dsRNA (500 bp). At concentrations of 1.5 mg/ml more than 80% of the electroporated embryos had incorporated the fluorochrome-labeled dsRNAs. The percentage of targeted cells per embryo was estimated at over 70% but the fluorescence intensity varied considerably among and within embryos for both types of dsRNA. Unsurprisingly, the degree of RNA uptake was substantially higher with the ULP1 siRNA, a molecule 24 times smaller than the RFP long dsRNA. By increasing the concentration of long dsRNA to 2 mg/ml, 100% of the electroporated blastocysts targeted with Alexa Fluor 594-labeled GFP long dsRNA (540 bp) incorporated the nucleic acid. Optical sectioning by confocal microscopy indicated that more than 90% of the cells of each blastocyst were targeted by the labeled dsRNA on average (minimum 70%) (Fig. 17.1; Soares et al., 2005). In all cases, the embryos were indistinguishable in morphology and developmental rate from non-electroporated control embryos cultured under the same conditions.

Successful delivery of plasmid DNA into pre-implantation embryos through electroporation, however, has not been achieved. Under a wide range of parameters, both *zona*-free and intact *zona* cleavage-stage embryos and blastocysts were impenetrable by a 4.7 kb GFP-expressing plasmid (pEGFP-C1; Clontech, Palo Alto, CA),

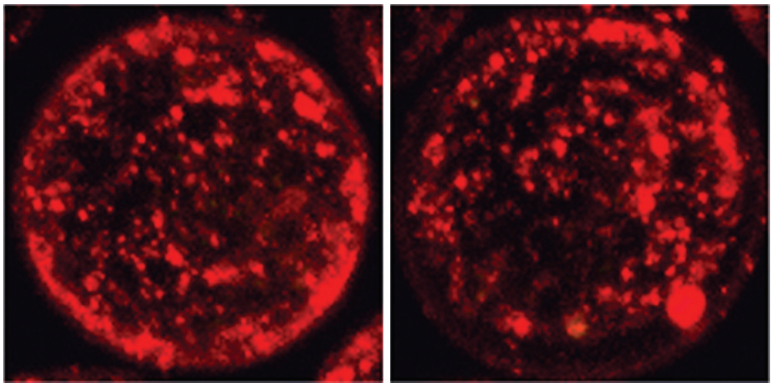

Fig. 17.1 Representative dark-field projections of the rendered z-stack of x–y confocal sections of blastocysts electroporated with AF594-labelled dsRNA, after 24 h of culture. One hundred percent of the blastocysts incorporate the dsRNA and an average of 90% of the blastomeres in each embryo (minimum 70%) are targeted. *Left*, the ICM is viewed from the top; *right*, the ICM is viewed laterally (Soares et al., 2005, courtesy of BioMed Central)

as determined by the absence of fluorescence after up to 2 days in culture. In contrast, GFP expression could be observed in embryos that had been injected with the same plasmid, as early as 6 h after injection (unpublished results). These observations indicate that the absence of GFP fluorescence in the electroporated embryos results from an ineffective delivery of the DNA, rather than from the embryos' inability to transiently express the plasmid. Considering that the same plasmid was effectively introduced into cells of E5.25 post-implantation embryos by electroporation (Soares et al., 2008), the impermeability shown by the pre-implantation cells to this DNA molecule is somewhat surprising.

The experimental setup for the electroporation of mouse morulae and blastocysts with dsRNA is depicted in Fig. 17.2. A microslide chamber with a 1-mm gap between electrodes (BTX Inc., San Diego, CA) is placed under a stereo microscope. Fifty microliters of chilled dsRNA solution in HBS at a concentration of 2 mg/ml are laid in a single drop of ~1 cm in length covering both electrodes. The embryos

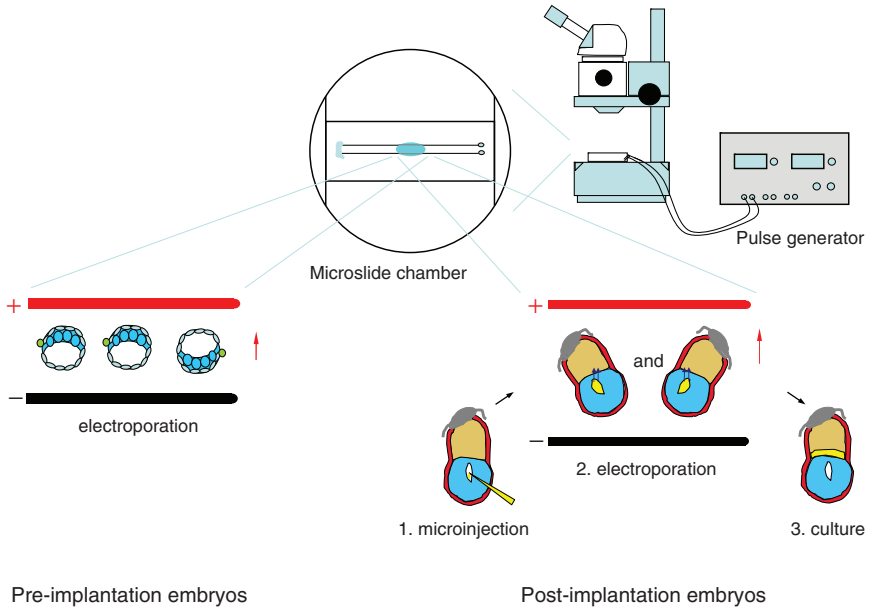

Fig. 17.2 Schematic representation of the electroporation of mouse blastocysts (*left*) and early post-implantation embryos following microinjection (*right*). Intact pre-implantation embryos are placed directly in a drop of dsRNA solution in HBS laid over the electrodes of a microslide electroporation chamber, and preferentially positioned with the ICM oriented towards either electrode. For tissue-specific delivery of dsRNA by directed electroporation, dissected post-implantation embryos are injected in the proamniotic cavity with AlexaFluor594-labeled dsRNA and transferred to a drop of HBS onto a microslide electroporation chamber. To achieve directed electroporation for the targeting of the extraembryonic ectoderm, the embryos are positioned with the ExE facing the cathode with a tilt of −45° during the first series of pulses, and +45° during the second series. Following electroporation, the embryos are cultured *in vitro* in a 5% CO_2 incubator

are washed in M2 medium (Quinn et al., 1982) and transferred to the dsRNA solution where they are aligned so as to form a row equidistant to both electrodes, preferentially with the inner cell mass (ICM) oriented directly towards either electrode. A square-shape pulse generator is used to deliver the electric pulses, as it has been shown that cell death and tissue damage are considerably lower than those resulting from bell-shaped electric pulses (Muramatsu et al., 1997). We used a 2001 Electro Cell Manipulator (BTX Inc.) to deliver three series of four pulses of 1 ms duration with 28–30 V intensity. Each series of pulses is separated by 1 min interval, during which the polarity of the chamber's electrodes is inverted. The inversion of polarity will ensure that the dsRNA is delivered into the embryos in a multidirectional manner, resulting in an increased uptake and a more even distribution of the nuclei acids. Each drop of dsRNA solution should be used for a single application only. This is mainly because the solution will be warmed up by the electric charge and the dsRNA concentration will be inevitable decreased. Immediately after electroporation, the embryos are washed in M2 medium and cultured in KSOM. A minimum of 2 h in culture is advisable before proceeding to the uterine transfer, if this procedure is required. During this time, the embryos recover from the electric insult and resume normal development.

The appropriate controls for the electroporation procedure can be divided in two groups: non-electroporated and HBS-electroporated control embryos. For the first group, embryos cultured together with the experimental counterparts are transferred to the dsRNA solution in the chamber – preferentially following one electroporation event – and returned to culture after 3 min. This group will serve as a control for the effects of the electric shocks on embryo development. The embryos of the second group are electroporated exactly as the experimental ones but in HBS only. Since they have been subjected to the same electric insult as the latter, these embryos will serve as a control for the general effects induced by the introduction of foreign molecules. One other crucial control group entails the electroporation of embryos with a dsRNA molecule targeting a sequence/gene that is not endogenous to the cells. This group, which does not fall under the same category as the two above, is nevertheless essential as it controls for the specific effects of the dsRNA on gene expression. It constitutes a first instance term of comparison for the assessment of the consequences of gene expression knockdown on the development of the experimental embryos.

The main advantages of the use of electroporation over microinjection in pre-implantation embryos are obvious: numerous embryos can be electroporated simultaneously and, importantly, multiple blastomeres are targeted in a single application. Consequently, the procedure is considerably faster than microinjection, which entails the manipulation of a single embryo at a time. However, electroporation requires a substantial amount of dsRNA (100 µg per electroporation with our protocol) and it is not suitable for the delivery of plasmid DNA under the parameters reported thus far. Whereas microinjection is valuable for following the loss or gain of gene function in single cells or their specific lineages, electroporation is most suitable for whole-embryo knockdown studies, especially from the 4-cell stage onwards.

2.2 *Culture and Electroporation of Early Post-implantation Mouse Embryos*

After implanting in the uterine wall, the mouse blastocyst undergoes a series of morphological changes and elongates to acquire a cylindrical shape. The different lineages inherited from the blastocyst now become clearly distinguishable from each other and display unique cellular properties (Fig. 17.3). The embryo also grows substantially during implantation and it almost triples in size in the course of a few hours going from ~57 µm long at E4.75 to ~136 µm at E5.0 (Perea-Gomez et al., 2007). Concomitantly, the number of cells in each embryonic region increases. Because of the complexity and rapidity of these changes, the need for adapted methodologies allowing loss-of-function (or gain-of-function) approaches for studying gene function in a specific time window is imminent. Albeit in reduced number, there are reports where the use of electroporation to induce gene silencing in post-implantation embryos has been documented (reviewed in Fujinaga, 2000; Takahashi et al., 2008). Calegari and colleagues (2002) established a methodology to target GFP mRNA into cells of the neural tube of E10 mouse embryos.

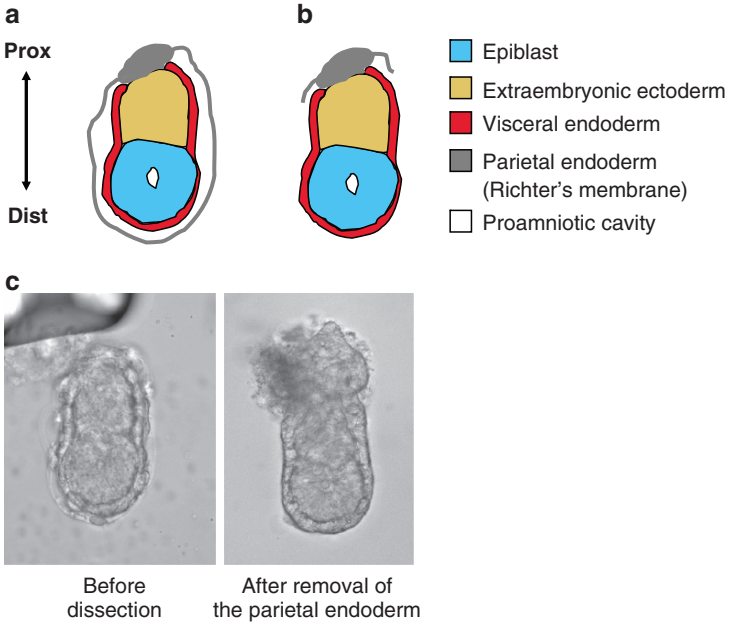

Fig. 17.3 Schematic representation and bright-field images of early post-implantation embryos. (**a, b**) Diagram depicting the different tissues of an E5.25 embryo. In (**b**) the embryo is depicted after removal of the parietal endoderm and ready to be experimentally manipulated. (**c**) Bright-field pictures of representative embryos corresponding to the diagram shown above. The orientation of the proximal-distal axis is shown on the *left*

This methodology proved to be very efficient in knocking-down GFP expressed from the Tis21 locus using whole embryo electroporation following microinjection of siRNA into the lumen of the neural tube, and subsequent culture. This approach has not yet been applied to any endogenous gene, so it remains to be seen whether the same efficiency can be achieved when targeting endogenous loci that are expressed at different levels than those of the Tis21-GFP reporter used in that study.

When working with earlier stages of post-implantation development, e.g. between E4.75-E6.5, the technical challenges are more stringent. One of the most critical steps of performing any manipulation of peri-implantation/post-implantation embryos (E4.75-E6.5) is the dissection of the embryo itself. Special care must be taken when 'freeing' the embryo from the extraembryonic tissues, namely from the parietal endoderm (Fig. 17.3). This is particularly important as keeping this tissue during culture will inhibit embryonic growth. Conversely, if removed completely, the risk of damaging the ectoplacental cone – hence leading to developmental defects – can arise. Two different tools have been routinely used to remove the parietal endoderm. Some groups have found that using tungsten needles (supplied by Fine Science Tools, for example) is most efficient. However, tungsten needles are extremely fragile and need to be sharpened each time after dissecting 7–10 embryos. The use of normal finest syringe needles has worked repeatedly and efficiently in our hands, with the advantage that these can be discarded easily when they become bent. During dissection, the medium must be at 37°C to avoid any damage or undesired defects in the developing embryo. The composition of the medium where dissection is performed is also an important factor to be considered. We have found that a rich cell culture medium (such as DMEM) supplemented with 10% Fetal Calf Serum is best. We do not recommend the use of PBS or HBS for dissection of peri-implantation embryos, as embryos tend to loose their shape and subsequently do not grow properly in culture.

The serum used to supplement the culture medium itself has also been an important aspect for efficient development of post-implantation embryos (Tam, 1998; Tam and Snow, 1980). Rat and mouse serum have been used most often, at concentrations that range between 20% and 100% depending on the stage cultured (Tam and Snow, 1980; Takahashi et al., 2008). Although more difficult to obtain, Human Cord Serum (HCS) has also been used for culture, especially for young embryos. A side-by-side comparison of the growth and developmental potential in different culture medium conditions revealed that embryonic development is between 20% and 30% more efficient when early post-implantation embryos are cultured in HCS compared to that of Rat Serum (our unpublished results). Human Cord Serum is prepared from a fresh placenta (obtained by patient's consent and under the appropriate Ethics Committee authorization). The use of syringes must be avoided throughout the procedure to prevent hemolysis. After collection of the serum from the cord, an immediate centrifugation step of ~5 min to pellet the blood cells is necessary. The serum (the supernatant) is immediately aliquoted and snap frozen in liquid nitrogen. HCS can then be stored at −80°C for long periods of time; however, when an aliquot is thawed, it should not be frozen again and should be used within 10 days. Upon thawing, the HCS must be heat inactivated at

56°C for 1 h. The HCS concentration to be used for culture can vary between 30% and 75%; higher concentrations of HCS proved to be detrimental for development (our unpublished results). Thus, we recommend the use of 45% HCS in DMEM for efficient culture of E4.75–E6.5 embryos. It is essential to pre-equilibrate the serum-supplemented medium for 2 to 10 h in the 5% CO_2 incubator that will be used for subsequent culture. Embryos can be then cultured without the need for rotation, i.e., in static culture (Tam and Snow, 1980; Jones et al., 2002; Richardson et al., 2006). Usually, 3–4 embryos are cultured per ~20 µl drop of medium in 35 mm diameter Petri dishes that are covered with mineral oil. Under these conditions, embryos have been successfully cultured and even imaged throughout culture between E4.75 and E5.75 (Richardson et al., 2006; Torres-Padilla et al., 2007).

For electroporation, a microslide chamber with 1 mm gap between electrodes is used (BTX Inc.). In principle, any tissue or region in the embryo can be potentially targeted. The use of this type of electroporation chamber is advantageous two-fold: (1) it is transparent, so it is easy to place the embryo and ensure that its orientation is correct and (2) different regions of the embryo can be targeted because it can be easily oriented in different ways between the electrodes. The chamber is directly placed under a dissecting microscope with under-stage illumination, in which all the manipulations are carried out (Fig. 17.2).

We have experimentally determined the conditions for efficient delivery of nucleic acids into specific embryonic regions without compromising embryonic development. Using a type 2001 Electro Cell Manipulator (BTX Inc.), we first assessed the effects of varying pulse number and electrical parameters (intensity and duration) on embryonic development, using a solution of rhodamine-labeled dextran in HBS. Concomitantly, the rate of incorporation of rhodamine-dextran into the embryonic cells was monitored by fluorescence microscopy. The best sets of electroporation parameters were subsequently employed to test the efficiency of delivery of a non-linearized CMV-driven GFP plasmid (pEGFP-C1, Clontech). To this end, E5.25–E5.5 embryos were placed between the electrodes of a microslide chamber in a drop of a chilled solution of pEGFP-C1 plasmid in HBS at a concentration of 1.5 mg/ml. Series of two to three pulses of 1 ms duration with 40–60 V intensity were delivered. After 12–14 h in culture, the embryos were imaged by fluorescence microscopy to monitor GFP expression. From these trials, the most efficient electroporation parameters were established as two series of three pulses of 1 ms duration with 30 V intensity (Soares et al., 2008). These settings proved to be reproducible and very efficient for the delivery of both the GFP plasmid and a DsRed plasmid of similar size (pDsRed2-N1, Clontech), and permitted normal embryonic development for ~48 h until E7.5, the latest time-point assayed. Thus, overexpression or ectopic expression studies of specific genes can be achieved using this approach. The effects resulting from ectopic expression of a particular gene *X* on the establishment of the body plan, for example, can be assessed. If *X* is normally expressed in the distal-most region of the embryo, one can ask whether ectopic expression of this gene in the proximal region by directed electroporation can induce the same patterning effects as those elicited by the gene in its original domain. Indeed, this can provide information on both the ability of a gene to induce

a response out of its original spatial context, and of the induced cells themselves to respond to the signaling effects of the gene. Yamamoto and colleagues (2004) have addressed similar questions by transfecting cells of the visceral endoderm of E5.5 embryos with expression vectors coding for small peptides of *Cer1* and *Lefty-1*. However, the vectors were delivered by liposome-mediated transfection following microinjection between the visceral endoderm and the epiblast, which is technically demanding. Furthermore, the use of vectors driving expression of bigger proteins or transcription factors for ectopic expression studies has not been reported.

As a means to implement loss-of-function approaches to study gene silencing, RNAi is now routinely used in a variety of systems. While it has been extensively employed in pre-implantation stages of the mouse development, the use of RNAi in functional studies has been less widely reported in the early post-implantation embryo. Mellitzer et al. (2002) have described the use of RNAi to knockdown *Otx2* and *Foxa2* genes by electroporating dsRNA into the node of E7.5 embryos, which were subsequently cultured for 24–36 h. We have recently reported the targeting of earlier stages of post-implantation development by knocking down *Bmp4* specifically in the extraembryonic ectoderm (ExE) of E5.25 and E5.75 embryos using long dsRNA (Soares et al., 2008).

Two different approaches can be used to induce RNAi in the post-implantation embryo by electroporation: one entailing the direct electroporation of the whole embryo in a solution of dsRNA (targeting the external-most layers of cells and tissues of the conceptus); and another where a microinjection step precedes electroporation. In the latter, the targeting of the proximal part of the extraembryonic ectoderm, for example, requires the microinjection of the nucleic acids into the proamniotic cavity prior to electroporating the embryo (Soares et al., 2008). This is because the proximal-most cells of this tissue are facing the proamniotic cavity, and not exposed to the culture medium. This approach can be used as early as the proamniotic cavity forms, at E5.25. Double-stranded RNA is prepared as described (Soares et al., 2008) and resuspended in HBS at a concentration of 1.5 mg/ml. To facilitate visualization of the dsRNA, the nucleic acid can be chemically labeled by coupling of a convenient fluorochrome such as AF594 (Molecular Probes, Eugene, OR, USA).

For direct electroporation, the experimental setup is the same as that described above for the pEGFP-C1 plasmid, whereby the embryo is placed directly in a drop of HBS with the dsRNA in solution. Conversely, in the protocol for combining microinjection with electroporation, the dsRNA solution is back-loaded into a glass needle at concentration of 1.5 to 2 mg/ml. In both cases it is useful to add a reporter expression vector to the mixture (such as GFP, YFP or DsRed), which will indirectly indicate which cells have incorporated the dsRNA. After dissection in DMEM supplemented with 10% FCS, the embryos are transferred to a microscope slide single concave onto a drop of dissecting medium covered with mineral oil. The injection needle is introduced into the proamniotic cavity and the nucleic acids are injected using air pressure from a dedicated air injector (e.g. an Eppendorf Transinjector). A slight swelling of the cavity – which is sustained even upon withdrawal of the injection needle – is normally a good indicator of a successful

procedure. After microinjection, the embryos are rinsed in HBS and immediately transferred to the electroporation chamber onto a drop of HBS (~200 µl) covering both electrodes. To ensure tissue-specific delivery, the embryos are positioned with the target region towards the cathode with a slight tilt (Fig. 17.2). Electroporation is carried out by delivering two series of three pulses of 1 ms duration with 30 V intensity (as above), after which the embryos are returned to a drop of pre-warmed DMEM medium supplemented with 10% FCS, under mineral oil to prevent evaporation. Finally, the embryos are cultured in HCS-supplemented medium pre-equilibrated at 37°C under a 5% CO_2 atmosphere, in groups of 3–4 embryos per drop for continued development *in vitro*. Because of the sensitivity of the early post-implantation embryos to medium conditions, it is important that the embryos are kept in warm DMEM medium supplemented with 10% FCS, once dissected. Embryos are handled one by one for electroporation and/or microinjection. The transfer between the microinjection rig, the electroporation chamber and the dissection medium is usually not longer than 2–4 min.

3 Application and Results

3.1 RNAi in Pre-implantation Mouse Blastocysts by Electroporation

The successful delivery of nucleic acids into whole embryos at late pre-implantation stages by electroporation prompted us to explore the effects of gene silencing by RNAi in peri-implantation development. In particular, we focused on the consequences of knocking down single and multiple genes by RNAi on the patterning of the early post-implantation embryo upon electroporation of pre-implantation blastocysts with long dsRNA (Soares et al., 2005).

The fully developed mouse blastocyst has three distinct lineages: the inner cell mass, the primitive endoderm (visually distinguishable from the ICM as an epithelial layer of cells at ~E4.0) and the enveloping trophectoderm. To assess the relative efficiency of knockdown in these lineages (at pre-implantation and in post-implantation derivatives), GFP dsRNA was delivered into intact embryos at the 8-cell and blastocyst stages of a transgenic line expressing the fluorescent fusion reporter histone (H2B)-EGFP (Hadjantonakis et al., 2004). After electroporation, the embryos were allowed to develop for 24 h in culture and the levels of fluorescence were investigated. At this time point, H2B-EGFP fluorescence was substantially decreased in all cell lineages (Fig. 17.4). By comparison to control embryos electroporated with *LacZ* dsRNA, the dsGFP-treated embryos showed a 73% overall reduction in fluorescence. Furthermore, the relative reduction of the fluorescence in the ICM and throphectoderm cells was of similar degree. After 24 h in culture following electroporation, a strong knockdown of the GFP mRNA levels was observed in the dsGFP-targeted blastocysts, as determined by RT-PCR

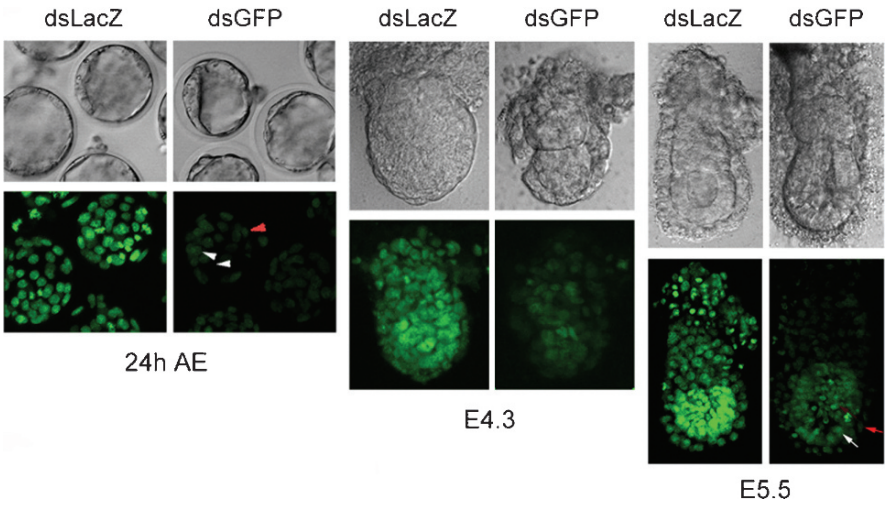

Fig. 17.4 Representative confocal bright-field images (*top*) and dark-field projections of the rendered z-stack of x–y confocal sections (*bottom*) of HB2-EGFP embryos electroporated with GFP dsRNA and LacZ dsRNA (control) at the 8-cell and blastocyst stages, followed by 24 h in culture (24 AE) and *in utero* development until the indicated stages (E4.3 and E5.5). *White arrowheads*, ICM cells; *red arrowhead*, trophectoderm cells; *white arrow*, epiblast cells; *red arrow*, visceral endoderm cells (Soares et al., 2005, courtesy of BioMed Central)

of 15 pooled embryos. The GFP message was reduced by 80% compared to the control embryos, demonstrating a good correlation between the levels of mRNA knockdown and the reduction in fluorescence.

Electroporated blastocysts were also transferred into the uteri of pseudo-pregnant females for continued embryonic development until later stages. The embryos were recovered at E4.3 and E5.5 and analyzed for GFP expression and mRNA abundance. These two time points correspond to ~40 and 72 h of development *in utero* following electroporation, respectively. The levels of histone-GFP fluorescence were reduced by an average of 65% at E4.3 and 52% at E5.5. This reduction was of a similar degree among the different lineages of the egg cylinder. Although the ICM-derived epiblast displayed an overall stronger fluorescence than the surrounding lineages, the degree of fluorescence reduction was equivalent to that of the visceral endoderm (derived from the primitive endoderm) and the extraembryonic ectoderm (derived from the throphectoderm) (Fig. 17.4). The levels of GFP mRNA at each stage were decreased by ~75% and 65% of those of the control embryos, respectively. Importantly, the electroporation procedure had no effects on the development of the embryos *in utero*, as no obvious differences in rate of development or morphology were detectable upon comparison with implanted non-electroporated controls. Furthermore, the embryos electroporated with GFP and LacZ dsRNA could develop to term and were indistinguishable from those of the non-electroporated control group (Soares et al., 2005). These observations show that the long dsRNA molecules do not have non-specific effects at peri-implantation

stages of the mouse development, and are very effective in eliciting the knockdown of gene expression by RNAi.

The technique was then applied to target endogenous genes belonging to key signaling pathways in peri-implantation development. BMP4, a member of TGFβ superfamily of secreted signaling molecules (Hogan, 1996), was chosen to determine if the effects of the electroporation of blastocysts with *Bmp4* dsRNA could recapitulate the phenotypes reported for its loss-of-function at gastrulation (Winnier et al., 1995). *Bmp4* is expressed pre-implantation in the ICM and polar throphectoderm of the blastocyst (Coucouvanis and Martin, 1999). After implantation and until the onset of gastrulation at E6.5, its expression becomes restricted to the extraembryonic ectoderm of the egg cylinder, particularly in the cells closest to the boundary with the underlying epiblast (Coucouvanis and Martin, 1999). Long dsRNAs targeting *Bmp4* (599 bp) and GFP (540 bp) were introduced into intact E3.5 blastocysts by electroporation. The embryos were allowed to develop in culture for up to 48 h and expression of *Bmp4* was investigated by RT-PCR 24 and 48 h after electroporation. The levels of transcript of the *Bmp4* dsRNA-electroporated embryos were as low as 20% of those of the GFP dsRNA-targeted controls at these time points (Fig. 17.5; Soares et al., 2005). In contrast, *Stat3* and *Gap3dh* mRNA levels were unaffected, indicating that the *Bmp4* dsRNA results in sequence-specific knockdown of *Bmp4* expression. Electroporated embryos transferred into pseudo-pregnant mice and recovered at E5.25 and E5.75 post-implantation showed a *Bmp4* mRNA reduction of ~70% and 44%, respectively (Fig. 17.5). The continued reduction of knockdown at these stages most likely reflects the dilution of

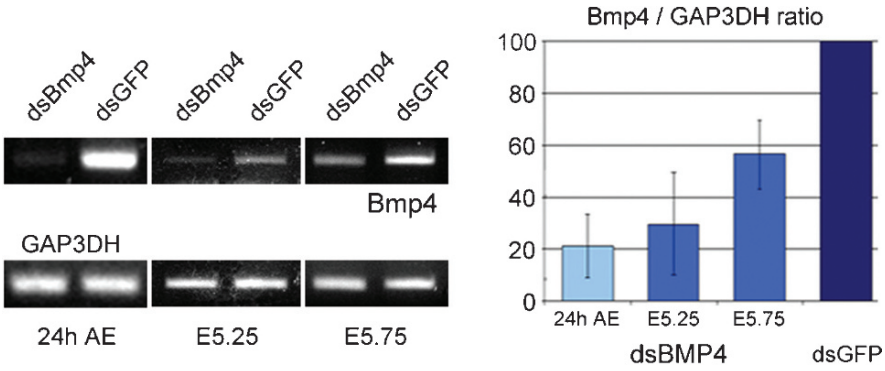

Fig. 17.5 RT-PCR of single embryos cultured *in vitro* for 24 h following electroporation, and embryos recovered at E5.25 and E5.75 following development *in utero*. Glyceraldehyde 3-phosphate dehydrogenase (GAP3DH) was used as an internal control for cDNA loading. Average Bmp4 mRNA levels (Bmp4/GAP3DH ratio) of embryos electroporated with Bmp4 dsRNA (dsBmp4) shown as a percentage of those of GFP dsRNA (dsGFP)-treated controls. Pale blue, 20 dsBmp4-electroporated embryos cultured *in vitro* for 24 h (control embryos: 20, dark blue). Light blue, 10 dsBmp4-electroporated embryos recovered at E5.25 and E5.75, respectively, following *in utero* development (control embryos: 10). Standard deviation bars are indicated (Soares et al., 2005, courtesy of BioMed Central)

the dsRNA molecules delivered before implantation. The degree of knockdown is considerable, nonetheless, and depending on the temporal requirement and turnover rate of the targeted transcript, it can be sufficient to affect the embryonic development of the conceptus. This appeared to be the case for *Bmp4*, as the embryos targeted with *Bmp4* dsRNA displayed a range of phenotypes reminiscent of those of the null embryos (Winnier et al., 1995). Although a proportion showed some development to later stages, many electroporated embryos arrested shortly after implantation. In sharp contrast to the GFP dsRNA-treated controls, of which only 1% were slightly delayed in development, over 70% of the embryos were either morphologically abnormal and/or defective in the expression of essential mesoderm and visceral endoderm genes, or died following *Bmp4* RNAi (Soares et al., 2005). These phenotypes are very similar to those reported for null mutant embryos both in form and variability. Nonetheless, it is important to emphasize that part of the observed pleiotropy is likely to result from the differential uptake of the dsRNA among and within the embryos.

Interestingly, a novel function for *Bmp4* in regulating the establishment of the anterior-posterior axis of the pre-streak embryo was uncovered. The expression of markers of the visceral endoderm was disrupted in *Bmp4* dsRNA-targeted embryos recovered at E5.5 and E5.75. Whereas in the control embryos the AVE had been properly established, as determined by the anterior localization of the AVE-specific markers *Cer1* and *Lefty-1*, in the *Bmp4*-targeted embryos these markers remained distally located and/or spread laterally, or were absent. These observations suggest that BMP4 signaling at peri-implantation stages regulates the expression domain of the AVE and controls its specific positioning. Importantly, the nature of this RNAi methodology, unlike the knockout approach, permits the definition of temporal intervals during which the expression of a given gene (or genes) is required for particular developmental processes. In this case, the influence of *Bmp4* in the patterning of the visceral endoderm seems to take place in a time window between E3.5 and E5.5, which corresponds to the period where the levels of *Bmp4* mRNA can be seen significantly downregulated.

Functional redundancy between genes and isoforms can also be studied by RNAi by targeting related genes independently or simultaneously in specific combinations. Studies of classical single and double genetic knockouts of the three murine *Dishevelled* genes have suggested functional redundancy between these genes (Lijam et al., 1997; Hamblet et al., 2002; Wang et al., 2004a). The Dishevelled proteins are members of the Wnt signaling pathway, which regulates cell fate and subsequent cell behaviour in metazoans (Moon et al., 1997). The three isoforms are expressed throughout pre-implantation development, with a surge from the morulae stages onwards, as seen for other members of the Wnt family (Wang et al., 2004b). We have used electroporation to deliver dsRNA targeting the *Dlv* genes – independently and simultaneously – into pre-implantation embryos. To ensure effective targeting and a timely downregulation of the surging *Dvl* transcripts, the embryos were electroporated in two distinct sessions (Soares et al., 2005). Upon simultaneous delivery of the three *Dvl* dsRNAs at the 8-cell stage and again at the blastocyst stage, a significant knockdown of the *Dvl* mRNAs (70%, 76% and 73%

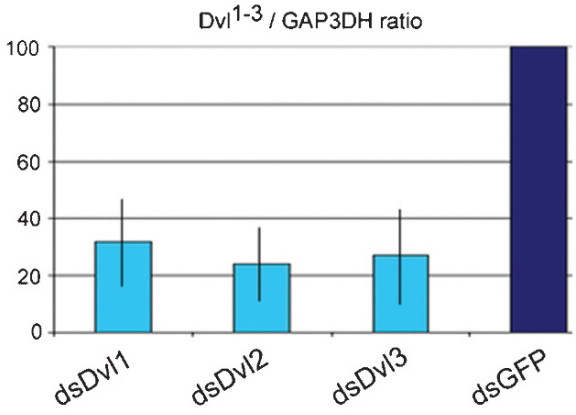

Fig. 17.6 Average *Dishevelled 1*, *2* and *3* mRNA levels ($Dvl^{1–3}$/GAP3DH ratio) of 10 embryos electroporated with $dsDvl^{1–3}$ shown as a percentage of those of 10 dsGFP-treated controls, following 24 h in culture. Standard deviation bars are indicated (Soares et al., 2005, courtesy of BioMed Central)

for each *Dvl* transcript, respectively) was observed 24 and 48 h after the second electroporation (Fig. 17.6). A similar knockdown efficiency was seen following electroporation of the dsRNAs independently, indicating that the simultaneous use of multiple dsRNA molecules does not saturate the cellular RNAi machinery. Importantly, only the levels of the corresponding *Dvl* mRNA were reduced upon delivery of each dsRNA, with the transcripts of the other two isoforms being unaffected (not shown). Following uterine transfer into pseudo-pregnant mice, the electroporated embryos were allowed to develop up to E6.5, E7.5 and E8.5. Whereas the embryos that had been electroporated with a single *Dvl* dsRNA did not display any morphological abnormality at any of these stages, nearly half of those electroporated simultaneously with the three *Dvl* dsRNAs were severely retarded in development or showed morphological anomalies including a failure to gastrulate. This phenotype is in agreement with the role of the Wnt signaling in the formation of the primitive streak, which is impaired in *Wnt3* mutants (Liu et al., 1999) and $LRP5^{-/-}$; $LRP6^{-/-}$ double mutants (Kelly et al., 2004). Notably, neural fold abnormalities and gross distortions of the midline were noticeable in a proportion of the *Dvl* dsRNA-targeted embryos analyzed at E8.0. This phenotype could represent the onset of the defective neural tube closure presented by the $Dvl1^{-/-}$; $Dvl2^{-/-}$ double mutants (Wang et al., 2004a). Surprisingly, the expression of essential mesoderm, visceral endoderm and epiblast markers was unaffected in some of the developmentally delayed embryos. This observation suggests that while the achieved degree of *Dvl* downregulation could delay embryo development, it was not sufficient to prevent gastrulation in a set of the affected embryos. The observed pleiotropy of phenotype following *Dvl* knockdown could also be a consequence of variable uptake of the dsRNA. Importantly, the simultaneous knockdown of the three *Dvl* transcripts uncovered an early functional redundancy between these genes since it elicited earlier developmental defects than those reported for the double knockout mutants (Wang et al., 2004a).

Taken together, the experiments described above demonstrate the potential of dsRNA electroporation in pre-implantation stages as a tool to uncover novel

phenotypes after implantation following RNAi. Moreover, the transient nature of this approach adds a temporal dimension to the analyses of gene function, which is often absent in classical knockout approaches.

3.2 RNAi in Early Post-implantation Embryos by Electroporation

The first step towards the analysis of potential developmental defects upon RNAi is to determine the efficiency and specificity of the knockdown achieved. This constitutes an essential aspect of the experiment and needs to be adapted to each experimental design depending on the approach, the gene under study and the region of the embryo that is being targeted.

To illustrate this idea, in this section we will focus on two studies that are available from the literature. Mellitzer and colleagues (2002) co-electroporated dsRNA for *Otx2* with a GFP expression vector into the node of E7.5 embryos. To analyze the degree of knockdown of *Otx2* expression, double immunostaining was performed for the two proteins after having cultured the embryos for different periods of time. In this way, positive cells for GFP were expected to be negative for (or have low levels of) the Otx2 protein if RNAi had been efficient, which can be easily determined by immunostaining. In this case, the experimental design involves a direct *in situ* observation of individual targeted cells. This is important when the gene that is being knocked down is expressed in a broader region than that targeted by electroporation. Indeed, the employment of techniques where the whole embryo is used (such as Western blot or RT-PCR) will result in a dilution effect and the efficiency of the RNAi can be underestimated leading to a misinterpretation of the results. Following this analysis, the authors determined that the effect of RNAi on Otx2 protein accumulation was dsRNA dose-dependent. In the course of their experiments, one side of the embryo was left intact (i.e., non-electroporated), which was used as a control region for any effects on development caused by the *in vitro* culture or electroporation conditions. Although controlling for non-specific effects resulting from *in vitro* culture conditions or the electroporation itself is essential, the use of non-specific dsRNA should also be systematically adopted. This is necessary to rule out side effects elicited by general responses to the RNAi process. An unrelated gene that is not expressed in the embryo such as *LacZ* or GFP, or any other gene that is not transcribed during the time of development under study, should be used as a negative control. Assessing this type of non-specific effects can prove burdensome when using a double immunostaining protocol to analyze changes in gene expression or protein accumulation because only one protein per embryo can be studied. Considering that expression of at least four or five genes should ideally be analyzed after RNAi, the number of embryos required for electroporation and processing for immunostaining and analysis increases exponentially. Adapting multi-color *in situ* hybridization or immunostaining procedures, whereby a number of different probes or antibodies can be employed per embryo, can help to partially overcome this problem.

In a different study, earlier stage embryos were electroporated with dsRNA targeting *Bmp4* at two different time points (E.25 and E5.75), by using a combined approach of microinjection and electroporation (Soares et al., 2008). Directed electroporation towards the extraembryonic ectoderm had been shown to efficiently deliver nucleic acids into this region as determined by the expression of an electroporated GFP plasmid (Fig. 17.7). In this study, *Bmp4* dsRNA was microinjected into the proamniotic cavity and subsequently delivered by directed electroporation. Because the aim was to *selectively* target the proximal-most region of the extraembryonic ectoderm, the embryos were placed between the electrodes of the electroporation chamber with the proximal region oriented towards the cathode and in a 'tilted' position in a ~45° angle relative to the cathode (Fig. 17.2). The embryos were then subjected to two series of pulses between which they were re-oriented to form a ~45° angle in the opposite direction, so that a comprehensive targeting of the proximal region of the ExE was achieved (Figs. 17.2 and 17.7). This approach resulted in a very efficient downregulation of *Bmp4* expression in the ExE, which is the only tissue of the conceptus where *Bmp4* transcripts are detected between E5.5 and E6.0 (Coucouvanis and Martin, 1999). The degree of knockdown achieved through this RNAi procedure was analyzed by semi-quantitative RT-PCR of whole embryos and was shown to be in the order of 80–95%. The embryos at both E5.25 and E5.75 responded equally well in downregulating *Bmp4* expression and showed the same extent of knockdown. The expression levels of two unrelated genes (*Oct4* and *Stat3*) were also assessed to rule out non-specific effects of the long dsRNA. This analysis revealed no changes in the mRNA levels of any of the two genes, suggesting that the delivery of *Bmp4* dsRNA resulted in sequence-specific knockdown of *Bmp4* expression (Soares et al., 2008).

Once the conditions for determining the efficiency of the knockdown elicited by the dsRNA are established and the adequate controls to rule out non-specific effects

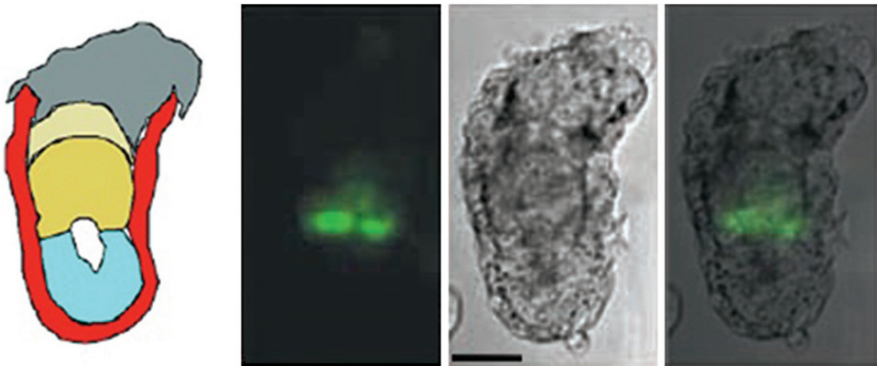

Fig. 17.7 Effective delivery of nucleic acids targeted to the distal extraembryonic ectoderm by directed electroporation. GFP expression in the ExE of a wild type embryo subjected to microinjection and directed electroporation of a GFP-expressing plasmid (pEGFP-C1), 8–10 h after electroporation. On the *left*, a diagram depicting the lineages of the embryo is shown. Color code is as in Fig. 17.3. Scale bar: 50 µm. GFP, brightfield and merge images are shown, respectively

have been set, the following step consists in the analysis of potential developmental defects. When a phenotype has a morphological outcome, it can be directly characterized through dissection and careful examination of embryonic structures. For example, the presence or absence of the Node in gastrulating embryos can be easily assessed. Similarly, the closure of the neural tube or the development of the correct number of somites can also be directly addressed. Changes in gene expression can be further analyzed by the use of transgenic lines carrying fluorescent or *LacZ* reporters, by *in situ* hybridization, by immunohistochemistry or a combination of the three. Using this kind of analysis, a localized effect on gene expression can be revealed since these techniques allow to precisely determine which group of cells express (or not) a number of marker genes and their position within the embryo. Semi-quantitative or quantitative RT-PCR, as well as Western Blot, can also be used but in this situation the analysis does not provide positional information of gene expression due to the nature of these techniques.

Mellitzer and colleagues (2002) performed a fine morphological analysis after *Otx2* and *FoxA2* RNAi in E7.5 embryos. It was concluded that downregulation of the Otx2 protein resulted in a severe reduction of the forebrain, which was in full agreement with results obtained from chimeric contribution studies of *Otx2* function (Rhinn et al., 1998). In turn, the study we have undertaken constitutes an example of the use of a transgenic line with a fluorescent reporter to monitor defects in embryonic patterning (Soares et al., 2008). The advantage of using a GFP reporter is that the effects of gene knockdown by RNAi on development can be monitored *in vivo* as the embryo develops without the need to sacrifice it for fixation. It is essential, of course, to establish in advance that the used transgenic reporter fully recapitulates the expression pattern of the endogenous gene. Using fluorescent markers driven by the promoter of a gene of interest also offers the possibility to directly assess the behaviour of cells expressing this gene using time-lapse imaging (Srinivas et al., 2004; Torres-Padilla et al., 2007). Notably, time-lapse analysis can be done in parallel in control and experimental embryos. To analyze the effects of the loss of BMP4 signaling on the formation of the anterior visceral endoderm, we have used a mouse line with a GFP reporter under the control of the *Cer1* gene promoter. The expression of GFP had been previously shown to faithfully mimic that of *Cer1*, making this transgenic mouse a good model to study the behaviour of the AVE cells, which express *Cer1* (Mesnard et al., 2004; Torres-Padilla et al., 2007). By analyzing transgenic embryos after microinjection and electroporation with *Bmp4* dsRNA, the position of the AVE was found to be severely affected upon downregulation of *Bmp4*. To confirm the validity of these findings, *Bmp4*$^{-/-}$ embryos (Winnier et al., 1995) were also studied. An abnormal pattern of expression of the AVE marker *Cer1* was also found in the *Bmp4* null embryos by *in situ* hybridization, indicating that BMP4 does indeed modulate the position of the visceral endoderm cells at E5.5–E5.75. Interestingly, the phenotype on the positioning of the AVE was observed solely in embryos electroporated at E5.25, and not in those electroporated at E5.75. Indeed, while the AVE position (as judged by the position of the GFP-expressing cells) was perturbed following *Bmp4* RNAi at E5.25, it was not so upon *Bmp4* knockdown ~10 h later, at E5.75.

These observations highlight the advantage of this experimental approach over standard genetic manipulations to address gene function from a temporal perspective. By following a genetic manipulation approach (either a classical or conditional knockout) it would have not been possible to ascertain such a short time window of action of the ExE BMP4 signaling to the visceral endoderm. It is conceivable that this type of experimental design (and associated outcome) will encourage other researchers to explore the role of other genes in even narrower time windows, particularly during peri-implantation development, when morphogenetic and signaling events are of an exquisite complexity.

4 Conclusion

Electroporation of nucleic acids is a powerful technique to study gene function. It has been largely applied to chick embryos, but it has been less utilized to study mammalian development. Several groups have used electroporation *in utero* applied specifically to retinal ganglion cells of the mouse embryo at E16 (Garcia-Frigola et al., 2007; Chen et al., 2008). In particular, electroporation techniques of neural progenitors and cortex cells have been the most developed (Holm et al., 2007). The recent efforts in developing electroporation methodologies suitable for earlier stage embryos should allow a broader use of RNAi or gain-of-function analyses during the peri-implantation stages of mouse development. It should be appreciated that RNAi and electroporation will not replace the classical genetic approaches of gene disruption and transgenesis. On the contrary, the limitations of these powerful approaches have only catalyzed the development of alternative experimental technologies aimed at addressing the function of genes in a complementary fashion. Targeting gene expression in a region-specific manner (e.g. in a subpopulation of cells within a tissue) and/or within a defined time window is not possible using standard genetic approaches. In fact, only very few efficient *Cre* recombination-mediated systems have been documented that are able to target a small group of cells within a tissue. Furthermore, no gene has been identified to date that displays restricted expression in individual blastomeres or regions of the preimplantation embryo that could potentially allow the use of a *Cre*-mediated conditional knockout strategy. As discussed in this chapter, these shortcomings have been circumvented by the use of electroporation of dsRNA to elicit RNAi at defined stages in development and in a tissue-specific manner.

Implantation is one of the most important events of the embryonic development in mammals. Indeed, a myriad of signaling pathways and gene regulatory cascades are in control of the morphogenetic changes that characterize this event. However, addressing gene function in a temporal- and region- specific fashion has been hampered by the lack of reproducible gene transfer techniques. Recent advances in electroporation methodologies as well as the improvement of culture techniques should allow the study of gene function over the implantation period with greater precision. Potentially, electroporation could also be used as a tool for transposon-mediated

gene transfer in the mouse embryo, as it has recently been shown to work in chick embryos (Sato et al., 2007). Transposon-mediated gene transfer should provide a more robust effect on gene regulation because of the ability of the electroporated transgene to retrotranspose, inducing more durable – if not permanent – effects. Finally, the development of high-resolution imaging techniques that permit *in vivo* tracking of molecules in single cells during development should also facilitate the study of the effects of gene missexpression on key developmental programs.

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Chapter 18
Gene Transfer into Mouse Haemogenic Sites, as a Mean to Functionally Approach the Control of Mesoderm Determination Towards a Haematopoietic Fate

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1 Introduction

It is now well established that in all vertebrate species the development of a functional haematopoietic system requires the sequential contribution of two independently generated pools of haematopoietic precursors. These two haematopoietic precursors waves are dedicated to specialized functions regarding haematopoiesis ontogeny. The first, which occurs in the extra-embryonic compartment, in the yolk Sac (YS) blood islands, rapidly produces the differentiated erythro-myeloid cells (erythrocytes, macrophages and megakaryocytes) necessary for developing tissue homeostasis. This extremely fast differentiation seems to occur at the expense of differentiation and maintenance potentials (see below). The second wave of precursors develops in the intra-embryonic compartment, in the aorta region, and gives rise to Haematopoietic Stem Cells (HSC), which are thought to be responsible for lifelong maintenance of haematopoiesis (Cumano and Godin, 2007).

In lower vertebrate species, such as Avian and Amphibian, the independent generation of extra- and intra-embryonic precursors pools, as well as the transient nature of the extra-embryonic one, was demonstrated through the use of interspecific chimaeras.

In the Avian model, the contribution of YS precursors to developmental haematopoiesis was assessed in chimaeras harbouring the intra-embryonic compartment of a quail embryo and the YS of a chick embryo, combined at stages anterior to the initiation of blood cell circulation (Dieterlen-Lièvre, 1975). The detection of quail-specific nucleole marker (Le Douarin, 1969) allowed the characterization of the nature, and thus of the embryonic origin, of blood cells in the chimaeras. The analysis of chimaeras at various development stages demonstrated that the precursors that first appear in the chicken YS do not display an extensive self-renewal capacity,

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as they are progressively replaced by a new population of Quail HSC generated in the intra-embryonic compartment (Dieterlen-Lièvre, 1975). The origin of intra-embryonic HSC was further ascribed to the region neighbouring the dorsal aorta, through cytological (Dieterlen-Lièvre and Martin, 1981) and *in vitro* (Cormier and Dieterlen-Lièvre, 1988) analyses, while *in vivo* reconstitution experiments demonstrated that aorta-derived cells maintain haematopoietic cell production till adulthood (Lassila et al., 1982).

In amphibian also, HSC responsible for definitive haematopoiesis were thereafter demonstrated to originate from a dorsal region that comprises the dorsal aorta, rather than from the ventral blood island (homologous to the amniotes YS) (Turpen, 1998).

In mammals, the characterization of the embryonic origin of intra-embryonic precursors identified in the early nineties relied on the development of organ culture of the presumptive haematopoietic site.

The presence of haematopoietic precursors in the intra-embryonic compartment has been evidenced from 8.5–9 days post-coitus (dpc) to 11.5 dpc (Godin et al., 1993; Medvinsky et al., 1993). From 8.5–9 dpc, the intra-embryonic haemogenic site, named para-aortic splanchnopleura (P-Sp), comprises the aorta and the splanchnic mesoderm. At later development stages, it is qualified as AGM as the Aorta, Gonads and Mesonephros further developed from the P-Sp. Haematopoietic precursors isolated from the P-Sp/AGM display the self-renewal and differentiation potential that characterize HSC, as they are multipotent (Godin et al., 1995, 1999) and able to reconstitute in the long term all the haematopoietic lineages of compromised recipients (Cumano et al., 2001; Medvinsky and Dzierzak, 1996; Müller et al., 1994; Yoder et al., 1997).

Precursors that display HSC features can only be detected after the development of a functional vascular network, an event that occurs at 8.25 dpc (4–5 somite stage) (McGrath et al., 2003), implying the possible mingling in the systemic blood of precursors that may originate from either the extra- or intra-embryonic compartments. To assess the capacity of the P-Sp/AGM to generate the HSC it later harbours, we isolated the presumptive P-Sp/AGM territory (the caudal intra-embryonic splanchnopleura: Sp), and the corresponding YS, at pre-circulation stages. The explants were maintained in organ culture for 2–4 days in order to allow the commitment of haematopoietic precursors, before analysing the presence of haematopoietic precursors in individual Sp explants and their YS counterpart. Whereas Sp explants give rise to a multipotent progeny, thus establishing its capacity to generate haematopoietic precursors, YS-derived precursors lack the ability to produce a lymphoid progeny (Cumano et al., 1996). When they are injected into RAG2$^{-/-}$/γc$^{-/-}$ double mutant recipients, that lack both lymphoid progenitors and natural killer cells (Colcucci et al., 1999), Sp-derived haematopoietic cells are able to reconstitute in the long term all the haematopoietic lineages of the recipient, and as such qualify as HSC. In this context, YS-derived precursors do engraft the recipient, but are unable to maintain, as a YS contribution to host haematopoiesis is no longer observed three months after transplantation (Cumano et al., 2001). These data, which result from experiments

performed before the initiation of blood circulation, and thus in the absence of cross-contamination by precursors of both haemogenic sites, led to the conclusion that definitive haematopoiesis initiated by HSC generated in the intra-embryonic compartment.

Do these two haematopoietic precursors populations differ in their generation? Experiments performed in the Amphibian model indicates that both extra- and intra-embryonic presumptive haemogenic sites initially display the same haematopoietic competence. Indeed, reciprocal grafts between the two territories demonstrated that, up to the neurulation stage, the presumptive territories gives rise to the progeny typical of the site where they are engrafted (Turpen et al., 1997). Provided that the suitable stage is targeted, it is thus conceivable to manipulate haematopoietic precursors potential and hence get information on the mechanisms that regulate their emergence and further differentiation and self-renewal potential. However, transduction protocol aimed to transduce mesodermal cells or haematopoietic precursors during early steps of development are sorely missing. Since the determination and further generation/amplification of haematopoietic precursors depends the maintenance of layer interaction/organ culture, the only transduction method currently available, which allows cells targeting within their environment and is compatible with organ culture, is *in situ* electroporation.

To comprehensively approach the haematopoietic determination, we designed *in situ* electroporation protocols suitable to transduce mesodermal/pre-haematopoietic precursors in both the extra- and intra-embryonic compartments. Our overall data establish that *in situ* electroporation will in the future constitute a suitable approach to analyse the qualitative effect of transduced gene in mouse haemogenic sites. However, additional effort must be undertaken to improve the quantitative efficiency of our protocols.

2 Principle

For both haemogenic sites, the basic transduction scheme involves injection/deposition of the plasmid towards the layer of interest in live embryo, followed by *in situ* electroporation. The whole presumptive haemogenic site is further explanted and kept in organ culture so that determination of the mesoderm toward a haematopoietic fate, leading to haematopoietic precursors generation, may occur.

The effect of *in situ* electroporation on the development of the explants is evaluated by comparing the viability and morphology of electroporated explants (or control explants electroporated without plasmid) to that of non-electroporated control, after the organ culture step. Transduction efficiency is assessed by flow cytometry analyses of GFP levels, whereas suitable targeting of haematopoietic precursors is assessed by characterising the co-expression of GFP and haematopoietic cell markers using flow cytometry analysis of electroporated explants after organ culture. The quality of haematopoietic progeny of GFP^+ sorted cells,

as well as the relative number of transduced precursors, is characterized using various *in vitro* assays.

3 Procedure

3.1 Animals and Embryo Recovery

All animal experiments are performed according to French and European institutional guidelines.

To generate embryos, Balb/c or C57BL/6 males are crossed with females and embryonic development is estimated considering the day of vaginal plug observation as 0.5 dpc. Pregnant females are sacrificed by cervical dislocation and embryos are collected in Phosphate Buffer saline solution (DPBS + $CaCl_2$, $MgCl_2$; Invitrogen). Presomitic embryos are staged according to the development of the allantois bud: OB (No Bud), EB (Early Bud) and LB (Late Bud) (Downs and Davies, 1993), whereas embryos at 8 dpc are staged by somite counting.

3.2 General Electroporation Procedure

The expression vector pEGFP-C1 (Clontech) was used throughout this study. The plasmid DNA solution at a 1 µg/µl concentration, supplemented with Fast Green (0.01%) to monitor the release of the construct, is deposited using a mouth driven capillary, nearby the targeted site.

Two 512 gold genetrodes (BTX) are positioned in such a way as to target the specified cell subset and *in situ* electroporation is performed (see Table 18.1 for electroporation parameters) using a square-wave pulse generator and monitor (ECM830, BTX).

Table 18.1 *In situ* electroporation parameters used to transduce haemogenic sites. *In situ* electroporation of 7 dpc YS (OB-LB stages) or 8 dpc Sp (Late Head Fold stage-4S stages) is performed using ECM 830 square wave electroporator and two gold genetrodes (BTX)

Hemogenic site	*Yolk Sac Mesoderm*	*Splanchnopleura Mesoderm*
Pulse intensity (V)	30	35
Pulse duration (ms)	50	50
Interval between pulses (ms)	500	500
Number of pulses	5	5
Distance between electrodes (mm)	4	4
Plasmid concentration (µg/µl)	1	1

3.3 Organ Culture

Immediately after electroporation, the YS or Sp are dissected from the embryo, as shown respectively in Figs. 18.1b and 18.2c, and transferred into 6-well plates (Falcon) containing "Complemented OptiMEM medium", i.e. OptiMEM with Glutamax (Invitrogen), 10% Fetal Calf Serum (Hyclone), 1% Penicillin-streptomycin (Invitrogen) and 0.1% β-mercaptoethanol (Invitrogen) and maintained in organ culture at 37°C, 5% CO_2. The duration of the organ culture step is 1–4 days for 8 dpc Sp-explants and 3 days for 7 dpc YS-explants, and is thereafter referred to as OrgD1-4 or OrgD3.

3.4 Expression Analyses

3.4.1 *In Situ* Hybridisation

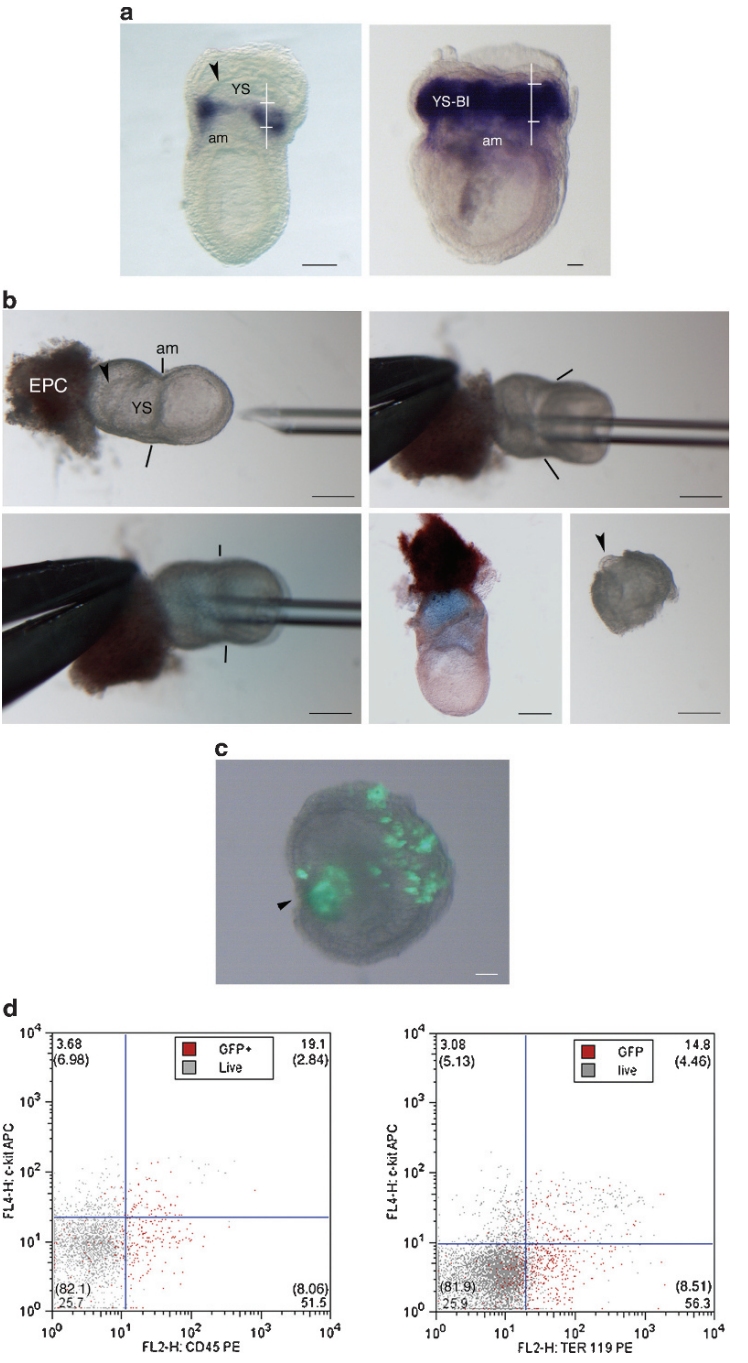
Explants are fixed in 4% paraformaldehyde in PBS overnight at 4°C and rinsed with PBS. *In situ* hybridisation is performed at 65°C, as previously described (Bertrand et al., 2005a). Digoxigenin (DIG)-labelled antisense Lmo-2 riboprobe, constructed by V. Lemarchandel, was obtained from a 450 bp PCR fragments subcloned into the Bluescript expression vector and synthesized using the DIG RNA Labelling Kit (Roche Diagnostics, Indianapolis, IN).

3.4.2 Immunostaining

To achieve confocal analyses, Sp-explants are maintained in "ibidy" chambers (μ-Slides 8 well, Biovalley) during organ culture. Explants are fixed in 4% paraformaldehyde in PBS for 2–4 h at 4°C, rinsed with PBS and then incubated in PBS, 10% foetal calf serum (2 h at room temperature) to prevent non specific antibody binding. Samples are incubated with purified Rat anti-Mouse EpCAM (Clone G8.8) overnight at 4°C, followed by incubation with Alexa 633 coupled-Goat anti-Rat (Molecular probes), for 1 h at room temperature. The EpCAM (Clone G8.8) monoclonal antibody developed by A. Farr was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. After blocking (30 min. at room temperature) with 1/10 rat serum, explants are incubated for 2 h with CD31-PE (Clone MEC 13.3, from Pharmingen), and washed before observation.

3.4.3 Microscopy and Photography

Wholemount *in situ* hybridised embryos or electroporated explants are observed on a Fluorescence-equipped Olympus SZX12 stereomicroscope, and images are acquired with the DP50 Olympus digital camera, using Olympus Cell* software. Confocal


a
YS
am
YS-BI
am
b
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am
YS
c
d
3.68
(6.98)
GFP+
Live
19.1
(2.84)
(82.1)
25.7
(8.06)
51.5
FL4-H: c-kit APC
FL2-H: CD45 PE
3.08
(5.13)
GFP
live
14.8
(4.46)
(81.9)
25.9
(8.51)
56.3
FL2-H: TER 119 PE

analyses are performed on a Zeiss LSM 510 Meta Laser Scanning Confocal microscope. Image stacks are collected using an x20/0.75 apochromat plan objective. Excitation wavelengths for GFP, PE and Alexa 633 are 488, 543 and 633, respectively. Images are acquired using BP505-530, BP560-615 and LP 650 filters, with the LSM Examiner software. Images are further processed with the Adobe Photoshop CS software.

3.5 Analysis of Haematopoietic Cell Production

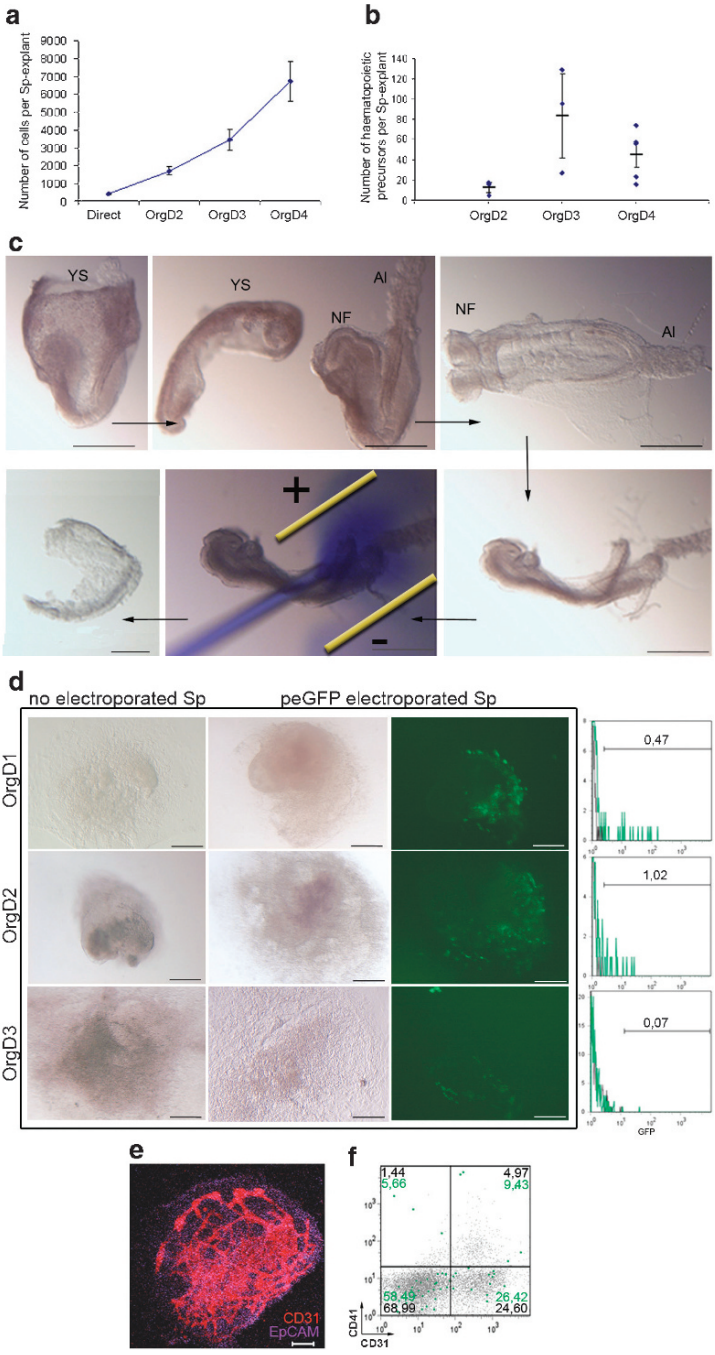
Prior to haematopoietic cell assays, YS or Sp-explants are dissociated by gentle pipeting and filtered to obtain a single cell suspension.

3.5.1 Culture on OP9 Stromal Layer and Limiting Dilution Assay

OP9 stromal cells (from S.-I. Nishikawa and T. Nakano, Riken Center for Developmental Biology, Kobe, Japan), are plated in 96-well plates (500–1000 cells per well) and cultured in "Complemented OptiMEM medium" overnight at 37°C in 5% CO_2/95% air.

Cells from YS or Sp explants are plated in "Complemented OptiMEM medium", further supplemented with Flt3-ligand, Stem Cell Factor and EPO (YS and Sp-explants) and Il-7 (Sp-explants) and cultured as previously described (Bertrand et al., 2005a). At various culture time points, cells are analysed by flow cytometry.

Fig. 18.1 *In situ* electroporation of 7 dpc YS. (**a**) Wholemount *in situ* hybridisation using lmo2 riboprobe. Abbreviations: am: amnion; BI: Blood Island. Lmo2 labelling allows the visualisation of the blood island (BI) and its allocation to the median third of the YS (*White bars*). The arrowhead points to the floor of the ectoplacental cavity. These observations lead to the appropriate positioning of the electrode in treated embryos. *Left panel*: Embryo at the EB stage. The prospective blood island forms a median ring in the YS. *Right panel*: Embryo at the LB stage. The blood island has greatly expanded. It already contains large amount of $lmo2^+$ haematopoietic cells. Magnification bar = 100 µm. (**b**) Scheme of *in situ* electroporation of 7 dpc YS. Abbreviations: am: amnion; EPC: Ectoplacental cone. Positioning of a LB stage embryo prior to plasmid injection (*upper left*). The arrowheads point to the floor of the ectoplacental cavity. The two lines flanking the amnion (*arrow*) indicate the variation of its position during the electroporation process. The capillary is inserted from the node region towards the floor of the ectoplacental cavity, through the am (*upper right*). The plasmid solution is released within the YS cavity (*lower left* and *median panels*) and pulses are applied. The YS is dissected from the embryo and placed in organ culture (*lower right*). Magnification bar = 100 µm. (**c**) Electroporated YS (EB stage) after 1 day in organ culture. Overlay of GFP and DIC images of a YS explant. GFP^+ cells are present at the site of adhesion to the culture dish (*arrowhead*) in large number, but also spread in the globular structure. Magnification bar = 100 µm. (**d**) Cytometry profile of electroporated 7 dpc YS performed immediately after the organ culture step. GFP^+ gated cells are displayed in red within the whole population, in grey. Most GFP^+ cells harbour haematopoietic markers, either myeloid (CD45, *left panel*) or erythroid (Ter119, *right panel*). Kit^+ precursors are also present within the GFP^+ subset. The percentages of cells within the GFP^+ population and the whole population (between bracket) are shown for the various subsets


a
Number of cells per Sp-explant
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Direct
OrgD2
OrgD3
OrgD4
b
Number of haematopoietic precursors per Sp-explant
140
120
100
80
60
40
20
0
OrgD2
OrgD3
OrgD4
c
YS
YS
AI
NF
NF
AI
+
−
d
no electroporated Sp
peGFP electroporated Sp
OrgD1
OrgD2
OrgD3
0,47
1,02
0,07
GFP
e
CD31
EpCAM
f
1,44
5,66
4,97
9,43
58,49
68,99
26,42
24,60
CD41
CD31

To perform limiting dilution assays, cells from whole OrgD3 7 dpc-YS are seeded at 100, 300 and 900 cells per well, whereas GFP$^+$ cells sorted from these explants are seeded at 10 or 30 per well. As for OrgD2-Sp explants, cells from the whole population are plated at 30, 100 and 300 cells per well, while GFP$^+$ sorted cells are plated at 1 or 10 cells per well. Cells are seeded in 36–48 wells from 96-well plates for each cell concentration, in culture medium supplemented with the appropriate cytokines (see above). After 5 days in culture for OrgD3-7 dpc YS-explants, or 7–10 days in culture for OrgD2-8 dpc Sp explants, analysis of the frequency of negative cultures for each cell concentration are performed to determine the frequency haematopoietic precursors, following the expected Poisson distribution.

3.5.2 Flow Cytometry Analyses and Cell Sorting

Cells are analyzed on a FACS Sort (Becton-Dickinson) as previously described (Bertrand et al., 2005a), and FACS data are analyzed with CellQuestPro (Becton-Dickinson) or FlowJo (Treestar) softwares. The following antibodies (all from Pharmingen) are used to reveal throughout this study: CD45 (Clone 30-F11), CD31

Fig. 18.2 *In situ* electroporation procedure aimed to target the mesoderm of the intra-embryonic haemogenic site before the onset of circulation. (**a**) Evolution of Sp-explants cells number during organ culture. Sp-explants initially contain around 400 cells per explant. After 2 days in culture, this number increases to 1700 cells per Sp-explant. This number doubles 24 h later and, after 4 days in culture, increases up to about 6500 cells per Sp. (**b**) Analysis of haematopoietic precursors number at different time of organ culture by limiting dilution. After 2 days in culture, Sp-explants contain around 14 precursors. One day later, this number increases to reach around 80 per explant, then decreases to 45 per Sp-explants at OrgD4. (**c**) Scheme of the *in situ* electroporation of 8 dpc Sp. Abbreviations: al: allantois, NF: Neural folds, YS: Yolk Sac. Embryos below the 4 and 5 S are separated from the YS and, after splitting of the amnion, extended flat on the culture dish. The Sp is then lifted up from the caudalmost part of the embryo and the plasmid solution deposited between the splanchnopleura and somatopleura. Pulses are applied and the Sp is dissected from the embryo and placed in organ culture. Magnification bar = 500 µm (excepted in the *lower left panel*: 200 µm). (**d**) Cytological and cytometry analyses of electroporated Sp-explants at different time of organ culture. Cytological analysis (*left panel*) Both non-electroporated control and electroporated Sp-explants show the same adherent morphology at OrgD1, 2 and 3, indicating a normal development and viability of electroporated Sp-explants after the organ culture step. GFP+ cells are mainly observed within the adhering layer (*right column*). Magnification bar = 200 µm. Cytometry profile (*right panel*) GFP$^+$ cells are detected in electroporated Sp during the 3 days of organ culture. The percentage of GFP positive cells represents about 0.47%, 1.02% and 0.07% in respectively OrgD1, D2 and D3 Sp-explants. (**e**) Structure of electroporated OrgD2 Sp-explants characterised by confocal microscopy analysis. Electroporated Sp-explants after 2 days of organ culture present CD31$^+$ vascular network nearby the EP-CAM$^+$ endodermal layer. Magnification bar = 100 µm. (**f**) Cytometry profile of electroporated OrgD2 Sp-explants. GFP$^+$ gated cells are displayed in green within the whole population represented in grey. CD31$^+$ CD41$^+$ (*upper right panel*) haematopoietic precursors represent 4.97% of the whole population while it corresponds to 9.42% of electroporated GFP$^+$ subset. This enrichment of haematopoietic precursors population in the GFP$^+$ subset obtained from electroporated OrgD2 Sp indicates a proper targeting of mesodermal layer during *in situ* electroporation step. The percentages of cells within the GFP$^+$ population (*green numbers*) and the whole population (*black numbers*) are shown for the various subsets

(Clone MEC 13.3) and Mac-1 (Clone M1/70) for myeloid cells, Ter119 (Clone Ly-76) for erythrocytes, as well as CD41 (Clone MWReg30) and c-kit (Clone 2B8), which, in combination with CD31, defines immature precursors. Cell sorting is performed with a FACS Diva cell sorter (Becton-Dickinson).

4 Application and Results

4.1 In Situ *Electroporation of 7 dpc YS*

We previously used wholemount *in situ* hybridisation analyses to pinpoint the stages when blood islands are enriched in immature mesodermal or pre-hematopoietic cells (Giroux et al., 2007). To do so, we compared the expression of lmo2, that identifies both immature blood islands haematopoietic precursors and their erythroid progeny to that of the embryonic globin βH1, which expression is restricted to differentiated erythroid cells, during early blood islands formation.

These expression pattern analyses reached two ends. They pointed to the OB-EB stages as the most enriched in immature precursors ($lmo2^{+}$ $\beta H1^{-/lo}$). They also allowed us to precisely affiliate early blood island position to the median third of the distance spanning from the amnion to the roof of the YS-cavity (Fig. 18.1a), thus ensuring an accurate positioning of the electrodes during the electroporation process.

4.1.1 Electroporation Procedure and GFP Levels and Evolution

In order to target early blood island precursors, embryos are dissected at the OB-LB stages. The Reichert membrane is removed, while the ectoplacental cone is kept in place, so that the YS cavity remains closed, thus avoiding the dispersal of the plasmid solution during the electroporation procedure.

The mesoderm constitutes the inner layer of the YS-cavity, so that the pEGFP plasmid solution is injected into the YS cavity. This is achieved by inserting the capillary from the node region and through the amnion, which leads to the collapse of the amnion towards the ectoplacental cavity. During the delivery of the plasmid within the YS cavity, an accurate injection is monitored by the fast green by the return of the amnion to its former position.

Then the electrodes (gold genetrodes 512 from BTX) are positioned parallel to the blood island ring, which position is inferred from expression pattern analyses, and pulses are applied (see Table 18.1 for electroporation parameters). After excision of the ectoplacental cone, the YS is dissected from the intra-embryonic compartment and placed in organ culture (Fig. 18.1b).

GFP can be visualized in electroporated YS after on average 3 h and is still detected in 7 dpc YS after 3 days in organ culture. The percentage of GFP positive

cells, analysed by flow cytometry, represents on average 0.5–1% of OrgD3-7 dpc YS, which corresponds to 50–100 cells per explant.

4.1.2 Viability/Tissue Organisation

After 3 days in organ culture, electroporated YS explants display the same morphology as control explants. They form a globular structure reminiscent of the *in vivo* YS structure, which is thicker at the pole that slightly adhere to the culture dish, where most haematopoietic cells are located, as ascertained previously by lmo2 and βH1 expression analyses (Giroux et al., 2007). Both electroporated explants and control YS develop a similar vascular network and clusters of red blood cells (Giroux et al., 2007). In electroporated explants, the vast majority of GFP^+ cells are usually found within the adhering site (Fig. 18.1c), which indicates an accurate targeting of the presumptive blood island.

4.1.3 Haematopoietic Precursor Targeting

Flow cytometry analysis of 7 dpc YS performed immediately after the 3 days in organ culture showed that most cells within the GFP^+ subset harbour haematopoietic markers such as c-kit, CD45 and Ter119. This indicates that mesodermal/pre-hematopoietic cells are successfully transduced and that their maturation proceeds normally during organ culture (Fig. 18.1d). Moreover, after 5 days in culture on OP9 stromal cells, sorted GFP^+ cells produce a haematopoietic progeny similar to control YS, namely $Ter119^+$ erythroid cells, $Mac\text{-}1^+/CD45^+$ macrophages, and few $c\text{-}kit^+/CD45^+$ haematopoietic precursors (Giroux et al., 2007). These differentiated cells no longer express the GFP after 4 days in culture, which gives an estimate of the duration of transduced gene expression.

Limiting dilution assay analyzed after 5 days on OP9 stromal cells established that the frequency of haematopoietic precursors in OrgD3-YS ranges from 1/450 to 1/500, which corresponds to about 10–15 precursors per YS. The electroporation procedure does not affect the recovery/viability of haematopoietic precursors, since similar numbers of haematopoietic precursors are obtained from control OrgD3-YS and OrgD3-YS electroporated without plasmid. The accurate targeting of the presumptive blood island using our *in situ* electroporation procedure is illustrated by the 3-fold enrichment in haematopoietic precursors frequency recovered from OrgD3-YS GFP^+ sorted cells (about 1/150), compared to control OrgD3-YS (Giroux et al., 2007) (Table 18.2).

However, at this development stage, the number of transduced mesodermal/pre-haematopoietic precursors is low (0.56 ± 0.18 per 0.5 YS-equivalent) and corresponds to about 1/10 of the precursors available for transduction in our unilateral electroporation procedure.

Table 18.2 *In situ* electroporation allows an accurate targeting of pre-haematopoietic mesoderm. The frequency of haematopoietic precursors from control explants (non-electroporated or electroporated without plasmid) and from GFP$^+$ cells sorted from electroporated explants is assessed using limiting dilution assays

	Precursor frequency	Number per Explant	Number of experiments
OrgD3-7 dpc YS			
Control	1/475	10–11	4
Electroporated without plasmid	1/453	10	3
GFP$^+$ cells	1/150 ± 57.62	0.56 ± 0.18 (1/2YS)	5

4.2 In Situ *Electroporation of 8 dpc Sp Mesoderm*

At 8 dpc, the Splanchnopleura (Sp), which associates the caudal endodermal and mesodermal layers, corresponds to the presumptive territory that will give rise to the region where HSC are generated, i.e. the para-aortic splanchnopleura at 9 dpc, then the AGM from 10 dpc. We previously showed that, while Sp anlagen is initially devoid of haematopoietic precursors, commitment toward a haematopoietic fate autonomously occurs in this territory, since cells that display all HSC features can be recovered from Sp-explants after 4 days in organ culture (Cumano et al., 1996, 2001). The developmental steps leading to haematopoietic determination from the extra-embryonic mesoderm begins to be unravelled thanks to the combined analyses performed during ES cell differentiation and during normal YS development. In contrast, very little is known about HSC determination from intra-embryonic mesoderm.

The transfer or modulation of candidate genes expression/activity, through *in situ* electroporation of Sp-explants appeared a promising approach to uncover the molecular mechanisms involved in HSC production from native mesoderm. However, plasmid expression of the gene of interest being transient, the number of GFP$^+$ available for characterisation after the 4 days in organ culture previously used was likely to be minimum.

Therefore, to assess for the feasibility of our *in situ* electroporation approach, we first analysed Sp-explants evolution during organ culture. To do so, we monitored cells expansion and haematopoietic precursors number at different time of organ culture.

Sp explants initially contain around 400 cells per explant. After 4 days in organ culture, this number increases up to about 6500 cells per Sp (Fig. 18.2a). To determine if haematopoietic precursors number increases in parallel to this expansion, we quantified these precursors at different time of organ culture by limiting dilution. After 2 days in culture, Sp-explants already contain 14 precursors, a number which further increases to reach around 80 per explant 1 day later. At 4 days in culture, precursors number decreases to 45 per Sp-explant (Fig. 18.2b). It appears from this study that haematopoietic precursors are present in Sp-explants as soon

as 2 days in organ culture, making them accessible to recovery and characterisation after *in situ* electroporation.

4.2.1 Electroporation Procedure and GFP Levels and Evolution

In order to target Sp cells before the connexion of the extra- and intra-embryonic vascular network, embryos are dissected before the 5-somite stage (Fig. 18.2c). After removal of the yolk sac, the amnion is slit and the embryo extended, with its back lying against the dish. The splanchnopleura is pulled away from the caudal part of the embryo up to the somite level. Sp explants, that harbour a horseshoe shape, typically encompass the caudal splanchnopleura and the lateral plate mesoderm and associated endoderm, as the innermost mesoderm, and the primitive streak region at early stages, remains attached to the embryo during the dissection process.

The embryo is placed so that it lies on its lateral side. The pEGFP plasmid solution, loaded with Fast Green to visualize construct deposition, is released between somatopleural and splanchnopleural layers, using a mouth-driven capillary. To electroporate the mesoderm layer, the electrodes (gold genetrodes 512 from BTX) are positioned parallel to the splanchnopleura so that the positive electrode faces the ventral side and pulses are applied. Then, the Sp explants are separated from the other embryonic structures and placed in organ culture (Fig. 18.2c).

4.2.2 Viability/Tissue Organisation

The evolution of electroporated Sp was systematically compared to non-electroporated control. After organ culture, both non-electroporated control and electroporated Sp-explants contain the same cell number, namely about 1500 cells per Sp-explants after 1 day in culture, between 1250 and 2600 cells per Sp-explants after 2 days in culture and between 2600 and 4200 cells, 1 day later. In our conditions, the expansion rate of transduced explants perfectly equals that of control Sp-explants (Fig. 18.2a), indicating that the electroporation process does not alter Sp-explant development during the organ-culture step.

A few hours after plating, Sp-explants start adhering to the culture dish. Upon organ culture, two distinct morphological evolutions may be observed. Sp explanted before the initiation of hindgut closure form a adherent layer that spreads with time, whereas Sp explanted after the beginning of somitic stages also harbour a central thickening which corresponds to the growing hindgut. Both non-electroporated control and electroporated Sp-explants similarly evolve during organ culture as both adhere to the plastic dish and further take on one of these structure organisations (Fig. 18.2d left panel). Both morphological examination and number cells count thus indicate a normal development and viability of electroporated Sp-explants during the organ culture step. Confocal microscopy analyses performed to characterise the

structure of OrgD2 Sp-explants indicates that they comprise a network of $CD31^+$ vascular network in close contact to the EpCAM-expressing endodermal layer. As shown in Fig. 18.2e, the electroporation process does not modify this tissue organisation.

GFP^+ cells can be visualized in electroporated Sp during the 3 days of organ culture. The percentage of GFP positive cells represents about 0.47% of Sp-explants cells after 1 day in organ culture. It reaches 1.02% at OrgD2 and then decreases to 0.07% 24 h later (Fig. 18.2d right panel). Taking into account the number of cells per Sp-explants, these percentages correspond respectively to approximately 8, 22 and 3 cells of the whole OrgD1, D2 and D3-Sp. Therefore, even while the highest number of haematopoietic precursors is obtained after 3 days of culture, as GFP expression levels drastically decreases between the second and third day of organ culture, we chose to further characterise electroporated Sp after 2 days in organ culture.

In order to determine if the electroporation process impairs the viability and further differentiation of haematopoietic precursors, we compared the precursor frequency in control and electroporated OrgD2-Sp by culture in limiting dilution on OP9 stromal cells for 7 days. In control OrgD2-Sp, an average 1 out of 102 cells give rise to a haematopoietic progeny, which corresponds to about 18.7 ± 1.3 precursors per Sp. Similar precursor numbers (13.8 ± 2.9) are obtained from electroporated OrgD2-Sp, indicating that the electroporation procedure does not significantly affect the recovery of haematopoietic precursors.

4.2.3 Haematopoietic Precursor Targeting

We next investigated whether *in situ* electroporation also efficiently targets Sp haematopoietic precursors. Haematopoietic precursors can be characterised by the expression of CD31 and CD41 markers (Bertrand et al., 2005b). In both non electroporated and electroporated OrgD2-Sp, $CD31^+CD41^+$ haematopoietic precursors represent around 3–5% of the whole population. These $CD31^+CD41^+$ haematopoietic precursors are two fold enriched in electroporated OrgD2-Sp GFP^+ subset compared to the whole population. This enrichment indicates the mesodermal layer, which will give rise to haematopoietic precursors is accurately targeted during the *in situ* electroporation step (Fig. 18.2f).

We next attempted to quantify the recovery of haematopoietic precursors derived from transduced mesodermal cells. Sorted GFP^+ cells were distributed at 1 to 10 cells per well on OP9 stromal cells and scored for the presence of a haematopoietic progeny 7 days later. In six out of the fourteen experiments performed, in which 120–360 GFP^+ cells were seeded, 1 or 2 wells contained haematopoietic cells. The recovered GFP^+ haematopoietic cells give rise to the multipotent progeny typical of intra-embryonic precursors as it includes Ter 119^+ erythroid cells, $Mac1^+$ myeloid cells and $CD19^+$ B-lymphoid cells.

However, the poor recovery of haematopoietic precursors which results from the low efficacy of transduction in this system coupled to the transduction of irrelevant

cell types, implies that *in situ* electroporation conditions need to be further improved so that we can functionally assess gene function during the determination of intra-embryonic HSC.

5 Comments

We here show that *in situ* electroporation may be used to target mesodermal/pre-hematopoietic precursors of both the extra- and intra-embryonic haemogenic sites. Furthermore, the process does not impair precursors viability and further haematopoietic commitment and differentiation upon organ culture. Indeed, the progeny typical of the YS and intra-embryonic haemogenic site is obtained from GFP^+ sorted cells. Our protocol ensures respectively three and two folds haematopoietic cells enrichments in transduced cells from YS and Sp-explants. However, our transduction approach obviously requires to be improved quantitatively for both sites, as the number of targeted precursors is extremely low (about 1 transfected mesodermal/pre-haematopoietic precursors per YS and even less for Sp explants). This poor outcome results from combined limitations:

1. The percentage of GFP^+ cells recovered from both haemogenic sites is low (0.5–1%), albeit consistent with those usually obtained following *in situ* electroporation of mouse embryonic tissues (Nakamura et al., 2002; Quinlan et al., 2006). It corresponds to 50–100 cells per OrgD3-YS and even less (about 10) for OrgD2 Sp-explants;
2. Only half of the YS can be targeted using our unilateral electroporation procedure, and unfortunately, the reiteration of pulse application with an inverted field, aimed to increase the targeted area, is unsuccessful as a decreased viability prevails over the increased GFP^+ cells recovery;
3. The number of haematopoietic precursors recovered from control haemogenic sites after the organ culture step is low (about 10 for OrgD3-YS and about 15 for OrgD2 Sp-explants). The quantity of precursors that develop from YS and Sp-explants seems somehow reduced compared to normal embryos of older stages. We here show that, in control Sp explants, the number of haematopoietic precursors culminates at about 80 per Sp at Org D3, whereas the maximal number of HSC (an average 100 per AGM) is reached *in vivo* at 10.5 dpc (Godin et al., 1999). This comparison suggests that our organ culture set up does not fully recapitulates normal embryonic development.

A better characterisation of haematopoietic development of extra- and intra-embryonic presumptive territories in organ culture may, beside improving our understanding of the early steps of haematopoietic commitment, especially in the intra-embryonic compartment for which no data is currently available, lead to an increased efficiency our *in situ* electroporation protocols. Indeed, we recently evidenced a limiting factor in the *in vitro* development of 7 dpc YS in culture. The modification of our organ culture condition leading to an explant development

closer to the *in vivo* situation also led to a three fold increase in the number of precursors recovered from control explants. These preliminary data open a way to improve *in situ* electroporation of early haemogenic sites, on one hand by increasing the number of targeted cells, and on the other hands, by performing gene function analyses in conditions that better mimic the development *in situ*.

Up to now, *in situ* electroporation constitutes the only possible gene transfer method to transduce mesodermal/pre-hematopoietic precursors within their environment and thus analyze the earliest steps of hematopoietic development.

Retrovirus-mediated gene transfer onto YS cells isolated from older embryos has been successfully implemented (Giroux et al., 2007; Kyba, et al., 2002), but proved inefficient to target 7 dpc YS-explants (Giroux et al., 2007), as well as Sp-explants. This may due to transcriptional inactivation of proviral particles, as shown during retroviral transduction of mouse embryonic stem cells (mESC) (Zaehres and Daley, 2006). In contrast, lentiviral vectors have been used successfully to express transgenes in mESC and in their *in vitro* differentiated progeny. Indeed, our preliminary results indicate that, when OrgD1-Sp-explants are exposed to lentiviral supernatant during 24 h, numerous GFP^+ cells may be observed in the explants. These results, which need do be further documented regarding the impact on the quantity and quality of transduced cells, provide an alternative approach to analyse the effects of candidate genes during haematopoietic determination. However, this protocol will not allow controlling spatio-temporal delivery of transgene, nor the transient expression of candidate genes, likely to mimic that occurring during normal development. Consequently, *in situ* electroporation of haemogenic sites deserves to be improved, in order to functionally approach the earliest stages of haematopoietic development.

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Chapter 19
Electroporation of Embryonic Kidney Explants

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1 Introduction

Metanephric kidney development in the mouse begins at embryonic day (E) 10.5, when the ureteric bud (UB), an outgrowth of the epithelial nephric duct, invades the neighboring metanephric mesenchyme (MM). The ureteric bud then undergoes a series of branching events to form the collecting duct network of the adult kidney (Fig. 19.1). As each ureteric bud tip forms, the adjacent undifferentiated mesenchyme is induced to epithelialize and form a nephron, the functional unit of the adult kidney that filters waste. Rodent embryonic kidneys can be dissected and cultured as explants such that branching morphogenesis and nephrogenesis can be observed *ex vivo* (Rothenpieler and Dressler, 1993; Vega et al., 1996; Piscione et al., 1997; Gupta et al., 2003).

The roles of signaling molecules and transcription factors during kidney development have been studied in explant cultures by introducing small interfering RNA (Davies et al., 2004), by treating with pharmaceutical agents (Tang et al., 2002), or by using viral transduction to overexpress mutant proteins (Favre et al., 2000). More recently, microinjection followed by electroporation has been used to successfully express plasmid DNA encoding proteins important for ureteric bud induction, branching morphogenesis, and nephrogenesis in the developing kidney (Gao et al., 2005; Self et al., 2006; Alie et al., 2007). This approach allows for both

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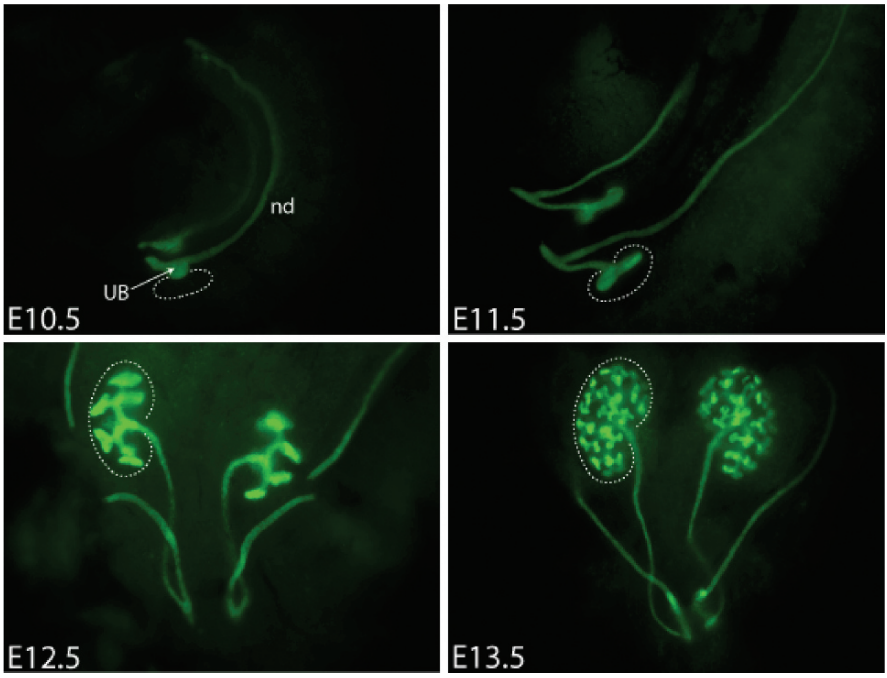

Fig. 19.1 Early stages of mouse metanephric kidney development used in microinjection and electroporation experiments. The *Hoxb7/GFP*$^{+/-}$ transgenic mouse can be used to monitor the morphological changes undergone by the ureteric bud during branching. The *Hoxb7* promoter drives the expression of green fluorescent protein (GFP) within the nephric duct (nd), the ureteric bud (UB), and the branched derivatives. The metanephric kidney arises at E10.5 (*top panel*, *left*) when the UB emerges from the nephric duct and invades the neighboring metanephric mesenchyme (MM), demarcated by the dotted white line. As a result of reciprocal signaling and induction by the adjacent MM, the UB proceeds to elongate and divide repeatedly in a process termed branching morphogenesis (*bottom panel*, *left and right*). The undifferentiated mesenchymal cells surrounding each UB tip undergo mesenchymal to epithelial transformation (MET), which is the first step in nephrogenesis. Microinjection and electroporation has been used to study the process of UB budding at E10.5–11.5 (*top row*) as well as nephrogenesis and branching from E12.5–14 (*bottom row*)

gain-of-function and loss-of-function experiments in a high-throughput, cost-effective manner. Tissue-specific effects can also be analyzed: DNA constructs can be delivered to either the metanephric mesenchyme or the ureteric bud lineages to evaluate the primary effects of genetic perturbation within a target cell population. This method can be used to evaluate the function of any gene of interest to determine whether it is then feasible to invest in the creation of more labour-intensive conditional knock-out or knock-in mouse lines. From expression profiling technologies such as microarrays and SAGE, a large number of genes have been implicated in kidney development (Meyer et al., 2004; Schmidt-Ott et al., 2005), yet for most of these genes, their function in organogenesis remains uncharacterized. Microinjection with electroporation therefore provides an economical means to rapidly evaluate the function of previously described genes, and to characterize the role of genes more recently implicated in kidney development.

2 Procedure

The method is based on microinjection of a DNA construct into specific regions of the developing kidney. The kidney is then electroporated by exposing the tissue to an electric field. This permeabilizes cell membranes by creating pores that permit DNA constructs to enter targeted cells.

2.1 Mouse Embryonic Kidney Cultures

Timed-pregnant CD1 mice (Charles River Laboratories, Wilmington, MA, USA) were sacrificed at embryonic day (E) 12, 13, or 14 to retrieve the embryos. *Hoxb7/GFP*$^{+/-}$ males (kindly provided by F. Costantini) were mated with CD1 females to obtain heterozygous offspring that expressed green fluorescent protein (GFP) in the ureteric bud and its branched derivatives. The mouse studies were performed in accordance with the rules and regulations of the Canadian Council of Animal Care. Using a M5A stereomicroscope (Wild Leitz®, Willowdale, ON, Canada), metanephric kidneys were dissected from the embryos, suspended in 20 µl of ViaSpan® (Barr Pharmaceuticals, Pomona, NY, USA), and cooled to 4°C prior to and during microinjection by placing them on a stage with a cooling system (20/20 Technology Inc., model BC-100, USA).

2.2 DNA Constructs

The expression vectors used in these experiments were pEGFP-C1 (Clontech Laboratories Inc., Palo Alto, CA, USA), containing enhanced green fluorescent protein (EGFP) as a reporter tag, and pcDNA3 containing mCherry red fluorescent protein (RFP) as a reporter tag. In both vectors, the human cytomegalovirus (CMV) promoter drives gene expression.

2.3 Microinjection and Electroporation

Microinjection was performed using an inverted microscope (Leitz Labovert FS, Foster City, CA, USA) with Nomarksi differential interference optics and a Leitz® micromanipulator. Microinjection needles were prepared from Pyrex® capillary tubing containing Omega Dot fiber (FHC Inc., Bowdoinham, ME, USA) and were pulled using the KOPF 720 needle puller (Narashige, Tokyo, Japan) and cut at the tips. Glass needles were connected via plastic tubing to an IM 300 microinjector (Narishige) which delivered the DNA expression vector at a concentration of either 2 or 5 µg/µl diluted in ddH_2O. The DNA was mixed with 1% FastGreen FCF dye (Fisher Scientific Inc., Ottawa, ON, Canada) in PBS in a 50:1 ratio. Each kidney was injected at a single site, receiving an average total volume of 0.025 µl. To

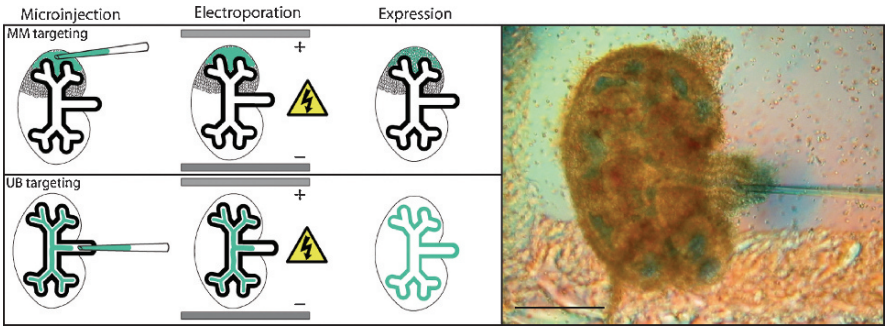

Fig. 19.2 The method of microinjection and electroporation in embryonic kidney explants. To target cells of the metanephric mesenchyme (MM) (*left panel*, *top row*), the needle is inserted in the cortex of the kidney and DNA is injected into a single injection site. After electroporation, the transgene is expressed within the region of injection. For ureteric bud (UB) targeting (*left panel*, *bottom row*), the needle is inserted into the lumen of the severed ureter to ensure that the plasmid DNA is dispersed throughout the branching UB. Microinjection of DNA and FastGreen dye into the ureter of an embryonic day 12 kidney is shown (*right panel*). The dye is seen dispersed throughout the ureteric bud and its branches. Scale bar: 0.5 mm

target the metanephric mesenchyme, the needle was inserted into the cortex of the explanted kidney (Fig. 19.2). To target cells of the ureteric bud, the needle was inserted into the lumen of the ureter such that after microinjection, the DNA solution was dispersed throughout the branching derivatives of the ureteric bud (Fig. 19.2). Following microinjection, kidneys were placed on a platinum plate Petri dish-base electrode (CUY701-P2E, Protech International Inc., San Antonio, TX, USA) and covered with an L-shaped platinum plate covered electrode (CUY701-P2L, Protech International). A BTX square wave electroporator (ECM830, Genetronics Inc., San Diego, CA, USA) was used to deliver five pulses of 175 V for 75 µs with a 1 s delay between pulses. Following microinjection and electroporation, kidney explants were grown on 0.4 µm pore size PET track-etched membranes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in DMEM/F-12 with 10% FBS and 0.1% penicillin/streptomycin (Wisent Inc., St.Bruno, QC, Canada). Explants were cultured in six-well plates at 37°C for up to 96 h.

3 Application and Results

3.1 Establishing Parameters for Microinjection and Electroporation

Most electroporation protocols in mammalian tissues have used conditions that were originally described in the chick consisting of a low voltage of 25 V applied in 1–5 pulses of 50 ms each, which is considered a long pulse time (Nakamura

and Funahashi, 2001). Microinjection and electroporation of the anterior tibialis muscle of adult mice has demonstrated that the parameters of high voltage, 900 V, combined with a short pulse, 100 µs, lead to higher and more sustained transgene expression (Vicat et al., 2000). Therefore, we screened a range of electroporation parameters to identify the optimal conditions for widespread gene expression and to minimize tissue necrosis in mouse embryonic kidney explants collected from E12–14 (Alie et al., 2007). After microinjecting the expression vector pEGFP-C1, we showed that kidney explants electroporated with a high voltage (250 V) and a long pulse time (100 ms) had high levels of EGFP expression, yet grew poorly with excessive cell death. Kidneys electroporated using a low voltage (25 V) and a long pulse time (50 ms) grew as well in culture as non-electroporated kidneys, however EGFP expression was significantly lower and more focal than in kidneys electroporated with a high voltage. The number of pulses was also important for gene expression: kidneys that received at least five pulses displayed the highest EGFP expression. The conditions for electroporation were therefore established such that kidneys were electroporated with five short pulses, 75 µs each, using a high voltage of 175 V. Kidneys that were microinjected and electroporated with these parameters grew well and underwent branching morphogenesis and nephrogenesis. The kidneys expressed the reporter gene as early as 2 h after electroporation, and maintained high expression for 96 h in culture.

3.2 Tissue-Specific Expression

Adjusting the site of microinjection allowed us to target either the metanephric mesenchyme or the ureteric bud within the developing kidney (Figs. 19.2 and 19.3). Peak levels of reporter gene expression, mCherry RFP, were detected 24 h after electroporation within the metanephric mesenchyme (Fig. 19.3). The metanephric mesenchyme was successfully targeted 95% of the time (n = 19/20 explants), by manually inserting the needle within the explant. The ureteric bud injections required more precision and were successfully targeted 75% (n = 30/40) of the time. Prior to microinjection, the ureter was severed at its connection to the renal pelvis, generating a short ureteral stump. Plasmid DNA was injected directly into the lumen of the cut ureter using a micromanipulator which facilitated the distribution of DNA throughout the branching ureteric bud via the lumen of the renal pelvis (Fig. 19.2). E14 kidneys were examined 24 h later, and reporter gene expression was detected within the ureteric bud branching network of the kidney explant (Fig. 19.3). We were also able to successfully target the metanephric mesenchyme or the ureteric bud when E12 or E13 kidneys were microinjected and electroporated (Fig. 19.3).

Localized gene expression after microinjection and electroporation allows researchers to compare a transgene's effect in regions of expression versus regions of no expression within the same kidney (Fig. 19.3). In this way, each kidney serves as its own control. Alternatively, two different kidneys with similar

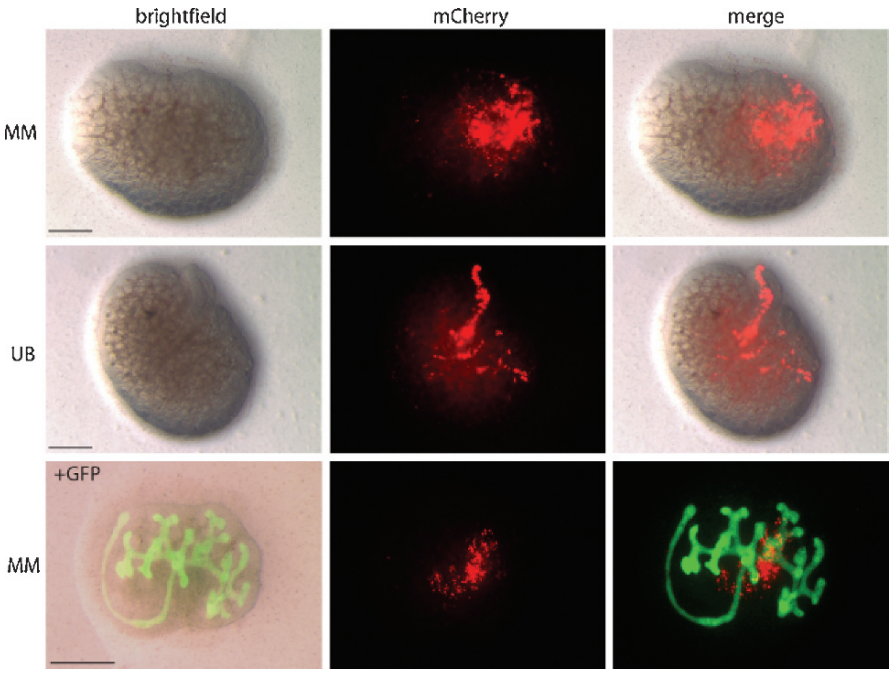

Fig. 19.3 Tissue-specific expression is achieved by targeted microinjection and electroporation of DNA constructs into different cell populations within mouse embryonic kidney explants. Embryonic day (E) 14 kidneys were dissected and then microinjected in either the metanephric mesenchyme (MM, *top and bottom rows*) or ureteric bud (UB, *middle row*) lineages with a plasmid encoding the mCherry RFP (red fluorescent protein) fluorochrome, and subsequently electroporated. Peak tissue-specific expression in the explants was detected after 24 h in culture. Kidneys derived from the *Hoxb7/GFP*$^{+/-}$ transgenic mouse line can also be used for microinjection and electroporation experiments (*bottom row*). An E12 GFP-expressing kidney was microinjected in the MM with mCherry RFP and then electroporated. The *Hoxb7/GFP*$^{+/-}$ explants can be used to monitor primary effects on UB branching by targeting the UB itself or the MM. Scale bar: 0.5 mm

amounts of fluorochrome expression can be paired for comparative analyses (Alie et al., 2007). Kidneys can be analyzed as whole mounts or they can be fixed and cryosectioned.

4 Comments

Microinjection and electroporation has been applied to a number of embryonic stages beginning at E10.5 when the UB first emerges, and continuing up to E14 when nephrogenesis is well established. Kidney explants taken at later stages of development are more difficult to study because they fail to grow adequately in culture. Several measures that have been shown to be beneficial in other sys-

tems were applied to this model to reduce tissue necrosis and improve transgene expression.

During dissection, kidney explants were suspended in ViaSpan®, a solution originally designed to preserve organs that have been harvested for transplantation. When cells are suspended in this solution and then electroporated with DNA, there is less toxicity (Baron et al., 2000). ViaSpan is enriched in potassium and magnesium, and this may reduce the loss of these ions from the intracellular compartment during electroporation, thereby reducing cytotoxicity.

When electroporation is performed at 4°C, cell viability is enhanced and the duration of cell permeabilization is increased, permitting greater DNA entry and higher expression (Rols et al., 1994). We therefore performed microinjection and electroporation at 4°C to minimize cytotoxicity and improve gene expression. Expression levels of reporter genes have also been enhanced when plasmid DNA is linearized prior to electroporation (Laursen et al., 1994). We investigated this, but found no significant difference in expression when linearized as opposed to circularized DNA was microinjected and electroporated in embryonic kidney explants (data not shown).

Although our current protocol leads to high levels of gene expression with minimal toxicity, additional measures may be beneficial. Transgene expression may be significantly increased if microinjected kidneys are exposed sequentially to two different electric fields: the first one containing a high voltage with a short pulse time followed by the second one containing a low voltage and long pulse time (Bureau et al., 2000). The initial high voltage permits a greater cell surface area to be permeabilized, while the subsequent long pulse time allows for increased DNA transport across the membrane (Satkauskas et al., 2002). Gene transfer and expression is also enhanced by increasing the proportion of the cell membrane that interacts with DNA. DNA travels in the direction of the anode when an electric field is applied. The area of the cell membrane that interacts with DNA can be increased when electric pulses are applied in different orientations and polarities (Phez et al., 2005). It is therefore possible to augment transgene expression in mouse kidney explants if the orientation of the kidneys is changed during electroporation, or if the polarities of the paddle and plate electrodes are reversed between pulses. Treatment with the enzyme hyaluronidase, which is known to degrade components of the extracellular matrix (ECM), has been shown to improve the expression levels of plasmid DNA injected into muscle fibers *in vivo* (McMahon et al., 2001). Degradation of the ECM enhanced cellular exposure to plasmid DNA and permitted the use of lower voltages which decreased cytotoxicity (Mennuni et al., 2002). It is possible that pre-treating kidney explants with this enzyme may also improve transgene expression and permit the use of lower voltages.

In our experiments, reporter gene expression began to weaken as early as 48 h post-electroporation, and was significantly diminished after five days in culture (Alie et al., 2007,). This may be due to the high level of cell proliferation and differentiation taking place in the developing kidney. Microinjection and electroporation of embryonic mouse brain *in utero* has shown that this technique can generate long-term expression, up to 6 weeks after treatment, when transfected

DNA is not diluted by cell division (Saito and Nakatsuji, 2001). Longer transgene expression may therefore be possible if terminally differentiated cells are targeted within kidneys at later stages of development (Tsujie et al., 2001; Takabatake et al., 2005).

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Chapter 20
Transfer of Foreign DNA into Aquatic Animals by Electroporation

Thomas T. Chen, Maria J. Chen, Tzu-Ting Chiou, and J. K. Lu

1 Introduction

Aquatic animals into which a foreign gene or a non-coding DNA fragment is artificially introduced and integrated in their genomes are called *transgenic aquatic animals*. Since 1985, a wide range of transgenic aquatic animal species have been produced mainly by microinjecting or electroporating homologous or heterologous transgenes into newly fertilized or unfertilized eggs and sometimes, sperm (for review, Chen and Powers, 1990; Hackett, 1993; Chiou et al., 2005). To produce a desired transgenic aquatic animal species, several factors should be considered. First, could the reproduction cycle of the aquatic animal species under consideration be completed in captivity? Second, a specific gene construct must be designed based on the special requirements of each study. For example, the gene construct may contain an open reading frame encoding a gene product of interest and regulatory elements that regulate the expression of the gene in a temporal, spatial and/or developmental manner. Third, an efficient method for delivering the transgene construct needs to be identified. Fourth, since not all instances of gene transfer are efficient, a screening method must be adopted for identifying transgenic individuals.

Since the development of the first transgenic fish in the mid 1980s, techniques of producing transgenic aquatic animals have improved tremendously. Among different methods for delivering gene constructs into aquatic animals, the electroporation method is considered to be the simplest but efficient method of gene delivery. In recent years, transgenic aquatic animals have been produced as valuable models for different disciplines of biological research as well as human disease modeling. In addition, transgenic technology has been used to produce aquatic animal species with beneficial traits, such as enhanced somatic growth and disease resistance, for

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aquaculture application. In this chapter, we will review the progress of producing transgenic aquatic animals by the electroporation method.

2 Principle

Electroporation is a successful method for transferring foreign DNA into bacteria, yeast, and plant and animal cells in culture (Neumann et al., 1982; Potter et al., 1984; Shigekawa and Dower, 1988). This method has become popular for transferring foreign genes into embryos or sperm of aquatic organisms in the past few years (Powers et al., 1992; Lu et al., 1996; Sin et al., 2000; Lu et al., 2002; Chiou et al., 2005 for review). Electroporation utilizes a series of short electrical pulses to induce formation of short-lived pores on the phospholipid bi-layer of the cell membrane, thereby permitting the entry of DNA molecules into cells (e.g., embryos or sperm), and once the electrical pulses are diminished, the pores on the cell membrane will be re-sealed rapidly (Tieleman, 2004). The patterns of electrical pulses can be emitted as a single pulse of exponential decay form (i.e., exponential decay generator) or high frequency multiple peaks of square waves (i.e., square wave generator). Figure 20.1 shows the commercially available exponential decay generator and square wave generator.

3 Procedure

While higher voltage can be delivered to samples by an exponential decay generator, the high heat produced by the generator can kill most of the electroporated samples. The advantage of using a square wave generator is that, though the generator delivers multiple short waves at high voltages, it produces low temperatures, thus facilitates the transfer of DNA molecules across the cell membrane without killing the cells or embryos. Therefore, more scientists have routinely adopted the square wave generator for delivering DNA into cells or embryos. More recently, a hand-held electrode probe has been developed by the BTX Company which allows delivery of square wave pulses to cells or tissues *in situ* (Fig. 20.1c). Similar designs have been reported by scientists to conduct *in situ* electroporation under a microscope (Rambabu et al., 2005; Hendricks and Jesuthan, 2007; Huang et al., 2007).

The basic parameters for electroporating foreign DNA into newly fertilized fish eggs are summarized in Table 20.1. Studies conducted in our laboratory (Lu et al., 1992; Powers et al., 1992) and those of others (Buono and Linser, 1992; Sin et al., 2000; Lu et al., 2002) have shown that the rate of DNA integration in electroporated fish embryos is on the order of 20% or higher in the survivors. Although the overall rate of DNA integration in transgenic fish produced by electroporation was equal to or slightly lower than that by microinjection, the actual amount of time required for handling a large number of embryos by electroporation is in orders of magnitude less than that required for microinjection. Several reports

Fig. 20.1 Commercially available electropors. (**a**) Exponential decay generator (Cell-Proator) from BRL, (**b**) a square wave generator from BTX, (**c**) hand-held electrode attach to the square wave generator from BTX

Table 20.1 Parameters of transferring foreign DNA into newly fertilized fish eggs by electroporation

Parameters	
Developmental stage	1 to 2 cell stage
Size of foreign DNA	<10 Kb
DNA concentration	100 mg ml^{-1}
DNA topology	Linear
Chorion barrier	Intact chorion
Electrical field strength	500 to 3000 V
Pulse pattern	Exponential / square
Pulse duration	Millisecond to second
Temperature	Room temperature
Medium	Phosphate buffered saline

have appeared in the literature describing successful transfer of foreign DNA into fish by electroporating sperm instead of embryos (Symonds et al., 1994; Tseng et al., 1994; Sin et al., 2000; Lu et al., 2002). An important factor of consideration in electroporating DNA into sperm of aquatic animals is to maintain the sperm in un-activated state while conducting electroporation, since addition of DNA solution into sperm can frequently lead to activation of the sperm. Once the sperm is activated, it can only remain active for less than 1 min, and after that the sperm is no longer able to fertilize eggs. Fortunately, sperm of most aquatic animals can remain un-activated even after addition of four volumes of PBS (130 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3), therefore, PBS has been used as a common sperm extender when electroporating sperm. With the success seen in electroporating DNA into sperm or eggs, this technique is considered as an efficient and versatile mass gene transfer technology.

4 Application and Results

The technique of electroporation has been used to transfer foreign DNA into newly fertilized eggs of medaka and zebrafish with good success. In an effort to transfer foreign DNA harboring a cecropin B or cecropin P cDNA into newly fertilized eggs of medaka in a Beckon 2000 electroporator (a square wave generator, conditions of electroporation: pulse frequency, 2^6; burst time, 0.4 s; cycle number, 5; pulse width, 160 μS; distance of electrode from surface to the buffer, 1 mm; voltage, 2.5 to 5.0 kV), Sarmasik et al. (2002) showed that about 50% of the electroporated eggs hatched, and of those about 5–10% of the animals were shown to carry the transgene at 4 months of age. In a series of separate studies, using the same conditions reported by Sarmasik et al. (2002), Chun and Chen (2004) reported that about 28% of the survived medaka fish that were electroporated at one-cell embryonic stage with a transgene containing rainbow trout IGF-I E-peptide cDNA contained the transgene. Although foreign DNA can also be introduced into zebrafish embryos by electroporation, the rate of success of DNA transfer is far less than that by microinjection. Using the electroporation conditions reported by Sarmasik et al. (2002) with a voltage setting of 4 kV to transfer foreign DNA into newly fertilized zebrafish eggs, Chun et al. (2006) reported that about 4.8% of the hatched fish carry the transgene, which is about five folds less efficient than by microinjection. From these results, it is clear that microinjection is a superior technique for introducing foreign DNA into zebrafish embryos than electroporation.

Foreign DNA can be routinely transferred into various fish species by electroporating the sperm instead of newly fertilized eggs (Symonds et al., 1994; Tseng et al., 1994; Lu et al., 1996, 2002; Sin et al., 2000). As mentioned earlier, fish sperm, in the presence of un-diluted seminal fluid, remains in the un-activated state. Upon dilution with a buffer or water, the sperm will be activated and remains active for about 30 s, and after that the sperm is no longer active, and is unable to fertilize eggs. In order to conduct electroporation in sperm, a suitable sperm extender that

prevents sperm to be activated after addition of the DNA solution needs to be identified. Fortunately, sperm of most aquatic animals can be maintained un-activated even after addition with four volumes of PBS (130 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3); thus PBS has been used as a common sperm extender for electroporation. Chiou et al. (in preparation) in our laboratory have attempted to introduce insect cecropin transgenes into rainbow trout sperm in order to produce strains of transgenic rainbow trout that confer resistance to infection by bacterial and viral pathogens. Figure 20.2 outlines the procedure of electroporating the cecropin transgene into rainbow trout sperm and the production of transgenic rainbow trout. About 300 µl of the undiluted sperm (~10^{10} sperm/ml) were mixed with 600 µl of DNA in a PBS buffer to give a ratio of 10^4 DNA molecules/sperm, the mixture was electroporated in a BRL's "Cell-Porator Electroporation System" under the following conditions: capacitance, 110 µF; voltage, 250–300 V; pulse number, 2. Under these conditions, about 50% of the sperm can be activated after electroporation. Results of PCR analysis of the genomic DNA isolated from the fin tissue of fish at 6 months of age showed that about 10–20% of the survived animals contain the cecropin transgene (Fig. 20.3). To date, we have established about nine transgenic families that exhibit resistance to infection by the bacterial pathogen, *Aeromonas salmonicida* and IHNV.

Like in insects, sperm produced by male crustacea are stored in a spermatophore, and upon copulation, the spermatophore is transferred from males to the reproduc-

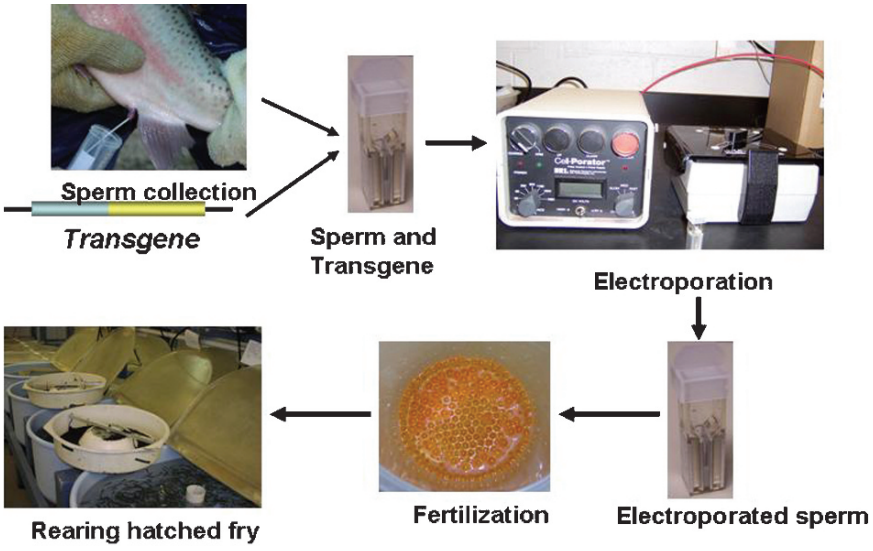

Fig. 20.2 Flow chart of producing transgenic rainbow trout by electroporating the transgene into sperm. Sperm is collected in a dry tube, diluted with sperm extender, mixed with transgene DNA to give 10^4 DNA molecules/sperm, and electroporated a Cell Proator under the following conditions: capacitance, 110 µF; voltage, 250–300 V; pulse number, 2. The electroporated sperm is used to fertilize rainbow trout and rear the hatched fry to adult hood

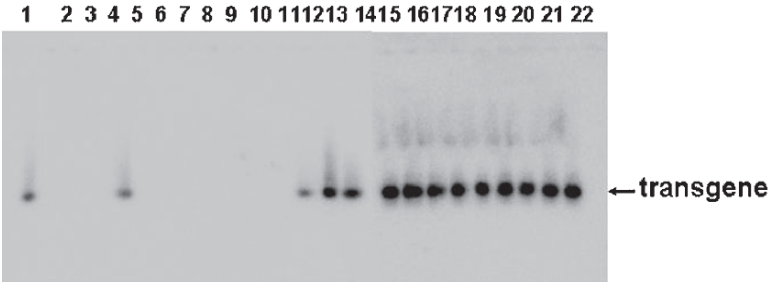

Fig. 20.3 Identification of transgene in the presumptive transgenic individuals. DNA samples were isolated from presumptive transgenic individuals and the presence of the transgene in the DNA samples were determined by PCR analysis using synthetic oligonucleotides specific to the transgene as amplification primer. The PCR products were resolved on 1.5% agarose gels by electrophoresis. DNA samples that show amplification of the transgene are from transgenic individuals

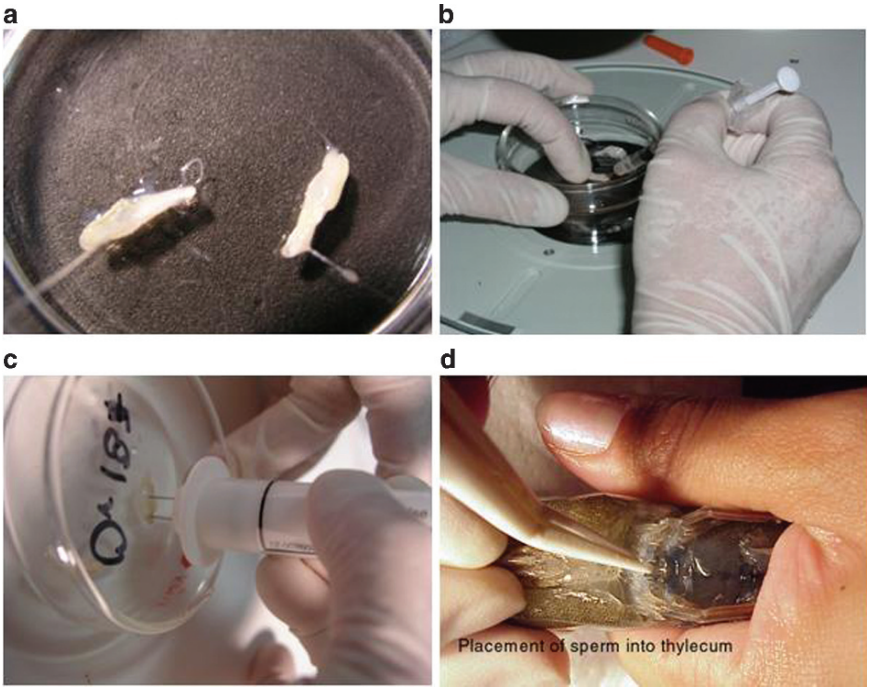

Fig. 20.4 Scheme of producing transgenic American white shrimp via electroporating transgene into spermatophore. (**a**) Intact spermatophores extracted from mature male shrimp, (**b**) injection of DNA into spermatophore, (**c**) electroporating foreign DNA into sperm, (**d**) extracting sperm from the electroporated spermatophore and inserted into the thylecum of gravid females

tive tract of females to fertilize eggs. By the time of spawning, the embryos are already developed into multiple cell stages, which are unsuitable for transferring foreign DNA into embryos by direct electroporation. However, we have found that, by using a hand-held electrode devise developed by BTX Company (Fig. 20.1c), the

sperm in the spermatophore can be electroporated with foreign DNA. Figure 20.4 depicts the procedures that were used to transfer insect cecropin cDNA into the American white shrimp, *Lipopenaeus vannamei*, via electroporating the sperm in the spermatophore. In this procedure, the intact mature spermatophore was extracted from males, injected with cecropin cDNA (20 µg of linearized DNA in a final volume of 20 µl) and electroporated in a BTX Square Wave Porter (conditions: voltage, 800 V; pulse length, 0.1 mS; pulse #, 2; pulse interval, 0.1 S). The sperm from the electroporated spermatophore was squeezed out with pair of forceps and transferred to the thylecum of the gravid females. Results of PCR analysis of the genomic DNA isolated from pleopods of 8 months old shrimp showed that about 30% of the survived animals contain the cecropin transgene (Fig. 20.5) under these electroporation conditions. We have also shown that electroporating the spermatophore that had been inserted in the thylecum of female tiger shrimp with the same device also resulted in successful transfer of the cecropin transgene. The same device can also be used to *in situ* electroporating foreign DNA into skeletal muscle cells in various aquatic animals.

Recently Hendricks and Jesuthan (2007) developed a micro-electroprating probe for *in situ* electroporation of foreign DNA into whole-mount of zebrafish brains. As described by Hendricks and Jesuthan (2007), this devise can easily electroporate brains of 100 zebrafish embryos in 1 h with no or little lethality and consistently high levels of transgene expression in numerous cells, and the transfected zebrafish brain cells are amenable to *in vivo* time laps imaging. Furthermore, with little modification of the designing of the electrodes, this device has also been

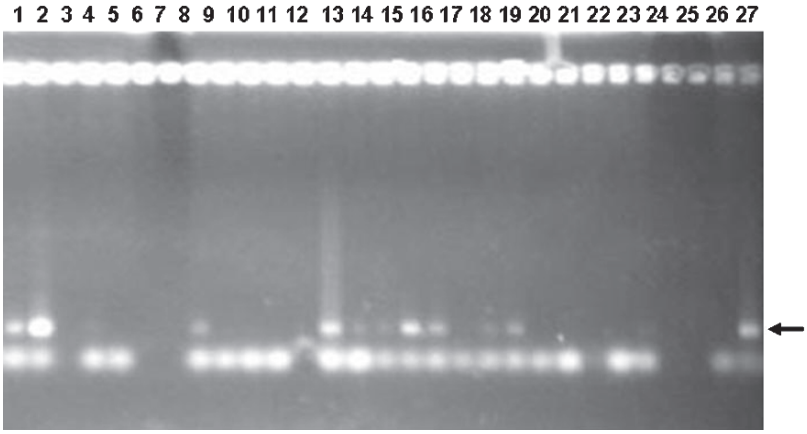

Fig. 20.5 Identification transgene in the presumptive transgenic shrimp. DNA samples were isolated from pleopods of presumptive transgenic individuals, transgenic amplified by PCR using synthetic oligonucleotides as amplification primers, and analyzed by electrophoresis on agarose gels (1.5%). *Arrow* indicates the amplified transgene. DNA samples that show amplification of the transgene are from transgenic individuals

used in *in situ* electroporation of foreign DNA, RNA and morpholinos into various zebrafish tissues with good success (Rambabu et al., 2005; Cerda et al., 2006; Thummel et al., 2006; Huang et al., 2007).

5 Conclusion

Transfer of foreign DNA into aquatic animals can be routinely achieved by electroporating DNA into sperm, one-cell stage embryos or somatic cells by using a square wave generator. Although the efficiency of DNA transfer by electroporation is similar to or slightly lower than the DNA transfer efficiency by microinjection, the time required for transferring a large number samples by electroporation is far shorter than that by microinjection. Based on studies conducted in our own laboratory and those in other laboratories, not only is the electroporation technique an efficient method of transferring foreign DNA into various fish species, it also is an excellent method of introducing foreign DNA into various aquatic invertebrate species such as oysters, clams and shrimp. With the use of a hand-held electrode probe or a micro-electrode probe, the technique can also be used in *in situ* to transfer foreign DNA into tissues or cells. Therefore, electroporation is considered as an efficient but simple mass gene transfer technology for producing transgenic aquatic animals.

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Chapter 21
Electroporation in the Regenerating Tail of the *Xenopus* Tadpole

Makoto Mochii and Yuka Taniguchi

1 Introduction

Xenopus laevis is a model system widely used to investigate embryogenesis, metamorphosis, and regeneration. The tail of the *Xenopus* tadpole is very useful in analyzing the molecular mechanisms underlying appendage regeneration (Slack et al., 2004; Mochii et al., 2007; Slack et al., 2008). It is transparent and suitable for whole-mount observation at the cellular level. The tail regenerates within 2 weeks of amputation. The conventional injection of blastomeres with mRNA, DNA, or antisense oligonucleotides is a powerful tool with which to study genetic mechanisms in early embryos, but it is not effective in late embryos or larvae. A transgenic approach has been used to analyze tail regeneration (Beck et al., 2003, 2006), but its success is largely dependent on the activity of the promoter used. There are limited numbers of promoters available that precisely regulate gene expression spatially and/or temporally. In vivo electroporation is an alternative method that can be used to manipulate gene expression in late embryos and larvae. The introduction of DNA or RNA into the cells of neurula and tailbud embryos has been reported (Eide et al., 2000; Sasagawa et al., 2002; Falk et al., 2007). Targeting larval tissues with in vivo electroporation also has been used to investigate neural networks, metamorphosis, and regeneration (Haas et al., 2001, 2002; Nakajima and Yaoita, 2003; Javaherian and Cline, 2005; Bestman et al., 2006; Boorse et al., 2006; Lin et al., 2007; Mochii et al., 2007). In this chapter, we report a procedure to introduce DNA into the tissues of the tadpole tail.

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2 Procedure

2.1 Animals

Xenopus laevis larvae were maintained at 22°C in 0.25% NaCl. The developmental stages were determined according to Nieuwkoop and Faber (1994). Tadpoles at stage 48 were anesthetized in 0.03% MS222 (Sigma) before the electroporation procedure or tail amputation.

2.2 Plasmids

The expression plasmid pCAX–AFP encodes a mutant form of green fluorescent protein (GFP) (Inouye et al., 1997) driven by the CAG promoter (Niwa et al., 1991). Plasmid DNA was prepared from an *Escherichia coli* culture with an alkaline sodium dodecyl sulfate method, followed by CsCl gradient centrifugation (Sambrook et al., 1989), and then resuspended in water at 2 µg/µL.

2.3 Injection of DNA

A glass capillary (3-000-203-G/X; Drummond) was pulled using a vertical pipette puller (Model PB-7; Narishige), and the tip was sharpened (tip inner diameter, 15–20 µm). The anesthetized tadpole was placed on a wet Kimwipe. The DNA solution containing 0.05% fast green was injected with a Nanoject II injector (Drummond). To label a single or a few cells, 2.3 µL of DNA was injected. The injection volume was increased to label more cells. The injected tadpole was rinsed in 0.1 × Marc's modified Ringer's solution (MMR) (100 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes buffer, pH 7.5) and then transferred to an electroporation dish, as described below. We used two dissecting microscopes, one equipped with an injector and the other used for the electroporation procedure.

2.4 Electroporation

A copper-plate anode (2 × 2 cm^2) was fixed on a plastic petri dish (6 cm in diameter) with hot melted plastic using a glue gun (Fig. 21.1). The anode was covered with a piece of paper towel (2 × 2 cm^2) saturated with 0.1 × MMR. The anesthetized and DNA-injected tadpole was placed on the paper towel and then covered with another piece of 0.1 × MMR-saturated paper towel (1 × 1 cm^2). The cathode (platinum stick or copper disk) was gently touched to the site on the paper towel covering the injected region, and electrical pulses were applied using an ECM

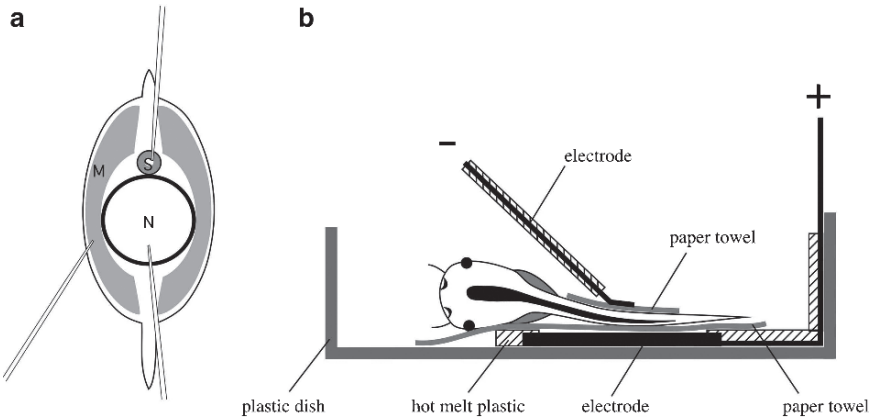


Fig. 21.1 Schematic representation of the electroporation procedure in the *Xenopus* tadpole tail. (**a**) Illustration showing the cross section of the tadpole tail and the micropipettes. To inject DNA into the muscle tissue, the pipette was inserted from the lateral side of the tail. DNA was injected from the dorsal and ventral sides to target the spinal cord and the notochord, respectively. S, spinal cord; N, notochord; M, muscle. (**b**) The anesthetized and DNA-injected tadpole was sandwiched between two pieces of 0.1 × MMR-saturated paper towel on the anode. The cathode was placed at the site on the paper towel covering the DNA-injected region. After pulses had been administered, the tadpole was rapidly transferred to the recovery solution

830 electroporator (BTX). Under the standard conditions, 10 pulses are applied at 30 V with a 5-ms pulse length at 100-ms intervals. After the pulses, the tadpole was rapidly transferred to 0.25% NaCl containing penicillin (50 U/mL) and streptomycin (50 µg/mL).

2.5 Observation

GFP expression was monitored under fluorescent light using an SZ12 dissecting microscope (Olympus). The electroporated tadpole was anesthetized and photographed with a digital camera system (DP50; Olympus).

2.6 Tail Amputation

The anesthetized tadpole was placed on a wet Kimwipe. The tail was amputated vertically using a razor (FA-10; Feather). To amputate the tail after electroporation, the tail was cut at a site slightly caudal to the GFP-labeled region under fluorescent light. The amputated tadpoles were allowed to recover and were maintained in 0.25% NaCl containing penicillin and streptomycin without feeding for 3 days and with feeding thereafter.

3 Application and Results

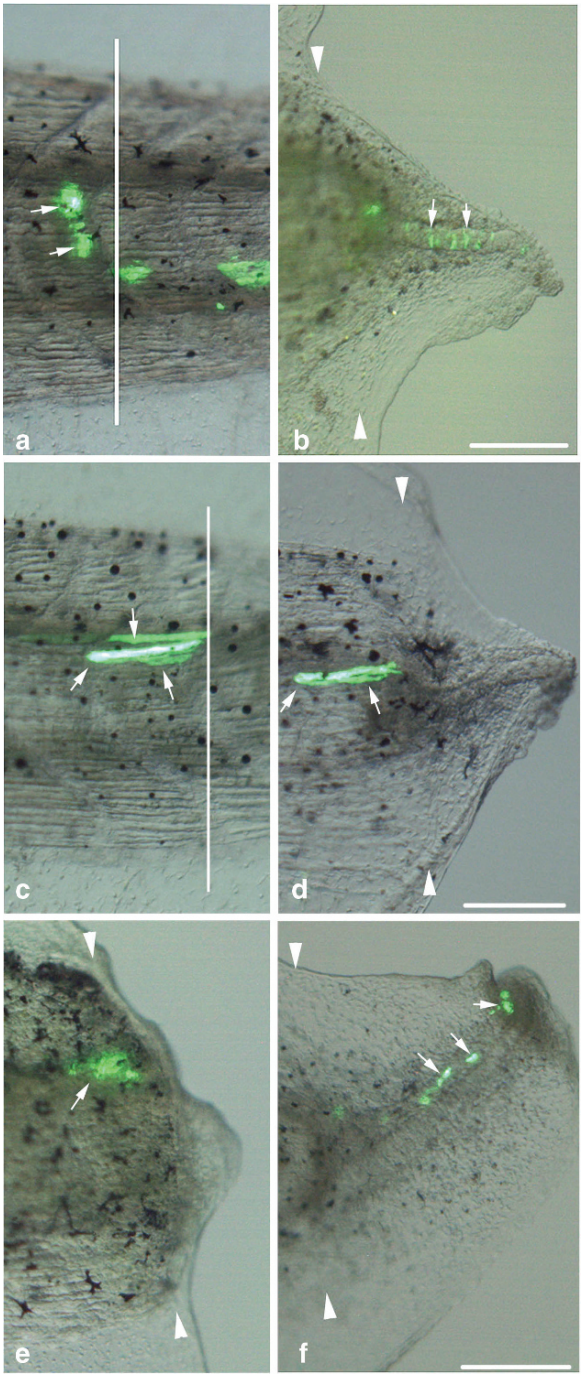
3.1 Summary of Tail Regeneration in Xenopus laevis

The major structures observed in the *Xenopus* tadpole tail are the notochord, the spinal cord, and muscle tissues (Fig. 21.1a). The notochord is an axial structure located in the center of the larval tail. It consists of large vacuolated cells and small cells, both of which are surrounded by a thick collagenous sheath. The spinal cord and a pair of sensory ganglia are located on the dorsal side of the notochord. Bilateral muscle masses cover the central notochord and the spinal cord. Most of the *Xenopus* tadpole tail regenerates after amputation. Within 2 days, notochord precursor cells accumulate to create a compact cell mass adjacent to the edge of the amputated notochord sheath. They proliferate and align in the proximal-to-distal direction to form an immature notochord by day 3 after amputation. The regenerating notochord cells finally differentiate into large vacuolated cells in the proximal region. The amputated spinal cord creates a terminal vesicle at its proximal end and then elongates caudally along the growing notochord. Myogenic cells accumulate in the mesenchymal region of the regenerating tail and differentiate into myofibers. We introduced plasmid DNA encoding GFP into the spinal cord, the notochord, and the muscle mass with an electroporation technique and traced the labeled cells after tail amputation.

3.2 Gene Transfer to the Notochord

To introduce the GFP plasmid into the notochord, a micropipette was used to prick the ventral side of the tail adjacent to the ventral fin. It then was inserted into the notochord through the ventral mesenchymal region, where neither spinal cord nor muscle tissues are located (Fig. 21.1a). The tip of the micropipette was sharpened because the notochord sheath sometimes prevents its insertion. The DNA solution containing fast green then was injected into the notochord. The high pressure within the notochord sheath (Adams et al., 1990) can interrupt DNA injection. Even in such cases, the DNA can be introduced into the notochord by retracting the inserted micropipette slowly. The injected tadpole was placed on a 0.1 × MMR-saturated paper towel covering the anode. The DNA-injected region, delineated with fast green dye, was covered with another piece of paper towel saturated with

Fig. 21.2 Lineage analysis during tail regeneration. *Arrows* in (**a**) indicate GFP-labeled notochord cells before amputation. (**b**) The same tadpole shown in (**a**) on day 4 after amputation. All the GFP-labeled cells were found in the regenerating notochord. *Arrows* in (**c**) indicate three muscle fibers labeled with GFP. (**d**) The same tadpole shown in (**c**) on day 5 after amputation. Two labeled cells were unchanged, whereas the other cell had degenerated and was not visible. (**e**, **f**) Spinal cord cells were electroporated after amputation and observed on day 2 (**e**) and day 7 (**f**). All the labeled cells were found in the regenerating spinal cord. The *white line* and the pair of *arrowheads* indicate the amputation planes. The dorsal side is up and the anterior side is to the left. Bar, 250 μm. From Mochii et al. (2007)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0.1 × MMR, which was touched with a platinum cathode to transmit the pulses (Fig. 21.1b). GFP fluorescence was first detectable after 12–24 h when we used the pCAX–AFP plasmid. In most cases, only the small notochord cells were labeled (Fig. 21.2a), although the large vacuolated cells also were labeled with faint fluorescence in some cases (data not shown). The labeling efficiency of the notochord cells was lower than that of the spinal cord or muscle cells, described below. The GFP-labeled small notochord cells had migrated caudally, proliferated, and differentiated in the regenerating notochord on day 4 after amputation (Fig. 21.2b).

3.3 Gene Transfer to Muscle

To target the muscle tissues, the DNA solution was injected into the segmented muscle mass from the lateral side of the tail (Fig. 21.1a). GFP-labeled myofibers with an elongated morphology were visible 12–24 h after electroporation (Fig. 21.2c). Some myofibers were labeled with GFP even in tadpoles that had been injected with DNA but had not been pulsed. The DNA may have been incorporated into damaged myofibers during their recovery process. The labeled myofibers degenerated within 24 h if they were injured by the amputation, whereas they did not change when the tail was amputated at a point distant from the labeled myofibers (Fig. 21.2d). No GFP fluorescence was observed in the newly formed myofibers that had been shown to originate from the muscle satellite cells but not from preexisting myofibers (Gargioli and Slack, 2004). Therefore, we think that the DNA was not introduced into the satellite cells under our experimental conditions. Alternatively, the GFP fluorescence may have been diluted to below the level of detection after multiple rounds of proliferation by the myogenic cells.

3.4 Gene Transfer to the Spinal Cord

To target the spinal cord, electroporation can be performed either before or after tail amputation. In the latter case, the tail was amputated first and the micropipette then inserted into the lumen of the spinal cord from the amputation surface, as described for the axolotl tail (Echeverri and Tanaka, 2002). If electroporation was performed before the amputation, the micropipette was inserted into the lumen of the spinal cord from the dorsal side of the intact tadpole tail, through the dorsal mesenchymal space between the muscle masses (Fig. 21.1a). GFP fluorescence in the spinal cord was first detectable after 12–24 h (Fig. 21.2e). The labeled cells moved caudally and proliferated in the spinal cord, which continued to elongate in the regenerating tail (Fig. 21.2f). Insertion of the micropipette into the amputated stump was much easier than insertion into the intact tail. However, the distance between the GFP-labeled region and the amputation plane was not precisely controlled in the former

case. Cutting the tail under fluorescent light after electroporation allowed more precise regulation of this distance.

4 Comments

The three major tissues in the *Xenopus* tadpole tail were labeled with GFP using this electroporation technique, and were traced during the regeneration process after tail amputation. Mesenchymal and/or epidermal cells adjacent to the targeted tissues were also labeled in some experiments, and the type of labeled cell could be identified by its shape and location in most cases. The simultaneous introduction of the *GFP* gene and a gene of interest should allow us to test the function of that gene product in the regeneration process. Morpholino oligonucleotides also should be effective in knocking down the expression of genes of interest, and already have been used to study tail regeneration in the axolotl (Schnapp and Tanaka, 2005) and fin regeneration in the zebrafish (Thummel et al., 2006).

The introduction of materials into large areas of target tissues is required to test their effects at the organ or tissue level. Increasing the injected DNA or using a disk-type cathode increased the number of labeled cells after electroporation (data not shown), although further modifications may be required to manipulate most of the cells in the tail.

In routine experiments, we observed GFP fluorescence a week after tail amputation. However, the GFP signals decreased day by day after a prolonged period or multiple rounds of proliferation by the labeled cells. Cotransfection of the GFP reporter and GAL4–VP16 constructs will enhance the level of GFP, permitting prolonged observation, as reported for the spinal cord regeneration of the axolotl (Echeverri and Tanaka, 2002). Alternatively, electroporation coupled to transposon analysis may overcome this problem. Coelectroporation of a transposon vector containing the *GFP* gene and a transposase-expressing plasmid resulted in the integration of the marker gene in planaria and in chicken embryos (González-Estévez et al., 2003; Sato et al., 2007). If these systems work in the *Xenopus* tadpole, we will be able to label cells stably for long periods.

Under our pulsing conditions, most of the tadpoles recovered and showed normal regeneration, whereas some damaged tadpoles died within several days. The electrical shock itself may influence cell behavior during regeneration. A higher voltage or repeated pulses often caused the tail to break at the pulsed site without cutting (data not shown). Atkinson et al. (2006) reported that the application of an electric field induces a dedifferentiation response, indistinguishable from the one that occurs following the amputation of newt appendages. Therefore, we must carefully evaluate the results of experiments using the electroporation technique.

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Chapter 22
Electrotransfer of Plasmid Vector DNA into Muscle

Satsuki Miyazaki and Jun-ichi Miyazaki

1 Introduction

Wolff et al. (1990) first reported that plasmid DNA injected into skeletal muscle is taken up by muscle cells and the genes in the plasmid are expressed for more than two months thereafter, although the transfected DNA does not usually undergo chromosomal integration (Wolff et al., 1991, 1992). However, the relatively low expression levels attained by this method have hampered its applications for uses other than as a DNA vaccine (Davis et al., 1995). There are a number of reports analyzing the conditions that affect the efficiency of gene transfer by intramuscular DNA injection and assessing the fine structures of expression plasmid vectors that may affect expression levels (Davis et al., 1993; Liang et al., 1996; Norman et al., 1997). Furthermore, various attempts were done to improve the efficiency of gene transfer by intramuscular DNA injection. Consequently, regenerating muscle was shown to produce 80-fold or more protein than did normal muscle, following injection of an expression plasmid. Muscle regeneration was induced by treatment with cardiotoxin or bupivacaine (Wells, 1993; Vitadello et al., 1994). We previously demonstrated that by combining a strong promoter and bupivacaine pretreatment intramuscular injection of an IL-5 expression plasmid results in IL-5 production in muscle at a level sufficient to induce marked proliferation of eosinophils in the bone marrow and eosinophil infiltration of various organs (Tokui et al., 1997). It was also reported that a single intramuscular injection of an erythropoietin expression plasmid produced physiologically significant elevations in serum erythropoietin levels and increased hematocrits in adult mice (Tripathy et al., 1996). Hematocrits in these animals remained elevated at >60% for at least 90 days after a single injection. However, improvements to this method have not been sufficient to extend its applications including clinical use.

Electroporation has long been used for DNA transfection of cells *in vitro* and it implies a physical process which exposes cells to a brief, high voltage that induces temporary poration to the cell membrane, allowing the influx of large molecules

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such as plasmid DNA. The entry of plasmid directly into cytoplasm may bypass the endosome–lysosome pathway, reducing the degree of DNA degradation. Its initial application *in vivo* was to transfer DNA into liver cells (Heller et al., 1996) and chick embryos (Muramatsu et al., 1997). We tried *in vivo* electroporation in skeletal muscle of mice by low voltage rectangular pulses, which had been successfully applied in chick embryos *in ovo* (Muramatsu et al., 1997; Funahashi et al., 1999). By applying electroporation, naked plasmid DNA can be delivered several hundred-fold more efficiently to muscle cells *in vivo* (Aihara & Miyazaki, 1998; Mir et al., 1999; Rizzuto et al., 1999). The resulting expression levels are considered sufficient to warrant further investigation of this method for human gene therapy for various diseases (Maruyama et al., 2000).

In vivo electroporation is now also called electrotransfer and has been tested in a broad range of target tissues and organs including for example: cardiac tissue (Harrison et al., 1998), bladder (Harimoto et al., 1998), carotid artery (Matsumoto et al., 2001), skin (Maruyama et al., 2001), brain (Inoue & Krumlauf, 2001), cornea (Blair-Parks et al., 2002), retinal ganglion cells (Dezawa et al., 2002), spinal cord (Lin et al., 2002) and tendon (Jayankura et al., 2003). Among these tissues and organs, skeletal muscle is the most widely targeted tissue for electrotransfer. Progressive improvements have also made electrotransfer the most efficient approach among non-viral and viral gene delivery systems in skeletal muscle. Skeletal muscle constitutes approximately 30% of the adult body mass and is easily accessible for various gene delivery approaches. Skeletal muscle has an abundant blood vascular supply. Thus, the major advantage of electrotransfer into muscle lies in its potential for the efficient transport system for the secreted protein into the systemic circulation. Skeletal muscle fibers are terminally differentiated cells and individual fibers are thought to persist for the lifetime. This nature may also support long-term expression of transgene.

Gene transfer by electroporation, which uses plasmid DNA as the vector, has several advantages over transfer using viral vectors. A large quantity of highly purified plasmid DNA is easily and inexpensively obtained. Gene transfer can be repeated without apparent immunological responses to the DNA vector. Although the gene expression is usually transient, there is less likelihood of recombination events with the cellular genome, eliminating the risk of insertional mutagenesis that is associated with the use of viral vectors. Since there are fewer size constraints than with current viral vectors, plasmid vectors can carry larger genes. It may also be possible to transfer a mixture of two or more different plasmid constructs into muscle by electroporation. Finally, new DNA plasmid constructs can be rapidly made and tested. Furthermore, gene transfer by electroporation also has advantages over other methods of gene transfer using nonviral vectors. Intramuscular electrotransfer strongly decreases the interindividual variability in expression usually observed after plasmid DNA injection into muscle (Mir et al., 1999). Moreover, plasmid DNA can be readily used without any modification, such as conjugation with liposomes.

Here we describe the protocol of *in vivo* electrotransfer of plasmid DNA into tibialis anterior muscles of adult mice. We demonstrate the expression of the *lacZ* genes in the muscle.

2 Materials

2.1 Experimental Animals

- Mice. We used eight-week-old female C57BL/6J mice purchased from CLEA Japan (Tokyo, Japan). Mice of other ages or strains or rats may be treated similarly.
- Anesthetic. 50 mg/ml pentobarbital sodium solution (Nembutal™; Abbott Lab., Illinois) was diluted to 6 mg/ml with a diluent containing 40% (v/v) propylene glycol and 10.5% (v/v) ethanol.

2.2 Plasmid DNA

The plasmid vector must include an expression unit that is active in striated muscles (*see* Comment 1). We have successfully used the pCAGGS vector (Niwa et al., 1991). To assess the efficiency of gene transfer, the following two constructs were convenient: pCAGGS-IL-5 (Tokui et al., 1997) and pCAGGS-lacZ (Niwa et al., 1991), which were constructed by inserting mouse IL-5 cDNA and the *E. coli lacZ* gene, respectively, into the unique *Eco*RI site between the CAG promoter and a 3′-flanking sequence of the rabbit ß-globin gene of pCAGGS. These plasmid vectors can be provided by J. M. upon request.

The pCAGGS plasmid is based on pUC13, a high copy number plasmid, and is easily grown in *E. coli* HB101, DH10B or other strains. Plasmid DNA is extracted by the alkaline lysis method, and purified using a plasmid DNA purification kit (PureLink™ HiPure Plasmid DNA Purification Kits; Invitrogen, Carlsbad, CA) according to the supplier's instructions (*see* Comment 2). Plasmid DNA is further purified by phenol and phenol/chloroform extraction, and ethanol precipitation. DNA is dissolved in pure water and its quantity and quality are assessed by optical density at 260 and 280 nm (*see* Comment 3). Prior to injection, DNA is diluted to its final concentration, 1–1.5 µg/µl in PBS (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). Because the salt concentration seems to affect the efficiency of gene transfer, the final DNA solution is made by adding 1 vol. of 10 x PBS to 9 vol. of DNA solution diluted with water.

2.3 Intramuscular DNA Injection and Electroporation

- Insulin syringe with a 27-G needle.
- Electrodes consisting of a pair of stainless steel needles, 5 mm in length and 0.4 mm in diameter, fixed with a distance (gap) between them of 5 mm (Fig. 22.1). These electrodes can be obtained from NEPA Gene (Chiba, Japan).
- Electric pulse generator (CUY21EDIT; NEPA Gene), which can produce square waves, i.e., the voltage remains constant during the pulse duration.

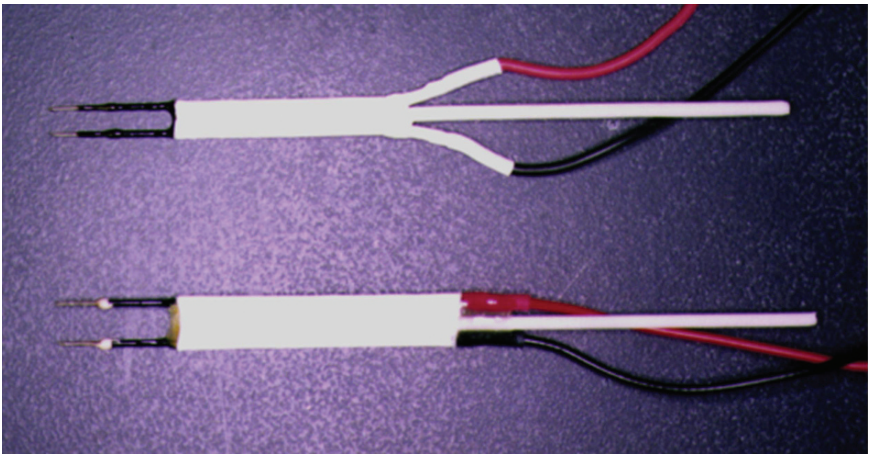

Fig. 22.1 Appearance of electrodes consisting of a pair of stainless steel needles, 5 mm in length and 0.4 mm in diameter, fixed with a distance (gap) between them of 3 mm (*upper*) or 5 mm (*lower*)

2.4 Assessment of the Efficiency of Gene Transfer

- Purified preparations of pCAGGS-IL-5 and pCAGGS-lacZ plasmid DNA at a concentration of 1.5 μg/μl in PBS.
- Murine IL-5 ELISA kit (Endogen, Woburn, MA).
- 4% Paraformaldehyde in PBS.
- 40 mM X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in dimethylsulfoxide (DMSO). This is diluted to 1 mM in PBS before use for staining.
- O.C.T. compound (Miles Inc., Elkhart, IL).
- Dry ice-acetone.
- Cryostat.
- Slide glasses coated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO).
- 1.5% Glutaraldehyde in PBS.
- Eosin.

3 Procedure

In the following section, we describe the method of gene transfer into tibialis anterior muscles of adult mice by *in vivo* electroporation. Some modifications will be required for application of this method to other muscles or other species (Vicat et al., 2000).

3.1 Intramuscular DNA Injection

1. Anesthetize mice by intraperitoneal injection of 0.1 ml/g body weight of 6 mg/ml pentobarbital sodium solution.
2. Inject the tibialis anterior muscles with 50 µg of purified closed circular plasmid DNA at 1.5 µg/µl in PBS using an insulin syringe with a 27-G needle (Fig. 22.2, left panel) (*see* Comments 4 and 5).

3.2 Electroporation In Vivo

1. Insert a pair of electrode needles into the muscle to a depth of 5 mm to encompass the DNA injection sites (Fig. 22.2, right panel) (*see* Comment 6). Monitor the resistance value. If the value is 1–2 k ohm, it shows that the electrodes are correctly inserted into the muscle. Otherwise, change the insertion site of the electrodes.
2. Deliver electric pulses using an electric pulse generator (*see* Comment 7). Three 50-ms-long pulses of the indicated voltage (50–100 V) followed by three more pulses of the opposite polarity are administered to each injection site at a rate of one pulse per sec.

3.3 Assessment of the Efficiency of Gene Transfer

Before introducing the gene of interest by electroporation, it is important to test the effectiveness of the experimental procedures using a positive control. This may be

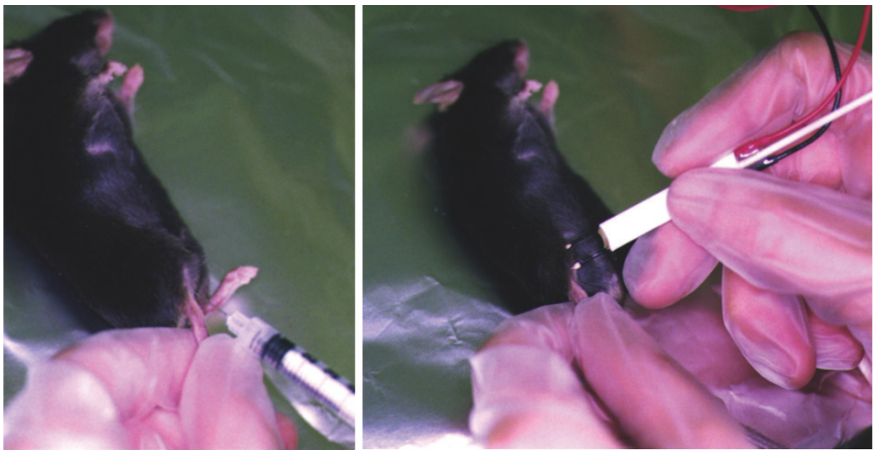

Fig. 22.2 Insertion of electrodes. A pair of electrode needles are inserted into the right tibialis anterior muscle to a depth of 5 mm to encompass the DNA injection sites

done using a plasmid that expresses some cytokine, e.g. IL-5, or β-galactosidase (*see* Comment 8).

3.3.1 IL-5 Expression

1. Inject the bilateral tibialis anterior muscles of anesthetized mice with 50 μg each of pCAGGS-IL-5 plasmid DNA at a concentration of 1.5 μg/μl in PBS, and deliver electric pulses at 100 V, as described above.
2. Five days after injection, obtain serum samples from the tail vein of the mice.
3. Assay the serum samples for IL-5 using an ELISA kit (Endogen, Woburn, MA), according to the supplier's instructions.

3.3.2 β-Galactosidase Expression

1. Inject the bilateral tibialis anterior muscles of anesthetized mice with 50 μg each of pCAGGS-lacZ plasmid DNA at a concentration of 1.5 μg/μl in PBS, and deliver electric pulses at 100 V as described as above.
2. Four or five days after injection, sacrifice the mice.
3. Fix the tibialis anterior muscles in cold 4% paraformaldehyde in PBS for 3 h, then wash in PBS for 1 h.
4. To detect *E. coli* β-galactosidase activity in whole muscle, stain the muscle sample at 37°C for 18 h in the presence of 1 mM X-gal (Fig. 22.3, left panel). Otherwise, go to the next step.
5. For transverse sections, embed the muscle in O.C.T. compound and freeze in dry ice-acetone.

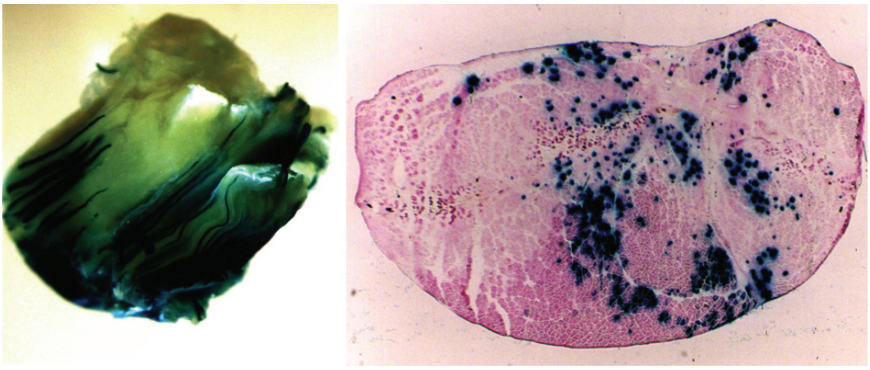

Fig. 22.3 Histochemical staining for β-galactosidase activity in the muscle after gene transfer of pCAGGS-lacZ DNA with electropulsation. The tibialis anterior muscle was injected with 50 μg of pCAGGS-lacZ plasmid DNA and treated with electric pulses of 100 V. Five days later, the muscle was excised. Transverse sections of the muscle sample were stained for β-galactosidase activity, and counterstained with eosin. Original magnification, × 40

6. Slice serial sections (15-μm thick) with a cryostat and place on slide glasses coated with 3-aminopropyltriethoxysilane.
7. Fix the slices in 1.5% glutaraldehyde for 10 min at room temperature, then wash three times in PBS.
8. Incubate the samples at 37°C for 3 h in the presence of 1 mM X-gal.
9. Counterstain the muscle sections with eosin.
10. Observe the sections with a microscope for X-gal staining (Fig. 22.3, right panel).

4 Application and Results

Electroporation-mediated gene transfer has been used effectively in the muscles of mouse, rat, rabbit, and monkey (Mir et al., 1999), and it has also been applied to gene transfer into cardiac muscle (Harrison et al., 1998). Thus, this method should have broad applications in physiological and pharmacological studies using experimental animals. It is likely that further improvement of this method will provide a new approach to gene therapy for human diseases. Among the potentially treatable human diseases are various autoimmune diseases, chronic inflammatory disorders, infections, malignancies, and acquired or inherited serum protein deficiencies. In addition to its potential use in gene therapy, *in vivo* electroporation provides also a powerful laboratory tool to study *in vivo* gene expression and function in muscle (Konig et al., 2002).

Immunogenic proteins expressed *in vivo* from plasmid DNA injected into muscle were shown to induce an immune response to them. This strategy of vaccination represents an attractive alternative to other methods of vaccination. The use of intramuscular injection of naked DNA encoding for an immunogenic protein that is called DNA vaccine is very effective in mice, but it needs to be improved in humans. In order to improve uptake of DNA by cells, electrotransfer was tested in mice, rabbit and guinea pigs, with hepatitis B surface antigen and HIV gag DNA vaccines (Widera et al., 2000). The results showed an increased antigen expression that led to rapid and high levels of production of antibodies.

4.1 In Vivo Electroporation

We investigated the applicability of *in vivo* electroporation for gene transfer into muscle, using plasmid DNA carrying the gene for IL-5, which is involved in the growth and differentiation of B cells and eosinophils (Takatsu, 1992). Fifty micrograms each of a control plasmid or an IL-5 expression plasmid were injected into the bilateral tibialis anterior muscles of mice. A pair of electrode needles were inserted into the muscle to encompass the DNA injection sites, and electric pulses were delivered using an electric pulse generator. A total of six 50-ms-long pulses of

50 V each were delivered to each injection site at a rate of one pulse per sec. Five days later, the serum IL-5 levels were measured by ELISA. In the mice injected with control plasmid, the IL-5 levels were below the detection limit of the assay (< 10 pg/ml), irrespective of the administration of electric pulses. The IL-5 levels in the mice injected with the IL-5 expression plasmid were 0.2 ng/ml without electropulsation, but 12 ng/ml with electropulsation. Histochemical analysis of muscles injected with a lacZ expression plasmid showed that *in vivo* electroporation markedly increased both the number of muscle fibers taking up plasmid DNA and the copy number of plasmids introduced into muscle cells. These results clearly indicate that *in vivo* electroporation is a much more effective means of introducing DNA into muscle than is simple intramuscular DNA injection (Aihara & Miyazaki, 1998). We also demonstrated that the muscle-targeted transfer of an erythropoietin expression plasmid into rats by *in vivo* electroporation produced significant elevations in serum erythropoietin levels and increased hematocrits (Maruyama et al., 2000).

4.2 Parameters Affecting the Efficiency of Gene Transfer into Muscle by Electroporation

Although a proposal exists that electropores are created by electric pulses, there are few data that describe the structural effects of electropermeabilization. The transmembrane potential difference at 200–250 mV is believed to lead to transient permeabilization of the membrane, which results in the exchange of molecules across the membrane. Conditions affecting the efficiency of gene transfer have been studied in detail for *in vitro* electroporation (Wolf et al., 1994). We examined various conditions for *in vivo* electroporation in muscle. To optimize the voltage of the electric pulses used for electroporation *in vivo*, we compared the serum IL-5 levels of mice subjected to electroporation at various electrode voltages. In this experiment, the pulse length (50 ms), number of pulses (six pulses), amount of DNA injected (50 µg per site), and DNA concentration (1.5 µg/µl in saline), which could all affect the efficiency of gene transfer, were fixed. Immediately after DNA injection, electrode needles with a 5 mm gap were inserted to encompass the DNA injection sites and electric pulses were administered at different voltages. Five days later, serum IL-5 levels were measured. As shown in Fig. 22.4, the serum IL-5 levels increased nearly proportionally with the voltage up to 100 V. However, administration of voltages higher than 100 V resulted in a marked decrease of the serum IL-5 levels, probably because of damage to the muscle cells. Optimal gene expression was achieved at 100 V, resulting in a serum IL-5 concentration of 25 ng/ml. The serum IL-5 concentration at 0 V was < 0.1 ng/ml. Therefore, gene transfer by electroporation *in vivo* is more efficient than the traditional method of intramuscular DNA injection by more than two orders of magnitude.

We examined the effect of different pulse length, number of pulses, DNA concentration, volume of injection fluid, and solution types on the efficiency of gene

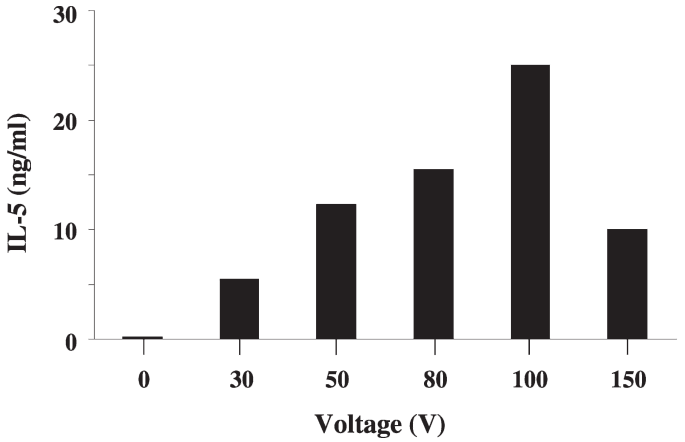

Fig. 22.4 Voltage dependence of the efficiency of gene transfer by electroporation *in vivo*. The bilateral tibialis anterior muscles were injected with 50 µg each of pCAGGS-IL-5 plasmid DNA. Electric pulses of the indicated voltages were delivered to the DNA injection site. Solid bars indicate serum IL-5 levels 5 days after electroporation. Each value represents the mean IL-5 concentration from three mice

transfer by electroporation. The optimal conditions of those tested were as follows: pulse length, 50 ms; number of pulses, 6; DNA concentration, > 0.5 µg/µl; DNA amount, > 50 µg/site; solution type, PBS. In these experiments, electric pulses were applied to the muscle immediately after DNA injection. However, our study showed that electric pulses may be applied up to 30 min after DNA injection without affecting the efficiency of gene expression. It should be noted that the optimal conditions for *in vivo* electroporation will be very different if this method is applied to other animals. Optimizing these parameters should permit the efficiency of gene transfer in other species to be improved. Vicat et al. (2000) showed that electroporation with high-voltage and short-pulse (900 V/100 µs) currents provides high-level and long-lasting gene expression in muscle. It will be necessary to study the relation between the conditions for *in vivo* electroporation and the time course of gene expression in detail.

We next examined whether the direction of the electric field relative to that of muscle fibers affects the efficiency of gene transfer. After intramuscular DNA injection, electric pulses of 60 V were delivered either in a longitudinal or a transverse direction relative to the muscle fibers. To fit the tibialis anterior muscles between a pair of electrodes, we used electrodes with a 3 mm gap. Five days later, serum IL-5 levels were measured to evaluate the efficiency of DNA transfer. There were no significant differences between electric fields that were applied longitudinal (31 ng/ml) or transverse (40 ng/ml) to the muscle fibers. However, before this topic is closed, the following issues must be considered. Because muscle cells are oblong, the number of cells lying between the electrodes is much larger in a transverse orientation than in a longitudinal orientation. Therefore, more cells should be

transfected in a transverse orientation. However, a higher voltage might be required for efficient electroporation in this orientation, since there are more cell membranes to be permeabilized between the electrodes. Further studies are required to determine how the direction of the electric field affects the efficiency of gene transfer. Because the tibialis anterior muscles of mice are small and it is difficult to insert a pair of electrodes into them in a transverse orientation, electric pulses were delivered in a longitudinal direction in the other experiments.

4.3 Duration of Expression

The time course of gene expression by electroporation *in vivo* was determined by following the serum IL-5 levels after electroporation at 100 V. As shown in Fig. 22.5, the serum IL-5 levels reached a maximum 5–7 days after electroporation and gradually decreased thereafter, followed by a decrease to approximately 10% of the peak value by three weeks after electroporation. We also examined the duration of expression when a rat erythropoietin expression plasmid was introduced into the thigh muscle of rats by *in vivo* electroporation. The serum erythropoietin levels reached a maximum 7 days after electroporation and gradually decreased thereafter. However, the erythropoietin levels were still approximately 30% of their peak value one month after electroporation. At present, we do not know the reason for this difference in the duration of expression between IL-5 and erythropoietin. It cannot be explained by the species difference between rats and mice, because other groups have reported long-term expression of luciferase in mouse

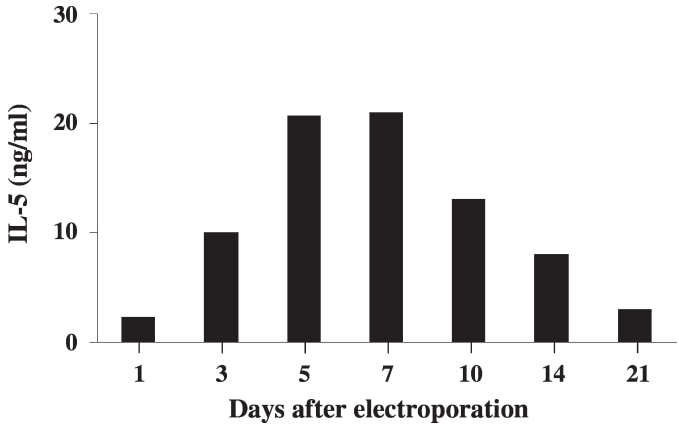

Fig. 22.5 Time course of IL-5 expression after transfer of pCAGGS-IL-5 plasmid by electroporation *in vivo*. The bilateral tibialis anterior muscles were injected with pCAGGS-IL-5 plasmid DNA. Electric pulses of 100 V were delivered to the DNA injection sites. Serum samples were obtained on the indicated days after electroporation and measured for IL-5 by ELISA. Each value represents the mean IL-5 concentration from three mice

muscle after DNA transfer by electroporation (Mir et al., 1999). Alternatively, local inflammation possibly caused by IL-5 expression might have reduced the duration of expression.

The duration and levels of gene expression by electroporation *in vivo* is also affected by the stability of the produced proteins, which may be increased by adding an immunoglobulin Fc portion to the gene product of interest, such as cytokines. When electrotransferred to muscle, such a construct, made with viral interleukin-10 (vIL-10) cDNA, led to the production and secretion of a fusion protein (vIL-10-Fc). This chimeric protein is very stable and bioactive. The use of the plasmid expressing this fusion cytokine led to a peak serum concentration 100-fold higher than with the non-fusion vIL-10 expression plasmid. Thus, the construction of expression plasmids producing fusion proteins appears to be a simple way to deliver enhanced levels of secreted proteins without altering their biological activities.

5 Comments

1. The selection of enhancers/promoters, poly A signals and other regulatory elements in an expression plasmid vector is extremely important for transgene expression. The ability of a vector to drive gene expression is a combined effect of all these elements and may vary among particular cells or tissues. Efforts have been made to construct highly efficient vector systems for transgene expression in muscle by searching for optimized combinations of these elements. We have used the pCAGGS plasmid (Niwa et al., 1991) as an expression vector. This vector contains the CAG (cytomegalovirus immediately early enhancer-β-actin hybrid) promoter, which is especially active in muscle. Other expression plasmids may be used if their promoter is very active in muscle cells: e.g., the VR1255 vector developed by Norman et al. (1997) for muscle-targeted expression contains the CMV promoter, enhancer, and intron, and the kanamycin resistance gene. Further improvements might include nuclear localization elements to enhance transport of plasmids into the nucleus as well as regulatory elements for export, stabilization and translation of transcripts. For gene therapy, it is sometimes desirable to regulate the expression of foreign genes introduced by *in vivo* electroporation. This may be attained by using a tetracycline (Tet)-inducible gene expression system, in which two expression units are required: one to express a Tet-regulatable chimeric transactivator and the other to express the gene of interest under the promoter regulated by the transactivator (Liang et al., 1996). Recently, it was reported that pharmacological regulation through the Tet-inducible system allows the regulation of serum erythropoietin and hematocrit levels (Rizzuto et al., 1999; Lamartina et al., 2002). This method is expected to provide a potentially safe and low-cost treatment for serum protein deficiencies.
2. The plasmid DNA preparation should be pure. Contaminating impurities may cause local immunological reactions, which may lead to early loss of gene

expression or affect the experimental results. To obtain highly purified plasmid DNA, plasmid extracted by the alkaline lysis method may be purified by two cycles of ethidium bromide-CsCl equilibrium density gradient ultracentrifugation followed by isopropanol precipitation, phenol and phenol/chloroform extraction, and ethanol precipitation.

3. Prior to the *in vivo* experiment, the DNA construct should be tested for transgene expression *in vitro* using a myoblast cell line, such as C2C12, which can be transfected easily by the lipofection method.
4. Prior to actual experiments, we recommend that you confirm that you can reproducibly inject the tibialis anterior muscles of anesthetized mice using some kind of dye.
5. The tibialis anterior muscle is a relatively small one and the maximal volume of DNA solution that can be injected into it is less than 50 µl.
6. Other types of electrodes may be used: external plate-type electrodes have been successfully used in our laboratory (Horiki et al., 2004).
7. We have used needle-type electrodes fixed with a distance (gap) of 5 mm. This distance should be altered to take into account the size of target muscle. The electric field strength (voltage/distance), but not the voltage itself, is assumed to be directly related to the efficiency of electropermeabilization. Therefore, if the electrodes have a gap of 10 mm instead of 5 mm, the voltage of the electric pulses must be doubled to obtain the same strength of electric field. However, higher voltages may cause very high local current around the electrodes, which may lead to irreversible damage to muscle and other tissues.
8. It was reported that X-gal histochemistry following the gene transfer of constructs encoding β-galactosidase may underestimate the anatomic extent of gene expression (Couffinhal et al., 1997).

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Chapter 23
Bone Formation by BMP Gene Transfection

Koshi N. Kishimoto and Yuji Watanabe

1 Introduction

An application of bone morphogenetic proteins (BMPs) has been expected to be a solution for fracture repair and bone regeneration ever since the discovery of their osteogenic potential (Urist, 1965; Reddi, 2000). Recombinant BMPs have been employed in in vitro and in vivo studies of bone induction. In vitro studies have revealed that BMPs cause the transformation of pluripotent mesenchymal cells obtained from bone marrow (Thies et al., 1992), fat (Dragoo et al., 2003), and muscle (Katagiri et al., 1994) into osteogenic cells. Clinical application of recombinant BMPs requires a high-quality recombinant protein and drug delivery system (DDS), which enables slow and continuous release of protein.

Vectors for gene transfer can be categorized into two groups: viral and nonviral. In vivo transfer of BMP gene by adenoviral vector could induce ectopic bone in muscle (Musgrave et al., 1999; Gonda et al., 2000; Chen et al., 2002). However, adenoviral vector causes an immune response. Obvious ectopic bone formation was detected in immunodeficient animals, but less so in immunocompetent animals (Musgrave et al., 1999; Li et al., 2003). Transplantation of adenoviral infected cells is more promising, and bone formation could be achieved in immunocompetent animals (Chang et al., 2003; Wang et al., 2003). Systemic administration (Okubo et al., 2000) and local administration (Kaihara et al., 2004) of immunosuppressant can decrease the immune response of the host and enables adenoviral infection and effective bone formation. The use of retroviral vector also is possible to transfect BMP gene and induce bone formation. Adenoassociated viral transfer (AAV) (Gafni et al., 2004) and helper-dependent adenoviral transfer (Abe et al., 2002) are less pathogenic and can be candidates for gene therapy of immunocompetent animals.

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The nonviral BMP gene transfer method is free from an immune response against virus. So the method to increase transfection efficiency with nonviral vectors has been investigated. The use of cationic liposome (Ono et al., 2004), in vivo electroporation (Kishimoto et al., 2002; Kawai et al., 2003, 2005, 2006; Kotajima et al., 2006) and in vivo sonoporation (Nakashima et al., 2003; Sheyn et al., 2008) using plasmid as the expression vector have been reported. These approaches may be more promising from a safety viewpoint. This chapter describes the current status and procedures of nonviral gene transfer of bone-inducing genes by electroporation and sonoporation. Also we will discuss ex vivo gene transfer of BMP. Ex vivo refers to extracorporeal gene transfer to the cells and transplantation of them. Gene-transferred cells could be transplanted in the form of a cell pellet or with a certain carrier. Nakashima et al. transferred the Gdf11 gene into dental pulp stem cells and transplanted the pellet of the cells into amputated dental pulp. The gene-transferred cells stimulated the regenerative dentin formation (Nakashima et al., 2004). This method should be the most promising technique for the clinical application of BMP gene transfer.

2 Procedure

2.1 Plasmid Preparation

Expression plasmid for electroporation is grown in *Escherichia coli,* and then extracted using a kit based on the alkaline lysis procedure. Endotoxins produced from *E. coli* could affect the efficiency of gene transfection and cell viability of the injection site. An endotoxin-free plasmid extraction procedure is recommended for steady results of electrotransfection. Extracted plasmid is dissolved in distilled water, saline (0.9% NaCl), or phosphate-buffered saline (PBS) for direct injection into animals. Saline or PBS can achieve higher gene expression products than distilled water. However, long storage in buffers except for TE may result in degradation of the plasmid. When using the same amount of plasmid, we can obtain more effective gene expression products by higher concentration of plasmid solution. Plasmid solution in high concentration is viscous. Viscous solution can stay longer in the injected site before electroporation, so it seems to be one of the reasons for its high efficiency (Wang et al., 2005).

2.2 In Vivo Electroporation

Ectopic bone formation refers to osteoinduction in the site where the bone should not exist. An animal model of ectopic bone formation in skeletal muscles could provide the important insights into bone formation in vivo. For electroporation, needle- or plate-type electrodes have been used. The representative parameters

for the needle-type electrodes are 5 mm gap, 100 V, 25 ms, 6 times (3 times each polarity) (Kishimoto et al., 2002; Kotajima et al., 2006). The representative parameters for the plate-type electrodes are 5 mm gap, 100 V, 50 ms, 8 times (4 times each polarity) (Kawai et al., 2003, 2005). The efficiency of electroporation is greatly affected by the conductance between electrodes. The conductance between plate-type electrodes depends on the skin contact. Fur removal, skin cream, and conductance monitoring are important for reliable transfection with plate-type electrodes.

Stimulation of electric pulse can induce electric burn. Skeletal muscle has a remarkable ability for regeneration from injury. During the recovery from electric burn, infiltration of mesenchymal cells could be seen around regenerating muscle fiber (Kishimoto et al., 2002). Muscle satellite cells may exist. Muscle satellite cells are pluripotent and enable muscle regeneration and also transdifferentiation into osteogenic or chondrogenic lineage. Pretreatment of muscles by high-concentration salt solution or by bupivacaine prior to electroporation increases pluripotent cells and allows more effective bone formation (Kotajima et al., 2006).

2.3 In Vivo Sonoporation

Experimental bone formation using sonoporation also was developed. A plasmid solution was mixed with microbubble and then injected directly into muscle. Microbubble reagent (Optison) is available from Molecular Biosystems Inc. (San Diego, CA). High gene transfer efficiency was achieved at 5–10% Optison. The representative parameters of sonoporator for in vivo ectopic bone formation are 5 W/cm2, 10 min, duty cycle at 50% (Sheyn et al., 2008). Sonoporation is less invasive than electroporation in general. For gene transfer aiming at secretion of gene products into systemic circulation, less invasiveness is an advantage of sonoporation. However, bone formation requires a source of stem cells which muscle damage provides in electroporation.

2.4 In Vitro and Ex Vivo Electroporation or Sonoporation

Ex vivo bone formation consists of several steps: collecting cells, primary culture, in vivo electroporation or sonoporation, and transplantation of cells (Nakashima et al., 2002, 2004). Mesenchymal stem cells from bone marrow, adipose tissue, and muscle could be used for bone formation. Gene transfer to cultured cells could be conducted. The parameters of electroporation and sonoporation have to be optimized for each cell type. There are many parameters, such as field strength [V/mm], pulse length, pulse number, buffer for electroporation, and concentration of plasmid and cells. In general, the smaller diameter cells require the stronger pulse. For gene transfer by electroporation into pluripotent murine cells, C3H10T1/2 and C2C12, 2.5~5 $\times$ 10^5 cells/ml in electroporation buffer was successful (75% cytosalts; 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4 pH 7.6,

5 mM $MgCl_2$; 25% Opti-MEM I) (van den Hoff et al., 1992). This buffer is similar to intracellular fluid which allows better survival rate despite the cell membrane breakage. We usually apply electric pulses by a square pulse generator, such as BTX-820, 830 (BTX), or CUY21 in vitro (Nepa gene), under the following parameters; 450~500 V, 2 ms, 1 Hz, and two pulses through 4-mm gap cuvette with 600 µl of cell suspension. The electroporated cells are allowed to recover in medium containing serum or serum-reducing medium such as Opti-MEM I (invitrogen).

3 Application and Results

After 3 days of electroporation in vivo, infiltration mesenchymal cells were observed. Immunohistochemical analysis revealed that BMP4 was expressed in muscle fibers. On day 14 after electroporation, calcified bone matrix that is positively stained by the von Kossa method and cartilage that is stained with alcian blue were detected. Bone marrow-like cells were seen in the bone matrix (Fig. 23.1).

After electroporation of control plasmid with GFP, no cartilage or bone matrix was found at any time. Nonspecific dystrophic calcium deposits between the muscle bundles were sporadically observed on day 7. The GFP expression was confirmed on the muscle fibers.

After in vitro electroporation, the survival rate of cells is about 50%. Overnight culture for recovery also allows viable cell selection. In vitro gene transfer by electroporation using a cuvette yield 30–60% of efficiency depending on the concentration of plasmid. In our experience, 50 µg/ml (30 µg/cuvette) of BMP plasmid allows enough efficiency for transdifferentiation of electroporated cells into chondrocytes. Pluripotent C3H10T1/2 cells that had been transfected with BMP gene in high-density culture turned into alcian blue-positive chondrocytes from 3 days after electroporation (Fig. 23.2).

4 Comments

Nonviral transfer of the BMP-expressing plasmid allowed secretion of the BMP protein and induced ectopic bone formation. So far, this method has not been able to induce enough ectopic bone for clinical application, so future investigation is necessary to enhance gene products. Formed bone started to be absorbed after 3 weeks. This not only is because BMP expression was decreased, but also ectopic bone in muscle lacks mechanical demanding. If there were mechanical demanding, formed bone would last longer. This should be confirmed with the orthotopic experimental model, such as bone formation after fracture or segmental bone loss in the next step.

The direction of differentiation by BMP treatment depends on the type of cells. The C2C12 myoblasts directly differentiate into osteoblasts that react to BMPs (Katagiri et al., 1994). Pluripotent C3H10T1/2 cells that have been transfected with BMP differentiate into chondrocytes. It requires further investigation to solve which

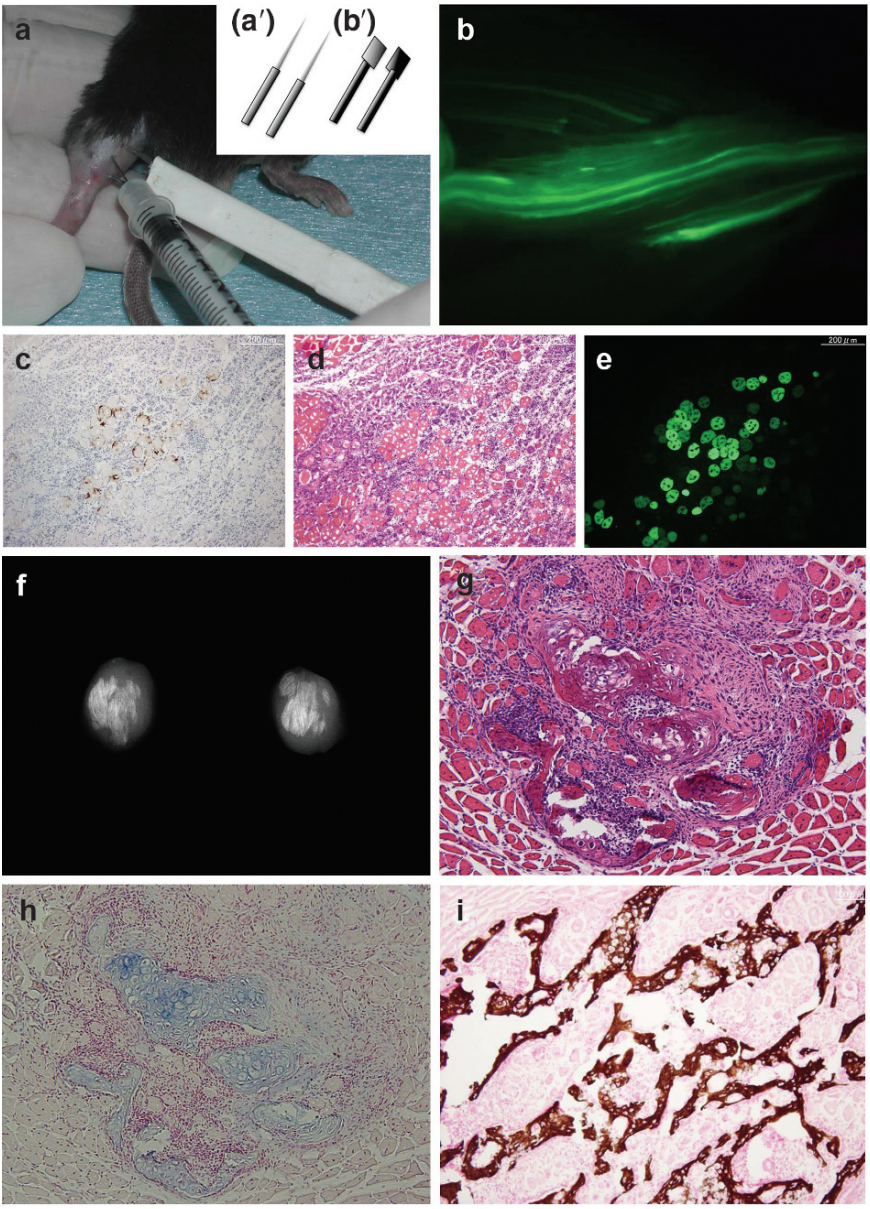

Fig. 23.1 Ectopic bone formation by in vivo electroporation of BMP4. (**a**) Electroporatic gene transfer into the calf muscle of mice. Needle-type electrodes were inserted. Plasmid solution was injected between electrodes (insertion). (**a´**) Needle-type electrodes; (**b´**) plate-type electrodes. (**b**) GFP expression in the muscle fibers (pEGFP-N1 promega). (**c**) Immunohistochemical detection (DAB brown) of BMP4 expression 7 days after coelectroporation of BMP4 and pEGFP-N1. (**d**) H&E staining of adjacent section of **c** and **e**. (**e**) GFP expression on the muscle fibers. (**f**) Soft X-ray examination of pCAGGS-BMP4 electroporated calf muscles at day 14 (150 µl of 3 µg/µl plasmid was used for each muscle). (**g**, **h**, **i**) H&E (**g**), alcian blue (**h**), and von Kossa (**i**) staining of ectopically formed bone at day 14

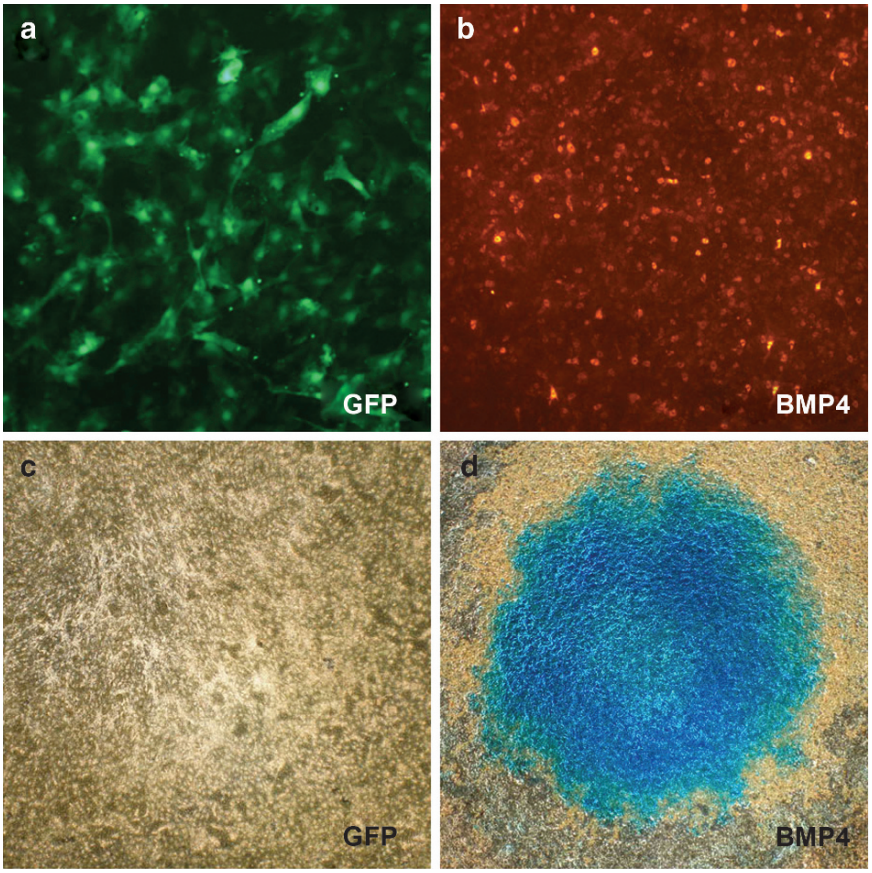

Fig. 23.2 Chondrogenesis by in vitro electroporation of BMP4 expression vector. A plasmid, pEGFP-N1 (**a** and **c**), or pCAGGS-BMP4 (**b** and **d**) were transferred into a murine mesenchymal cell line, C3H10T1/2, by electroporation in cuvette. (**a**) GFP expression at 3 days after electroporation. (**b**) Immunocytofluorescence for BMP4 at 3 days after electroporation. Cells were cultured in high-density micromass. One hundred thousand cells in 10 µl medium were dropped in the center of a 12-well plate. After 90 min of incubation, wells were gently filled with culture medium. (**c** and **d**) Alcian blue staining of micromass showed negative for control plasmid (**c**) and positive for BMP4 (**d**) at 7 days after electroporation

is the more beneficial method for clinical application: transplantation of osteogenic cells or of chondrogenic cells. But we think it more advantageous to transplante chondrogenic cells because large long bones are formed from a model of cartilage that arose from condensation of mesenchymal cells in the development.

If in vivo electroporation were applied to clinical practice, electric pulses could affect the heartbeat, especially for the patient with arrhythmia. For future human application, extracorporeal gene transfer would be beneficial (Kimelman et al., 2007). Also ex vivo gene transfer allows a wide choice of transplanting cells which

can be both the target of transfection and the source of differentiation, such as bone marrow mesenchymal stem cells, muscle-derived cells, and fibroblasts. The ex vivo method claims a complex technique, so there are many problems to be solved. However, its flexibility is advantageous for future clinical application.

5 Troubleshooting

So far, mice and rats have been used as experimental animals for ectopic bone formation in the skeletal muscle. Electrical burn can induce dystrophic calcification in the skeletal muscle. Calcification lacks bone-specific micro-architecture and is totally different from bone. The incidence of the dystrophic calcification depends on the species of mice (Ivandic et al., 1996). Because the dystrophic calcification disturbs the analysis of bone formation, it is quite important to select the species.

As an experimental site, the muscles that are encased in a compartment such as lower leg and forearm muscles seem to be free from leakage of plasmid solution and suitable for in vivo electroporation.

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Chapter 24
Electroporation of the Testis

Kentaro Yomogida

1 Introduction

The mature mammalian testis is a marvelous organ that produces numerous sperm cells during its reproductive phase. This biologically significant process consists of three steps: stem cell self-renewal and differentiation, meiosis and genetic recombination, and haploid cell morphogenesis into sperm (Russell et al., 1990). The first step provides a good model for investigating the molecular mechanism of stem cell regulation. Currently, the mechanism underlying sperm cell production is a very exciting topic in regenerative medicine (Lensch et al. 2007; Okita et al., 2007). The spermatogonial stem cell system has several advantages, including the easy histological identification of stem cells (Russell et al., 1990), a clear relationship between stem cells and the supporting Sertoli cells, which provide a stem cell niche (Tadokoro et al., 2002; Yomogida et al., 2003), and a transplantation assay for stem cell activity (Oatley & Brinster, 2006). Although germline stem (GS) cells derived from the gonocytes in newborn testis constitute a suitable *in vitro* system for investigating the properties of spermatogonial stem cells (Kanatsu-Shinohara et al., 2003, 2004), studies using living mammalian testes continue to provide information regarding the roles of the stem cell niche. *In vivo* electroporation of the supporting cells in the testis will expand our ability to study it.

The last two steps in sperm cell production are important for species preservation and evolution. In mammals, meiotic cells at all stages of division and haploid cells are found continuously only in the testis. Indeed, most of our knowledge of the molecular mechanisms underlying these two processes has been obtained using mutant and genetically engineered animals, such as transgenic or knockout mice, because there are currently no suitable *in vitro* systems (Lau & Chan, 2007). However, the use of such mice requires significant time, money, and labor. The *in vivo* electroporation of mammalian testes could reduce this burden because it would allow a gene of interest to be inserted into the cells, and their behavior could be followed directly.

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2 Principle

2.1 Targeting the Cells in the Testis

As mentioned above, germline cells should be the target of *in vivo* electroporation. To supply mature sperm continuously, spermatogenesis occurs along the walls of the seminiferous tubules, which connect to the epididymis via the rete testis and efferent ducts (Figs. 24.1 and 24.2). Completed sperm are released into the lumen and are transported to the epididymis for their maturation through these ducts. A DNA solution can be injected into most of the lumen via this route, as in germ cell transplantation (Ogawa et al., 1997). In mature testes, numerous germ cells at various stages of differentiation may be found on the inner side of the seminiferous tubule wall. Foreign DNAs injected into the lumen may be easily transferred into the germ cells given an adequate electric charge. However, few genes can be electroporated into the spermatogonia that exist outside the testis-blood

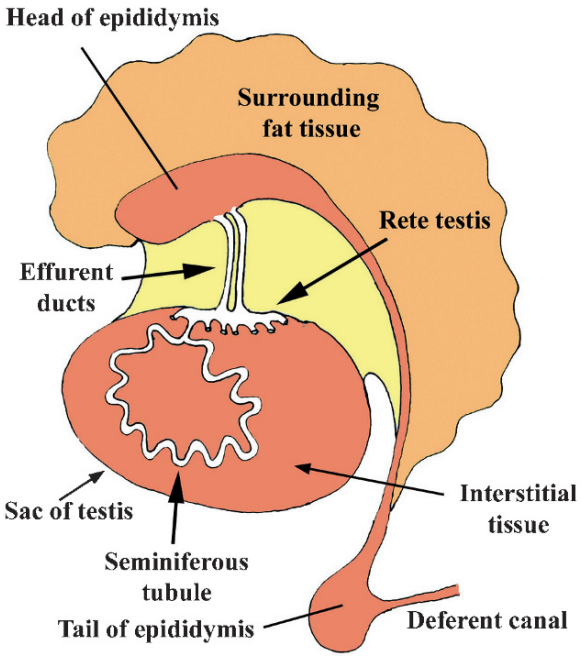

Fig. 24.1 Structure of the mouse testis and its accessories. The testis is enclosed in a tight collagen sac that consists of closed interstitial tissue and many seminiferous tubules connected to the rete testis. Spermatogenesis occurs continuously and cyclically on the seminiferous tubule wall. Completed sperm are transferred to the epididymis via efferent ducts for maturation. Mature sperm are stored in the tail of epididymis and are ejaculated from an efferent canal. Testicular blood vessels run parallel to the efferent ducts and epididymis. The testis can be pulled out with its surrounding fat tissue

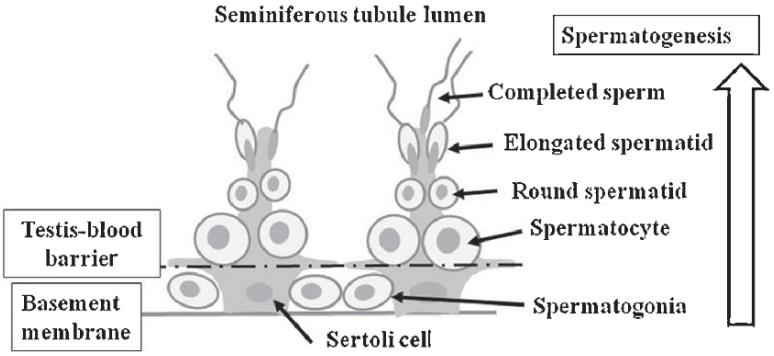

Fig. 24.2 Schema of spermatogenesis on the seminiferous tubule wall. Spermatogenesis occurs on the seminiferous tubule wall from the outside to the lumen. Injected DNA can be transfected into the cells on the inner side of the testis-blood barrier, which is made up of supporting Sertoli cells

Table 24.1 Developmental emergence of differentiated germ cells in the mouse testis

Age (days)	Undifferentiated spermatogonia	Differentiated spermatogonia	Early spermatocyte	Late spermatocyte	Round spermatid	Elongated spermatid	Completed sperm
5	→						
7	→	→					
14	→	→	→				
21	→	→	→	→			
28	→	→	→	→	→		
35	→	→	→	→	→	→	→

barrier formed by the supporting Sertoli cells (Fig. 24.2). Therefore, the various differentiated germ cells on the inner side of the testis-blood barrier should be good targets for *in vivo* electroporation. To efficiently introduce genes into germ cells at a specific stage of differentiation, testes should be used at the first emergence of the target cells (Table 24.1).

Supporting Sertoli cells, which make up the seminiferous tubule wall, play important roles in spermatogenesis (Russell et al., 1993). Since foreign genes in the lumen access the wall cells directly, they are also good targets for this method. Sertoli cell maturation occurs during testis-blood barrier formation. Since the most mature cells can no longer divide, circular foreign genes in mature Sertoli cells are expressed permanently. To introduce genes exclusively into these cells, testes at the start of germ cell differentiation (i.e., those with few differentiated germ cells on the inner wall) should be selected as target organs.

The stroma surrounding the seminiferous tubules is a closed, cramped space with vessels, immunocytes, and hormone-secreting Leydig cells (Russell et al., 1990). A DNA solution could feasibly be injected into the space by punching through the wall of the rete testis; however, since a large volume of solution would compress the vessels and cause tissue damage, these interstitial cells are not suitable targets for *in vivo* electroporation.

3 Procedure

In this section, the *in vivo* electroporation of mouse testis is described in detail as a typical example. Depending on the target animal, this protocol should be modified.

3.1 Equipment

- Electroporator (Fig. 24.3a) with a foot switch and forceps-type electrodes (Fig. 24.3b). The electrodes must be of the proper size for the testis. A current direction selector is useful for efficiently energizing the testis (Fig. 24.3c).
- Stereomicroscope with a zoom range of 7–70X.

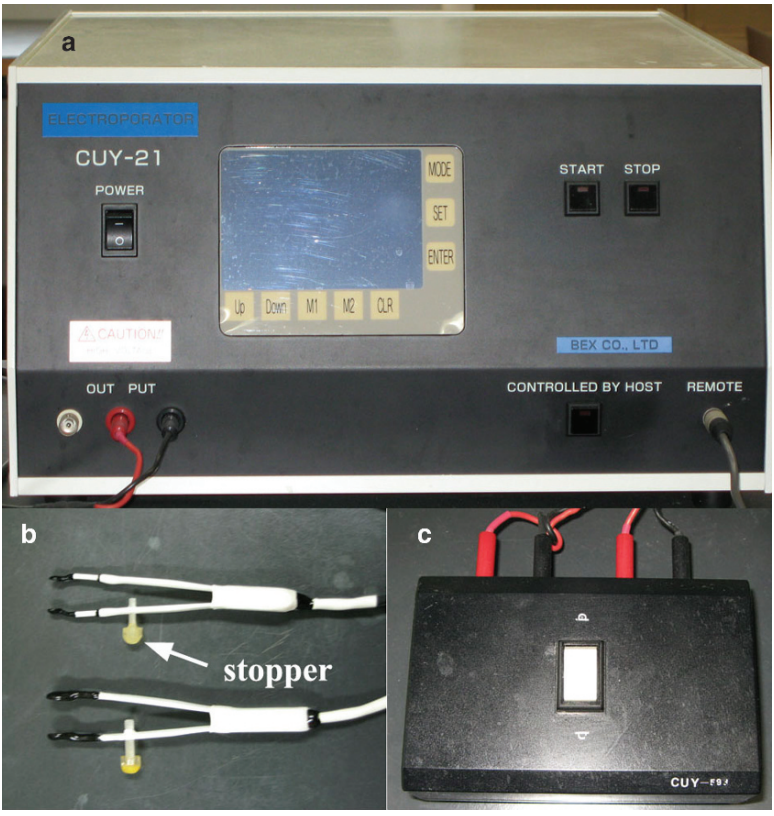

Fig. 24.3 Electroporator and forceps-type electrodes. (**a**) An electroporator. A pedal switch is helpful for energizing the testis. The diameter of the forceps-type electrodes must match the size of the target (**b**). The stopper helps avoid rupturing the testis due to excessive pressure. A current direction selector is recommended for changing the direction of the current (**c**)

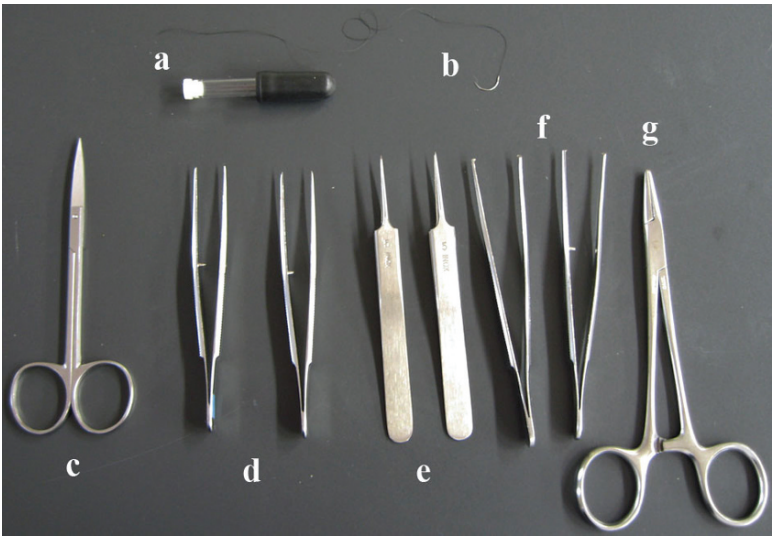

Fig. 24.4 Surgical tools for *in vivo* electroporation of testis. (**a**) A rubber-bulb syringe is needed to fill the glass needle with the DNA solution. (**b**) 5-0 Silk thread with a round needle for suturing the wound. (**c**) A pair of ophthalmic scissors. (**d**) Two sets of straight-tipped forceps for pulling out the testis. (**e**) Two sets of exact tweezers for controlling the efferent ducts for electron microscopy. (**f**) Two sets of hooked forceps for opening and closing the abdominal wall. (**g**) A needle holder

- Surgical tools: two sets of exact tweezers for electron microscopy (INOX5), two sets of forceps, two sets of hooked forceps, one pair of ophthalmic scissors, a needle holder, and 5-0 silk thread with a round needle for angiorrhaphy (Fig. 24.4).
- Injector

An expensive microinjector system is unnecessary. A suitable apparatus can be set up using a disposal 1-ml syringe, about 40 cm of silicon tubing (ca. 7 mm diameter), a Pasteur pipette, and a rubber-bulb syringe (Fig. 24.5a).

- Glass needles

Glass needles may be produced from borosilicate glass (O.D. 1.0 mm, I.D. 0.75 mm, 10 cm in length; Sutter Instrument [Novato, CA, USA]) using a micropipette puller (Model P-97/IVF, Sutter Instrument; pulling conditions: heat = 730, Pull = 0, vel = 50, and time = 200). Grinding of the needle tip is unnecessary; it can be broken at the desired point with exact tweezers before use (Fig. 24.5b).

3.2 Reagents

- DNA solution in TE buffer containing 0.4 mg/ml trypan blue (final concentration 1 mg/ml DNA).
- Anesthetic for intraperitoneal injection. We usually use Avatin, which has a wide safety margin.

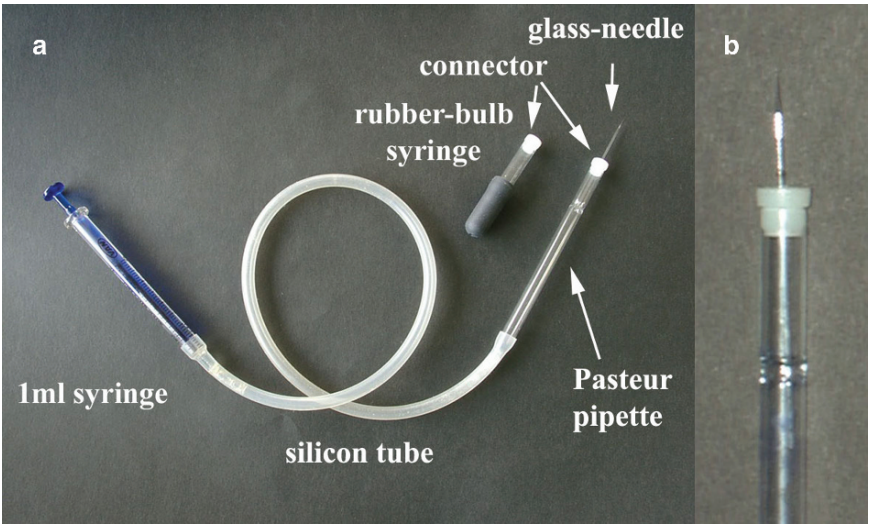

Fig. 24.5 Hand-made injector. An expensive microinjector system is unnecessary. An injector can be constructed from common laboratory items (**a**). (**b**) A glass needle in the injector

- Physiological saline.
- Seventy percent ethanol for sterilization.

3.3 Protocol

1. Break the needle tip at the desired point using exact tweezers under a stereomicroscope. Fill the glass needle with the target DNA solution from the bottom using a rubber-bulb syringe. About 20 μl of solution will fill a single needle. Insert the glass needle into the injector (Fig. 24.5b).
2. Set the anesthetized mouse on its back under the stereomicroscope (set at a low magnification). Make a transverse skin incision about the size of the testis above the umbilicus with ophthalmic scissors. Open the peritoneal membrane with a vertical incision along the Hunter line. Grasp one side of the fat tissue surrounding the testis with forceps and pull it over to the opposite side to eliminate distortion (Fig. 24.6).
3. Follow the rete testis to the area where the blood vessels and efferent ducts are attached. Pick up the efferent ducts using exact tweezers with your non-dominant hand. Hold the injector with your dominant hand and insert the glass needle into the rete testis via the lumen of the efferent duct (Fig. 24.7a).
4. Push the inner cylinder of the injector. If the tip of the needle has been adequately placed, you will see the blue DNA solution enter the seminiferous tubules via the

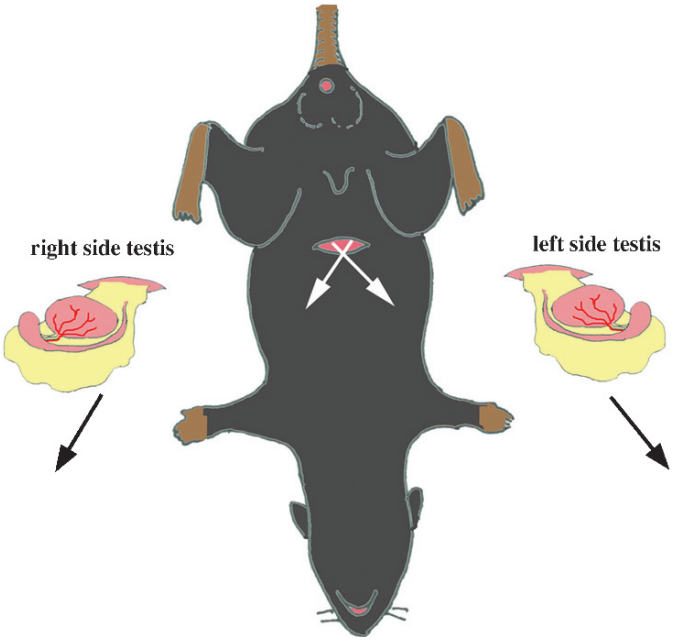

Fig. 24.6 Testis exposure for eliminating distortion. The testis is pulled upward toward the opposite side (in the direction of the arrow) with the surrounding fat tissue

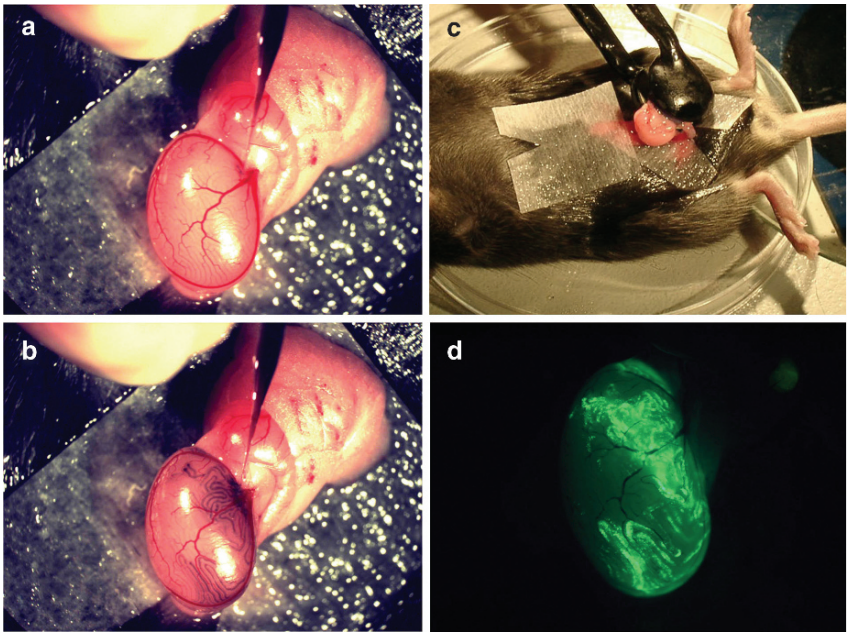

Fig. 24.7 *In vivo* electroporation of germline cells. The glass needle is inserted into the rete testis via an efferent duct (**a**). The injected DNA in the seminiferous tubules can be traced with trypan blue (**b**). The injected testis is nipped with forceps-type electrodes and energized (**c**). Transfected germline cells can be detected by green fluorescence protein (GFP) expression from the transgenes (**d**)

rete testis (Fig. 24.7b). The seminiferous tubules are contained in a closed space enveloped by a tight collagen sac (Russell et al., 1990). As the lumen fills, the testicular volume will increase slightly against the sac. Younger mature testes should be selected for this method because the sac becomes less flexible with age. Although 10–20 µl of solution can be injected into normal younger mature testis, only 1.5–2 µl of solution are needed for 2-week-old immature testis.

5. Remove the needle from the rete testis and damp the electrodes with physiological saline. Gently nip the testis with the electrodes to avoid rupture (electrodes with a stopper are recommended; Fig. 24.7b). Energize the testis two times with adequate voltage (40–50 V for adult testis, 20 V for 2-week-old testis) at a 50-ms interval with one push of the foot switch. (The use of insufficient voltage will lower the transfection efficiency; however, too much voltage will damage the cells of the testis.) Conduction of the electrical current will produce air bubbles between the electrodes and testis. Reverse the direction of the current using the current direction selector (Fig. 24.3c) and reenergize as described above. Place the electrodes at a right angle, re-nip the testis, and energize four times in the manner described above (Fig. 24.8).
6. Restore the testis to its original position to eliminate distortion. The remaining testis can now be treated.

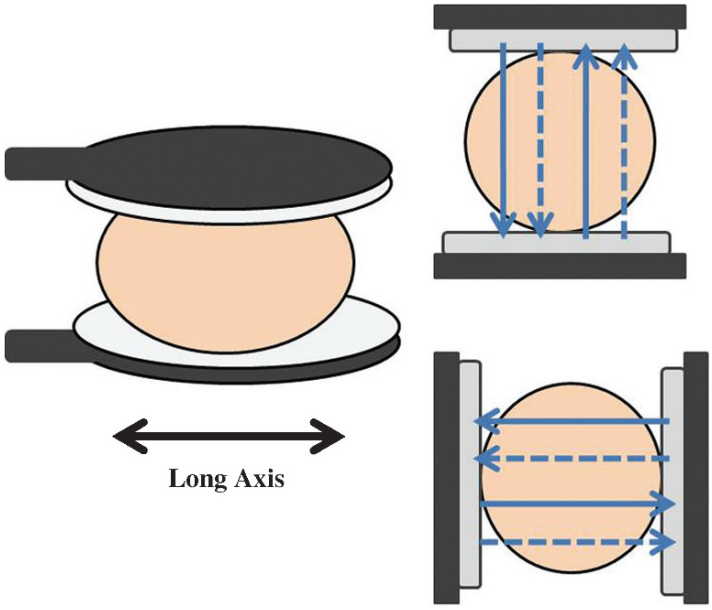

Fig. 24.8 Current directions used to energize the testis. Nearly all testicular cells on the inner side of the testis-blood barrier must be electroporated efficiently; thus, energizing should be done two times in four different directions as illustrated

7. Close the peritoneal wound and close the incision with sutures.
8. Within about 24 h, you should be able to detect expression of the electroporated gene in the cells of the testis (Fig. 24.7d) and be able to analyze their behavior.

4 Application and Results

4.1 Study of Mammalian Meiosis, Genetic Recombination, and Haploid Cell Behavior

At present, there is no suitable *in vitro* system for investigating mammalian meiosis or haploid cell behavior. To establish a useful system, it is important to learn more about these processes. As mentioned above, meiotic spermatocytes and haploid spermatids are good targets for *in vivo* electroporation. This method will provide a useful tool with which to study sperm cell production. Using adequate expression vectors, promoter assays (Ike et al., 2004), tagged-protein tracing assays (Huang et al., 2000), gain-of-function assays, and RNA interference assays (Shoji et al., 2005) will be possible.

4.2 Study of the Roles of the Supporting Cells and Stem Cell Regulation

Sertoli cells support various differentiated germ cells and show cyclic gene expression during spermatogenesis (Russell & Griswold, 1993). As mentioned above, circular expression vectors are expressed permanently in mature Sertoli cells (Fig. 24.9; Yomogida et al., 2002). To understand the mechanism of spermatogenesis, *in vivo* electroporation of the testis will provide useful information about the roles

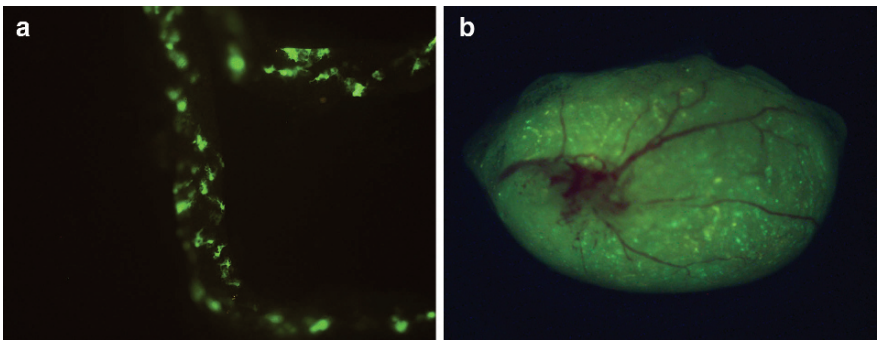

Fig. 24.9 Electroporation of Sertoli cells. Sertoli cells are good targets for *in vivo* electroporation. Electroporated Sertoli cells can be observed to constitute the unraveled seminiferous tubule wall with GFP (**a**). Transgene expression may be observed about one day after electroporation (**b**)

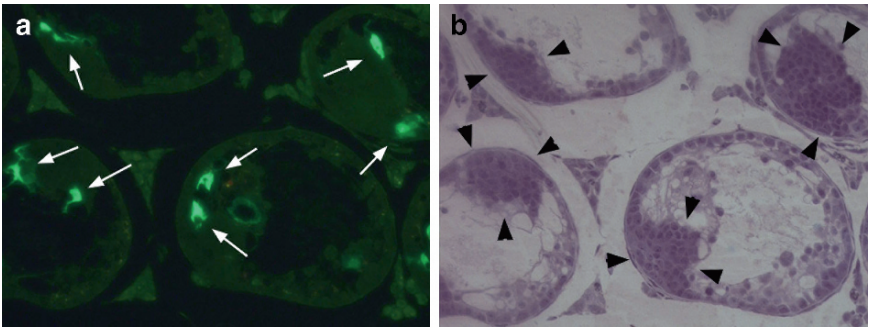

Fig. 24.10 Expansion of spermatogonial stem cells by electroporated gene expression. Ectopically expressed glial cell line-derived neurotrophic factor (GDNF)-expanded spermatogonial stem cells. Transfected Sertoli cells carrying GDNF and GFP expression vectors are shown (**a**: *arrows*; *fluorescence view*). Many round spermatogonial stem cells can be observed around the transfected Sertoli cells (**b**: *arrowheads; hematoxylin and eosin [H&E] staining*)

of Sertoli cells. Spermatogonial stem cell behavior is also controlled by extrinsic signals provided by Sertoli cells. However, little is known about the regulatory mechanism. *In vivo* gene transfer to Sertoli cells may help clarify the regulation of stem cells (Fig. 24.10; Yomogida et al., 2003).

4.3 Clinical Applications

Infertility is becoming a social problem in developed countries (Ferlin et al., 2007). Nearly half of all infertility cases may be due to a problem with spermatogenesis. Investigating spermatogenesis using the method described here may provide clues as to the causes of infertility, as well as direct solutions. If infertility is due to a genetic dysfunction in Sertoli cells, infertility could be treated without ethical problems by transferring a normal gene by *in vivo* electroporation into the testis (Fig. 24.11; Yomogida et al., 2002).

4.4 Transgenic Animal Production

Gene transfer to germline cells is considered a good method for creating transgenic animals (Yamazaki et al., 1998, 2000). *In vivo* electroporation of the testis is currently possible, but the efficiency is low. Transfected mature sperm from a single electroporation event comprise only a small proportion of ejaculated semen. To create the desired offspring using this method, *in vivo* fertilization (IVF) or intra-cytoplasmic sperm injection (ICSI) with transfected spermatids or sperm is required (Huang et al., 2000). Thus, at present, established methods using fertilized eggs are used to produce transgenic animals.

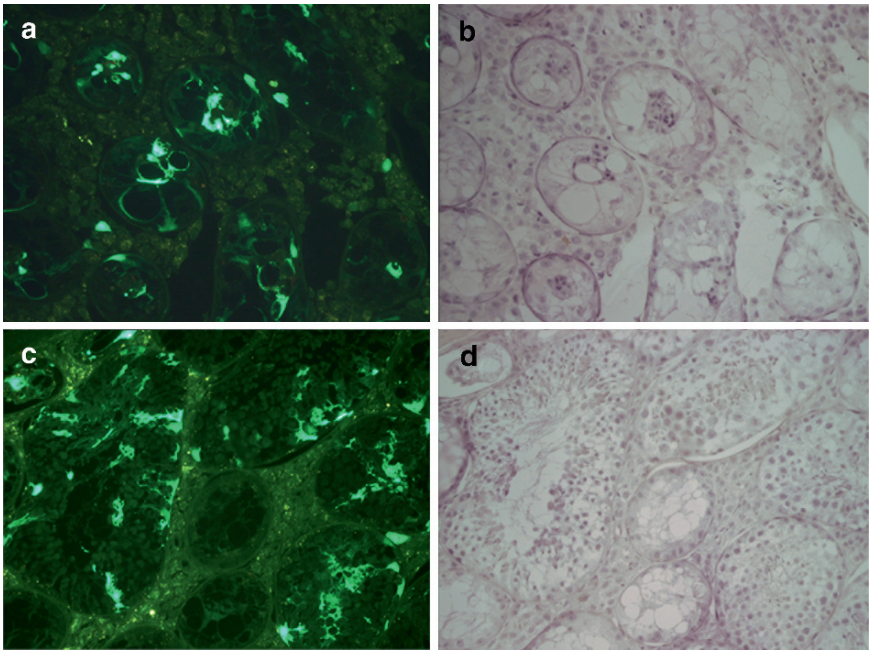

Fig. 24.11 Rescue of spermatogenesis in Sertoli cell-defective testis. The Sertoli cells in Sl^{17H}/Sl^{17H} mutant mice carry a defective *kit-ligand* gene; thus, they cannot support normal spermatogenesis. Although the electroporation of a GFP expression vector into the cells cannot rescue spermatogenesis (**a**: *fluorescence view*; **b**: *H&E staining*), those Sertoli cells transfected with GFP and wild-type kit-ligand expression vectors can support normal spermatogenesis (**c**: *fluorescence view*; **d**: *H&E staining*)

4.5 Other Possibilities

As described above, the injection of DNA into the seminiferous tubules via the rete testis is similar to germ cell transplantation. The effects of drugs or antibodies on spermatogenesis may be directly tested using this method.

5 Troubleshooting

- Testis is congested with blood.

  The testis may have been handled too roughly with the forceps, or the spermatic cord might be twisted.
  With the testis pulled out of the abdominal lumen, grasp the surrounding fat tissue softly to eliminate distortion.

- The blood vessels adjacent to the rete testis were damaged by the glass needle.

  If the injury is mild, you can stop the bleeding by applying pressure with a Kimwipe.

- The seminiferous tubules are not receiving the injected DNA solution.

  The tip of the glass needle should be in the interstitial space.
  If the DNA solution is injected into the interstitial space, the situation is irreversible; thus, it is important to avoid stabbing the wall of the rete testis. By inserting the glass needle into the efferent duct and releasing a drop of solution (push the inner syringe slightly), you can check the location of the needle tip.

- The solution is trapped in the needle.

  The tip of the glass needle may be in contact with the wall of the rete testis.
  Pull the glass needle out slightly to adjust its position.

  The air pressure is too low.
  Check for air leaks at every joint in the injector.
  Before inserting the needle, ensure that the inner syringe is sufficiently retracted.
  The diameter of the needle tip may be too narrow; ensure that the break is made at an appropriate site to yield an opening about 50–100 μm in diameter.

  The glass needle is stopped up with crystals of trypan blue.
  Use fresh trypan blue without crystals.
  If the particles are small, break the bottom side of the needle slightly and retry the injection.

- The electricity is not working.

  The surface of the electrode may be coated with proteins.
  Polish the surface softly with kitchen cleanser.

  The lead wire might be broken at the junction with the forceps bottom.
  Do not rotate the forceps-type electrodes too much when nipping the testis.

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Chapter 25
Direct Gene Transfer into Plant Mature Seeds via Electroporation After Vacuum Treatment

Takashi Hagio

1 Introduction

A number of direct gene transfer methods have been used successfully in plant genetic engineering, providing powerful tools to investigate fundamental and applied problems in plant biology (Chowrira et al., 1996; D'halluin et al., 1992; Morandini and Salamini, 2003; Rakoczy-Trojanowska, 2002; Songstad et al., 1995). In cereals, several methods have been found to be suitable for obtaining transgenic plant; these include bombardment of scutellum (Hagio et al., 1995) and inflorescence cultures (He et al., 2001), and silicon carbide fiber-mediated DNA delivery (Asano et al., 1991) and *Agrobacterium tumefaciens* transformation (Potrykus, 1990). Electroporation of cereal protoplasts also has proved successful but it involves prolonged cell treatments and generally is limited by the difficulties of regeneration from cereal protoplast cultures (Fromm et al., 1987). Many laboratories worldwide are now using *Agrobacterium* as a vehicle for routine production of transgenic crop plants. The primary application of the particle system (Klein et al., 1987) has been for transformation of species recalcitrant to conventional *Agrobacterium* (Binns, 1990) or protoplast methods. But these conventional methods can be applied to the species and varieties that are amenable to tissue culture (Machii et al., 1998). Mature seeds are readily available and free from the seasonal limits that immature embryo, inflorescence, and anther have. This method enables us to produce transgenic plants without time-consuming tissue culture process.

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2 Procedure

Day 1

1. Mature seeds are precultured one day prior to gene transfer in germination buffer, which is composed of:

   - 0.2% Polyvinylpyrrolidone (PVP)
   - 0.2% Sodium hypochlorite solution (active chlorine: approx. 0.001%)

Day 2

2. The seeds are washed with sterile distilled water several times.
3. The seeds are soaked in 2 ml electroporation buffer in 6-cm petri dish.

Electroporation buffer composition:

- 910 µl Sterile distilled water
- 500 µl 1% Polyvinylpyrrolidone (PVP) solution
- 30 µl 10% Tween 20 solution
- 100 µl 0.1 M spermidine
- 200 µl Plasmid DNA solution(1 µg/µl TE buffer)
- 100 µl 10% Cellulase Onozuka RS(Yakult Honsha. Minato-ku Tokyo Japan)
- 160 µl 2.5 M Calcium chloride dihydrate

The total amount is 2000 µl (2 ml).

4. The petri dish containing the seeds soaked in the electroporation buffer is placed in a vacuum chamber (Fig. 25.1). The petri dish is placed on ice in the vacuum chamber.
5. The vacuum pump is turned on for about 10 min. The vacuum pump is turned off and the valves of the vacuum chamber are closed. The vacuum degree is about 0.09 MPa. The sample is kept under the vacuum condition for at least 1 h.
6. The valves of the vacuum chamber are opened and the petri dish is taken out. The seed and buffer are transferred into the electroporation chamber (Fig. 25.2). The width of the chamber is 1.0 cm and contains 1.0 ml of electroporation buffer. The electroporation chamber is placed on ice and connected with the electroporation device using the designated cables.
7. Electrical pulses are added (Figs. 25.1 and 25.3).

Electrical conditions:

- Voltage: 50–100 V/cm
- Pulse duration: 50–99 ms
- Pulse interval: 50–99 ms
- Pulse number: 50–99 times

8. After the electroporation, the chamber is placed on ice for at least 2 min. The seeds and buffer are transferred into a 6-cm petri dish. The sample is incubated under an appropriate condition depending on the plant species. For the stable transformation of wheat, the sample is incubated at 10°C in dark.

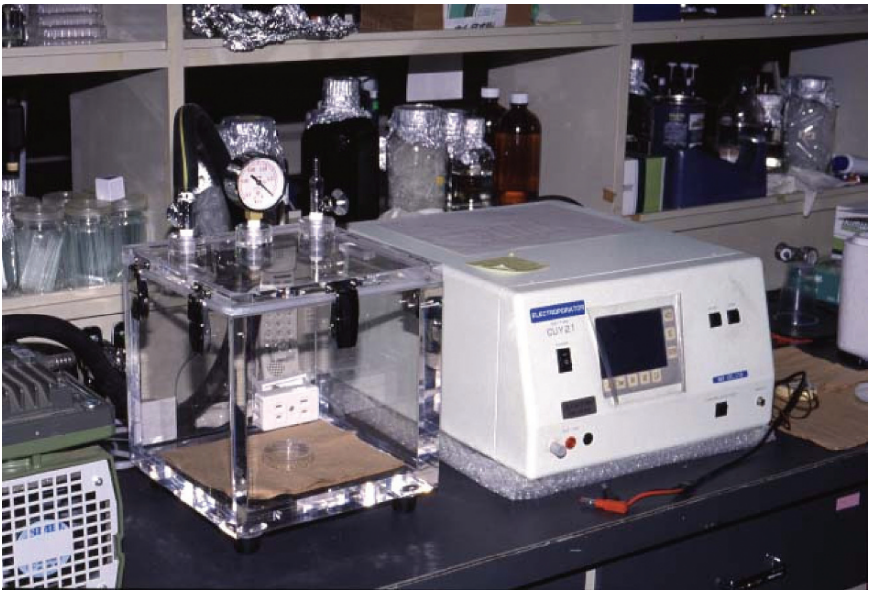

Fig. 25.1 A power supply for electroporation (*right*) and a vacuum chamber (*left*)

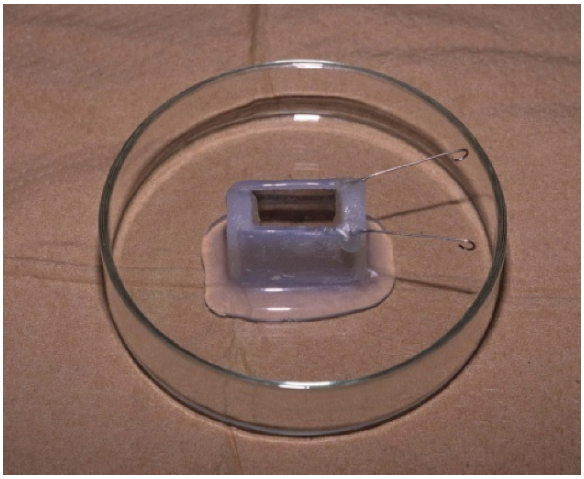

Fig. 25.2 Electroporation chamber. Electrodes are made of platinum. Approximately 20 wheat seeds and 30 rice seeds can be treated

Day 3

9. The incubation of the sample is continued.

Day 4

10. The assay of the transient gene expression is started by adding X-Gluc solution if GUS gene is introduced (Jefferson, 1987; Jefferson et al., 1987). If NPT

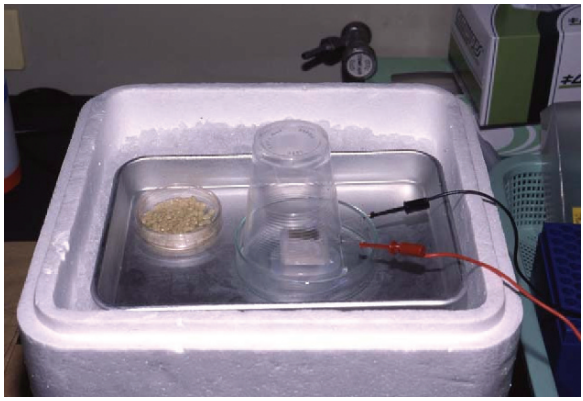

Fig. 25.3 Electroporation chamber placed on ice

II gene is introduced to obtain stably transformed plants, seeds are incubated in the selection medium containing approximately 200 ppm G418 (Chawla, 2002). A toxicity test is recommended to determine the optimum concentration of G418. The strength of G418 varies depending on the manufacturer and/or manufacturing lot. Stably transformed wheat plants were produced using this procedure.

3 Application and Results

To determine the physical parameter, plasmid pWI-GUS, which was fully described by Ugaki et al. (1991), was used. It has a β-glucuronidase (GUS) gene as a reporter. Typically, 20 mature seeds of wheat were soaked in germination buffer and incubated at 10°C in the dark overnight. Then the seeds were incubated in an electroporation buffer containing pWI-GUS plasmid for 3 h with or without the condition of reduced air pressure. Electroporation was carried out with an electroporation device (CUY-21, NEPA GENE, Chiba Japan) (Figs. 25.2 and 25.3) that generates rectangular electric pulses, in a 1.0-cm-wide cuvette containing 1.0 ml of electroporation buffer. Rectangular wave pulses with various field strength, pulse length, and pulse numbers were applied to samples. The optimal electrical conditions for DNA delivery into wheat was estimated to be around 50 V/cm, 50 ms pulse duration, and 99 pulses. After electroporation, the seeds were incubated in a germination buffer for 2 days, after which they were transferred to a GUS assay buffer (Jefferson, 1987). When the seeds were electroporated after depressurization in a vacuum chamber, significantly higher GUS activity was detected (Fig. 25.4). Depressurization treatment prior to electroporation is crucial because it may enhance transport of the buffer into the intercellular space (Figs. 25.4 and 25.5).

To obtain stably transformed wheat and rice plant, pWI-H5K plasmid (Ugaki et al., 1994) was used. The plasmid was derived from pWI-GUS, which is fully

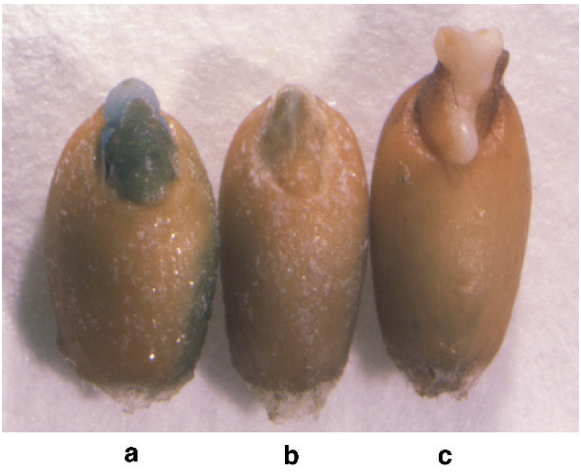

Fig. 25.4 GUS gene expression on wheat mature seeds. pWI-GUS Plasmid was introduced. (**a**) Depressurization pretreatment was made prior to electroporation. (**b**) Depressurization pretreatment was not made prior to electroporation. (**c**) Nontransformed control

described by Ugaki et al. (1991). The GUS gene cassette was removed from pWI-GUS and neomycin phosphotransferase NPT II gene was inserted into the plasmid as a selective marker. One hundred mature seeds of wheat were soaked in germination buffer and incubated at 10°C in the dark for 2 days. Then the seeds were incubated in electroporation buffer for 3 h under the condition of reduced air pressure. In one treatment 20 seeds were placed in an electroporation cuvette and electroporation was performed. Buffer composition and the physical parameters generally were the same as in the transient expression experiment mentioned above. After electroporation, the seeds were incubated in germination buffer for 2 days at 10°C Then the seeds were transferred to a selection medium containing 200 ppm geneticin sulfate (GIBCO) and incubated for 2 weeks in a growth cabinet. After the selection culture, germinated seeds were transplanted into pots with soil and were grown in growth chambers. During the heading period, fresh leaves were collected and genome DNA was extracted for PCR and Southern analysis to detect the introduced NPT II gene. In the wheat experiment, approximately 10,000 seeds were treated and 53 geneticin-resistant plants were regenerated. PCR analysis showed that 32 plants showed positive. Of these, 32 transformants, DNA samples from eight plants, to date were tested in Southern hybridization (Fig. 25.7) . There are high copy numbers of transgene integration in some plants. This may indicate that multiple copies of the transgene were present in the integration locus. The eight plants normally reached maturity and self-fertilized seeds have been obtained (Fig. 25.6). Two independent T_1 lines were examined for the transmission of NPT II gene by PCR analysis (Fig. 25.8). For each line, ten seeds were randomly chosen and seeded in a pot. Genome DNA was extracted from the seedlings and PCR analysis was made. Positive response was observed (Fig. 25.8) in all 20 samples. This confirmed the transmission of the introduced NPT II gene. These experiments have demonstrated that DNA has been delivered into the wheat mature embryos of mature seeds via electroporation with the help of depressurization treatment, and transgenic plants have been recovered. In rice experiments we obtained similar results (data not shown).

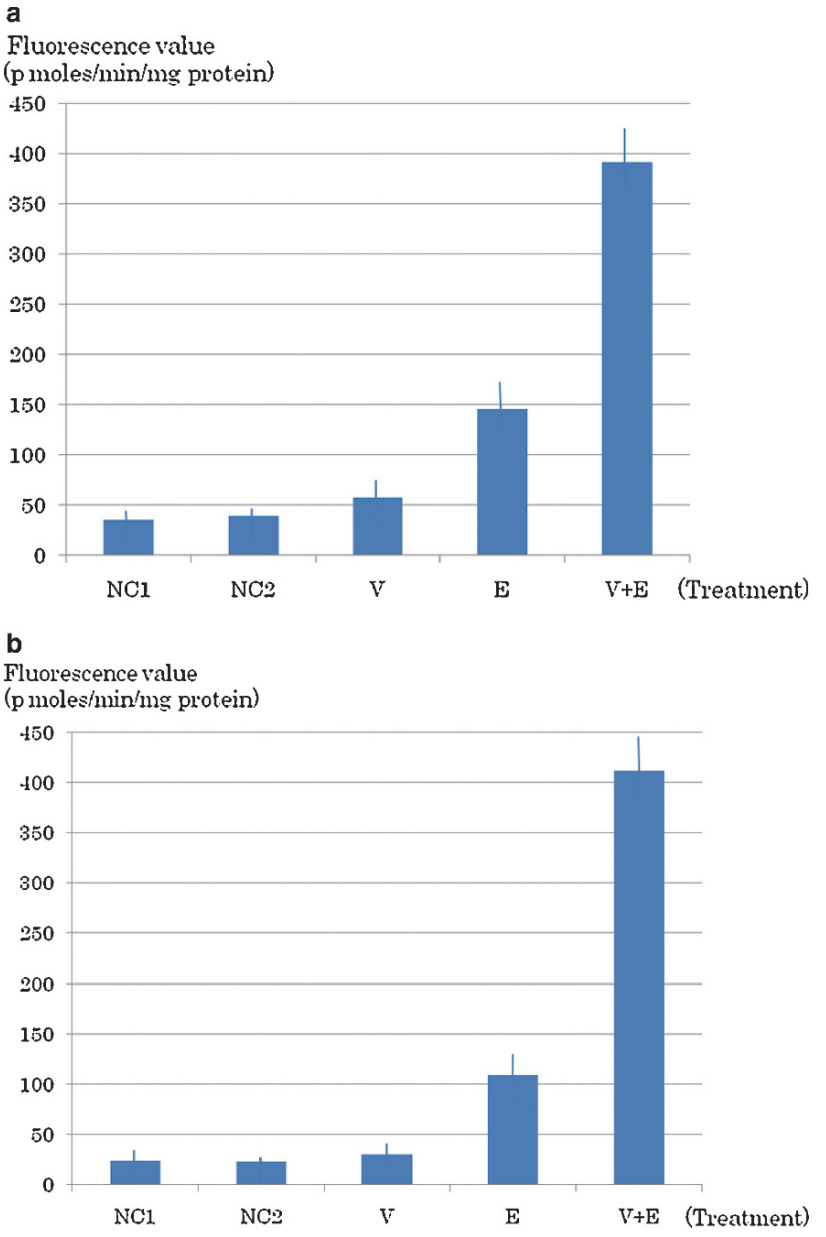

Fig. 25.5 Fluorometric assay of GUS gene expression in wheat (**a**) and rice (**b**). NC1: Negative control No.1 (the seeds were soaked in the electroporation buffer without containing plasmid DNA). NC2: Negative control No.2 (the seeds were soaked in the electroporation buffer containing plasmid DNA). V: The seeds were soaked in the electroporation buffer containing plasmid DNA and the vacuum treatment was made. E: The seeds were soaked in the electroporation buffer containing plasmid DNA before electroporation. V + E: The seeds were soaked in the electroporation buffer containing plasmid DNA and the vacuum treatment was performed before electroporation. Bar indicates standard error

Fig. 25.6 Fertile transgenic wheat

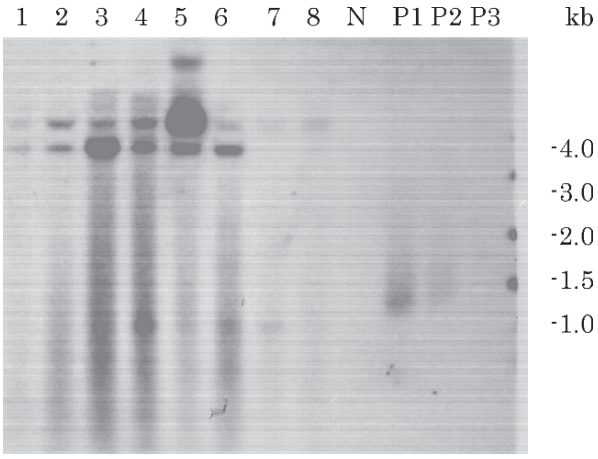

Fig. 25.7 Southern hybridization analysis of T_0 plants. Lane 1–8: DNA of T_0 plants digested with Xho I N: DNA of nontransgenic plant digested with Xho I ; P1–P3: fragment of NPT II (approx 1.0 kb) gene derived from the plasmid pWI H5K; P1: approximately equivalent to 10 copies of NPT II fragment; P2: approximately equivalent to two copies of NPT II fragment; P3: approximately equivalent to 1 copy NPT II fragment

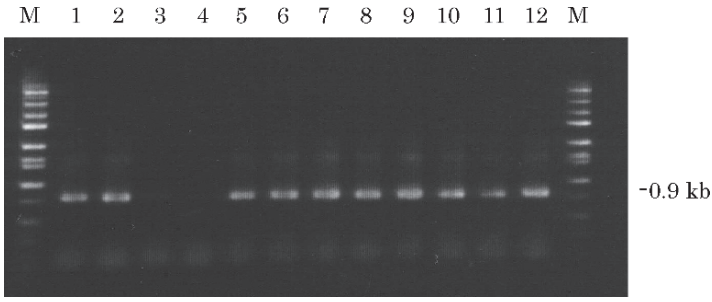

Fig. 25.8 PCR analysis of NPT II gene in the presence of T_1 plants. M: marker, lane1: positive control with plasmid pWI-H5K; lane 2: positive control with plasmid pWI-H5K and nontransgenic plant DNA; lane 3: negative control with water; lane 4: negative control with nontransgenic plant DNA; lane 5–8: T_1 plants from T_0 line No.1; lane 9–12: T_1 plants from T_0 line No.2

We also observed transient GUS gene expression in seeds of soybean, tomato, and *Brassica*, using pUC-derived plasmids (Chawla et al., 2002). Conditions of electroporation and the buffer contents were the same as in the case of wheat and rice. In about 10% of soybean seeds and 15% of *Brassica* and tomato seeds, blue loci, which were the indicative of transient GUS expression, were visually observed mainly in the shoot tip area.

This simple method of gene transfer in plants will be a promising direction for basic research and biotechnological applications. It has good potential for gene transfer to walled cells. A number of DNA delivery techniques have been developed for cereal transformation (Walden and Wingender, 1995). They include direct gene transfer by particle bombardment and *Agrobacterium*-mediated transformation. Successful results have been obtained by using these methods, but the methods are still not sufficiently reliable to be applied to a wide spectrum of plant species and/or varieties. Electroporation-mediated direct gene transfer has been developed and has been used in generating transgenic plants (Sorokin et al., 2000). So far, mostly protoplasts have been the target of direct gene transfer, and fewer reports on *in planta* transformation have been published (Sinclair et al., 2004). Target tissues of *in planta* transformation were immature embryos, nodal buds, inflorescences, and so forth, but using mature seeds with the help of depressurization treatment has yet to be reported.

Transgenic plant regeneration has been established using mature seeds of wheat and rice. Wheat and rice are the first- and/or second-ranking cereals by weight (FAO, 2002). Cereal breeding programs will benefit from new procedures for the generation of varieties with improved disease resistance, enhanced stress tolerance, and increased yield. Genetic manipulation will make significant contributions to these programs. The wheat transformation procedure presented here does not require the establishment of genotype-dependent tissue culture of immature embryos (Machii et al., 1998). To obtain immature embryos, plants need to be grown in fields or greenhouses for 5 to 8 months. This method may be especially useful for DNA delivery into the plant species that have poor tissue culture response. At present, this technique is dependent on a combination of factors including pretreatment of the targeted materials, plasmid constructs, and optimization of electroporation conditions. This method is simpler

and more rapid than conventional techniques, and it can be applied to a wide range of commercial rice and wheat varieties. These results also indicate that this technique has a potentiality of transferring foreign DNA into tissues of a wide range of organisms. Further study is in progress to improve this method in our laboratory.

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Chapter 26
RNA Interference in Chicken Embryos

Nick J. Van Hateren, Rachel S. Jones, and Stuart A. Wilson

1 Introduction

The chicken has played an important role in biological discoveries since the 17th century (Stern, 2005). Many investigations into vertebrate development have utilized the chicken due to the accessibility of the chick embryo and its ease of manipulation (Brown et al., 2003). However, the lack of genetic resources has often handicapped these studies and so the chick is frequently overlooked as a model organism for the analysis of vertebrate gene function in favor of mice or zebrafish. In the past six years this situation has altered dramatically with the generation of over half a million expressed sequence tags and >20,000 fully sequenced chicken cDNAs (Boardman et al. 2002; Caldwell et al., 2005; Hubbard et al., 2005) together with a 6X coverage genome sequence (Hillier et al., 2004). These resources have created a comprehensive catalogue of chicken genes with readily accessible cDNA and EST resources available via ARK-GENOMICS (www.ark-genomics.org) for the functional analysis of vertebrate gene function.

The chicken embryo is conveniently packaged in an egg shell and it is a relatively straightforward process to create a window in this shell. This allows access to the embryo at any stage of development and facilitates manipulation of the embryo. After this procedure, the window can be resealed and the egg incubated for a suitable time period prior to analyzing the results of the manipulation. Such manipulations traditionally involved "cut and paste" experiments in which tissue is excised and transplanted to ectopic locations in the embryo or from quail embryos, which are readily distinguished histologically, to chick embryos to generate chimeras. Whilst these studies have led to many important discoveries (Brown et al., 2003), there has recently been an increase in direct genetic manipulation approaches that can be applied to the chick embryo.

Manipulation of embryonic gene expression frequently involves over expression of wild type or dominant negative forms of cDNAs and, most recently, RNA inter-

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ference (RNAi) has been used to selectively knockdown the expression of genes. Both procedures involve the introduction of nucleic acids into the embryo which can be achieved using a variety of techniques. Retroviral vectors are commonly used for gene delivery, in particular the replication competent avian splice (RCAS) vectors, originally developed in Stephen Hughes' laboratory. These vectors are able to replicate in the embryo leading to widespread and stable delivery of a gene. Stocks of the virus can be prepared by transfection of the DF-1 cell line and then harvesting the viral supernatant for infection of embryos. Alternatively, the viral vector DNA can be directly introduced into the embryo, allowing subsequent expression of viral proteins and replication. The reader is directed to the Hughes lab website (www.retrovirus.info/RCAS/) for more detailed information about the RCAS system.

As an alternative to using RCAS viral supernatants for gene delivery, in ovo electroporation is now widely used for delivering nucleic acids to tissues in the chicken embryo (Itasaki et al., 1999). This technique uses 1–6 short (50–100 ms) pulses at low voltages and results in high levels of gene delivery and embryo viability. This technique has been used to introduce DNA into a wide range of embryonic tissues including neural, ectoderm, trunk, limbs and placodes (Itasaki et al., 1999). One of the easiest tissues to electroporate in the embryos is the developing neural tube which at early stages comprises an open tube, reminiscent of the well in an agarose gel, into which DNA can be readily injected and held prior to electroporation. Positioning of the electrodes for a neural tube electroporation is also straightforward and for these reasons, in ovo electroporation is very widely used to investigate neural tube development.

Some tissues prove difficult to electroporate and alternative methods for DNA transfection have been developed. An example is the hypoblast of the early embryo which structurally consists of a loose assembly of cells and lacks any form of lumen to act as a receptacle for DNA solutions prior to electroporation. To circumvent this difficulty, Albazerchi and colleagues have recently described a method using lipofectamine-mediated transfection to introduce DNA into the chick hypoblast (Albazerchi et al., 2007). An alternative to lipofectamine-mediated transfection is sonoporation where ultrasonic sound waves are used to transfect tissues. Ohta et al. have used this technique to transfect chick embryonic limb buds with GFP (Green fluorescent protein) and LacZ and have demonstrated efficient gene transduction (Ohta et al., 2003).

RNA interference is an extremely useful tool for the selective silencing of genes in higher eukaryotes; however, a significant barrier to carrying out RNAi in mammalian cells is the interferon response. This can be induced by the introduction of long (>50 bp) double stranded RNA into cells, a technique commonly used to elicit RNAi in *Caenorhabditis elegans*. Induction of the interferon response leads to widespread non-specific gene silencing, however the use of short interfering RNAs (siRNAs) bypasses this response in mammalian cells allowing efficient and specific gene silencing (Elbashir et al., 2001).

In ovo gene knockdown by RNAi was first reported six years ago and involved electroporation of siRNA duplexes into the developing neural tube. The siRNAs targeted the Rous sarcoma virus genome and inhibited retroviral infection (Hu et al., 2002).

It was subsequently reported that long double stranded RNAs could be used to selectively inhibit gene expression in the chicken embryos (Pekarik et al., 2003), which was surprising given that the interferon response was first discovered in chickens and chick embryos (Isaacs and Lindenmann, 1957).

Since the first reports of successful RNAi in the chicken embryo, a number of different plasmid and retroviral based systems have been described, utilizing a variety of promoters of mammalian and chick origin to drive expression of short hairpin RNAs (Katahira and Nakamura, 2003; Bron et al., 2004; Chesnutt and Niswander, 2004; Dai et al., 2005; Kudo and Sutou, 2005; Harpavat and Cepko, 2006).

In our laboratory we have developed a suite of plasmid and retroviral vectors specifically tailored for RNAi in the chicken (Das et al., 2006). The system involves the use of a chicken U6 promoter which we have shown is more efficient than mammalian U6 promoters in the chick embryo. In order to ensure optimal processing by DROSHA and incorporation of siRNAs into the RISC complex (RNA-induced silencing complex) we engineered a naturally occurring microRNA operon to accommodate synthetic hairpins based on human microRNA30 (Fig. 26.1d). We have made direct comparisons between conventional short hairpin RNAs and synthetic miRNAs for silencing several genes, driven from the same chick U6 promoter and find consistently that synthetic miRNAs based on miRNA30 work better in the chick embryo. Using this vector system we were able to demonstrate ~90% silencing of luciferase expression in the chicken cell line DF-1 and in the chick neural tube (Das et al., 2006).

The original publication describing our RNAi vectors mainly used the plasmid pRFPRNAiA. However, we have since developed a modified vector pRFPRNAiC which allows more efficient cloning of synthetic miRNAs (Fig. 26.1d and Shiau et al., 2008). This vector also carries a red fluorescent protein (RFP) marker which allows simple *in vivo* tracking of cells which have received the plasmid.

It may be desirable to knockdown two genes simultaneously to mitigate problems associated with gene redundancy. pRFPRNAiC is designed to allow the incorporation of a second synthetic miRNA into a single vector. Two different hairpins to a single gene can be cloned into a single vector to ensure optimal silencing. Alternatively, two different miRNAs to a single gene can be cloned into separate vectors and the plasmids can be combined for co-transfection or co-electroporation. In many cases generating two vectors in parallel is easier and faster than sequential cloning of miRNA hairpins into the same vector.

Whilst this chapter focuses on the use of pRFPRNAiC for silencing expression in the chick embryo it is worth noting that the entire miRNA expression cassette can easily be extracted from pRFPRNAiC on a *Not*I-*Cla*I fragment and subcloned into alternative backbone plasmid vectors or the RCASARNAi or RCASBRNAi retroviral vectors (Das et al., 2006). The use of retroviral vectors means RNAi can be carried out in tissues which are not readily electroporated and further can lead to stable long term gene silencing. The reader is directed to (www.retrovirus.info/RCAS/) for more details and protocols regarding the use of RCAS.

In this chapter we describe a series of protocols for the design, construction and electroporation of RNA interference vectors into the developing chick neural tube.

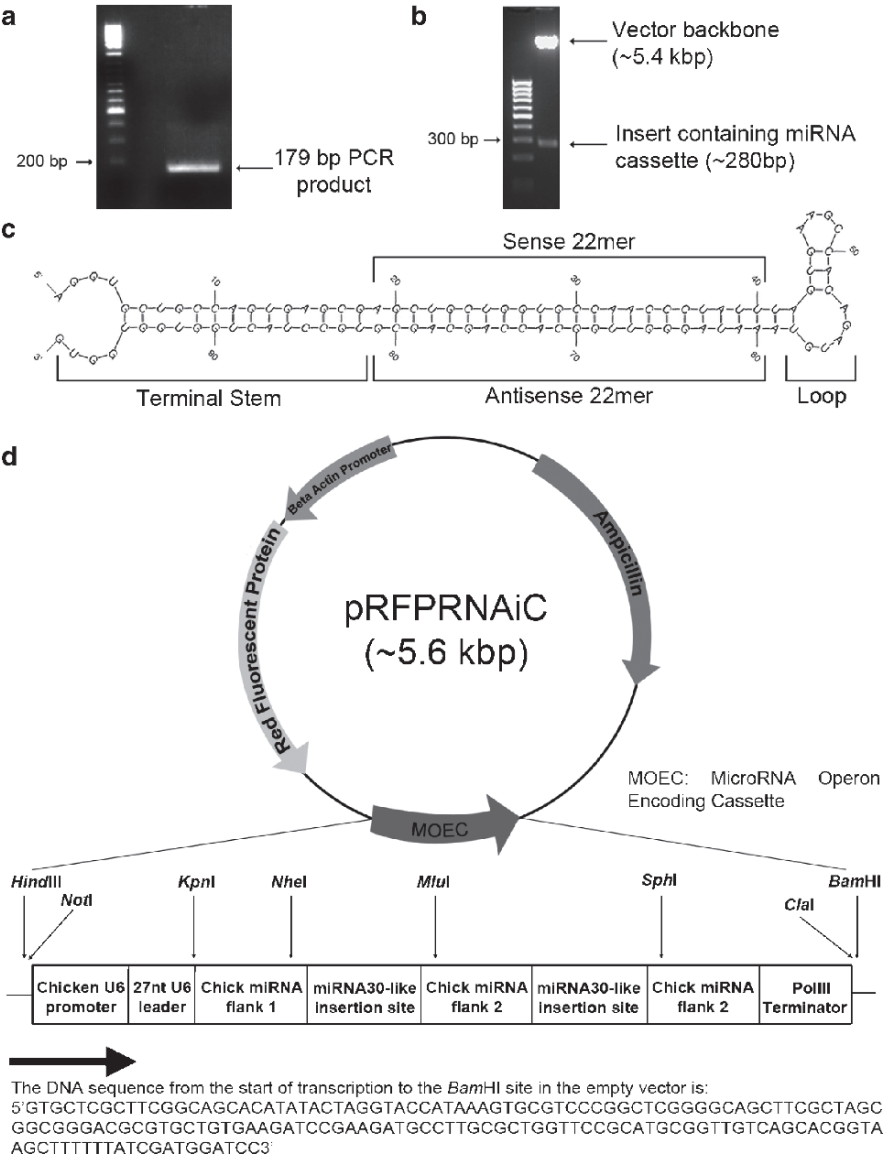

Fig. 26.1 Sub-cloning miRNAs into pPFPRNAiC. (**a**) PCR products obtained following annealing of universal and gene specific oligonucleotides. (**b**) Insert released after digestion of clones with *Bam*HI and *Kpn*I. (**c**) Structure of miRNA hairpin targeting firefly luciferase expression showing sense and antisense strands as well as common loop and terminal stem sequence. (**d**) Map of pRFPRNAiC showing the microRNA operon expression cassette (MOEC) and the sequence from the start of the MOEC to the *Bam*HI site

We also describe two methods for analyzing the results of gene knockdown after electroporation: wholemount *in situ* hybridization and immunohistochemistry. These methods of RNAi-mediated gene knockdown produce a significant knockdown of the target gene which is transient but is likely to persist for ~96 h post electroporation. For many developmental biology applications in the chick, this is a sufficiently long time frame.

2 Procedure

2.1 Generation of RNAi Vectors

2.1.1 Target Sequence Choice and Oligonucleotide Design

1. The nucleotide sequence of the target gene may be obtained from various sources including the Ensembl database (www.ensembl.org) which is an automated annotation system for eukaryotic genomes that includes the chicken genome. As the annotation of genes is automatic, there may be discrepancies between different releases of the database, especially for recently sequenced organisms such as the chicken. For this reason, it is advisable to search for an EST or cDNA clone of the gene of interest which will allow verification of the Ensembl data. This can be achieved by searching databases such as NCBI (www.ncbi.nlm.nih.gov) or the collection of chicken ESTs and cDNAs available at www.chick.umist.ac.uk (Boardman et al., 2002), bearing in mind that only a partial cDNA sequence is required to generate RNAi vectors.
2. The miRNA target sequences for the gene of interest can be obtained using an online RNAi target finder program such as the one provided by Genscript (www.genscript.com/ssl-bin/app/rnai). This target finder program uses complete or partial cDNA sequences and allows target sequences to be chosen within the open reading frame or the untranslated regions (UTRs). The use of target sequences in the UTRs is advantageous because it allows subsequent rescue experiments to be performed using a cDNA expression vector which lacks the UTR sequences. The target sequence selected should be 22 bases long with a GC percentage between 30% and 60% and these options can be selected using the Genscript program. In addition, the program performs a BLAST search with each miRNA target sequence against the genome of the chosen organism to ensure target sequences do not recognize other genes, causing off-target effects. The relevant organism should be selected to ensure the BLAST search generates reliable target sequences. The output of the program is a list of possible target sequences which are scored and ranked according to how efficient they are likely to be for gene silencing. We recommend using the highest scoring sequences for designing vectors.
3. Once a candidate target sequence has been identified the gene should be checked for single nucleotide polymorphisms (SNPs) contained within this sequence. SNPs can be identified using ENSEMBL which derives its data from a large

scale SNP discovery project generated from sequencing various chick genomes (Wong et al., 2004). Further SNP information can be obtained from the chick EST/cDNA website at www.chick.umist.ac.uk whose SNP data is derived from comparison of multiple ESTs. The presence of a SNP in the region of a target sequence could result in reduced efficiency of knockdown in embryos with a certain allele for the SNP and therefore such target sequences should be avoided.

At least three and ideally four different target sequences should be chosen to maximize the probability of generating vectors that mediate efficient knockdown. Therefore, the top four target sequences (lacking SNPs) generated by the target finder program should be used to design gene specific oligonucleotides to generate miRNA coding regions. The miRNAs that are to be cloned mimic human microRNA30, and consequently contain a mismatch in the first base of the sense strand. Therefore if the first base at the 5′ end of the target sequence is A, it should be changed to C and *vice versa*. If it is G, it should be changed to T and *vice versa*. The gene specific oligonucleotides contain common sequences that form part of the miRNA flanking sequences and also the common loop and terminal stem sequences (see Fig. 26.1c). For the first cloning site of pRFPRNAiC, the gene specific oligonucleotides can then be designed as follows:

Forward
5′-AGGTGCTGCCAGTGAGCG <insert target sequence with mismatch>
TAGTGAAGCCACAGATGTA-3′

Reverse
5′-CACCACCACCAGTAGGCA <insert target sequence>
TACATCTGTGGCTTCACT-3′

An example for a miRNA hairpin targeting luciferase expression is given below.
Target sequence:
5′-CGCTGCTGGTGCCAACCCTATT-3′

Mismatch sequence:
5′-AGCTGCTGGTGCCAACCCTATT-3′

Luc Forward
5′-AGGTGCTGCCAGTGAGCG **AGCTGCTGGTGCCAACCCTATT**
TAGTGAAGCCACAGATGTA-3′

Luc Reverse
5′-CACCACCACCAGTAGGCA **CGCTGCTGGTGCCAACCCTATT**
TACATCTGTGGCTTCACT-3′

The gene specific oligonucleotides for the second cloning site of pRFPRNAiC can be designed as follows:

Forward
5′-GTTCCTCCGCAGTGAGCG <insert target sequence with mismatch>
TAGTGAAGCCACAGATGTA 3′

Reverse
5′-GAAGACCAGCAGTAGGCA <insert target sequence>
TACATCTGTGGCTTCACT 3′

4. In addition to the gene-specific oligonucleotides, two universal oligonucleotides are required to amplify the miRNA hairpin sequences. These oligonucleotides are common for all target genes.

The universal oligonucleotides for cloning into the first site are as follows:

U1F
5′ GGCGGGGCTAGCTGGAGAAGATGCCTTCCGGAGAGGTGCTGCCAG
TGAGCG 3′

U1R
5′ GGGTGGACGCGTAAGAGGGGAAGAAAGCTTCTAACCCCGCTATTC
ACCACCACCAGTAGGCA 3′

The universal oligonucleotides for the second cloning site are as follows:

U2F
5′ GGCGGGACGCGTGCTGTGAAGATCCGAAGATGCCTTGCGCTGGTTCC
TCCGCAGTGAGCG 3′

U2R
5′ CGCCGCGCATGCACCAAGCAGAGCAGCCTGAAGACCAGCAGTAGGCA
3′

The universal oligonucleotides contain unstructured chicken miRNA flanking sequences which are known to be important for processing of the miRNA precursors (Zeng and Cullen, 2005).

2.1.2 Vector Construction

Materials
- 10 mM dNTP mix (containing dATP, dCTP, dGTP and dTTP)
- Agarose
- Plasmid Miniprep kit (Qiagen)
- Gel extraction kit (Qiagen)
- pRFPRNAiC – available from www.ark-genomics.org together with other RNAi vectors published in (Das et al., 2006)
- Oligonucleotides as described above
- Restriction enzymes: *Nhe*I, *Mlu*I, *Kpn*I, *Bam*HI, *Sph*I (Roche)
- *Pfu* DNA polymerase (Promega)
- T4 DNA ligase (Roche)
- Calf intestinal alkaline phosphatase (Roche)
- BigDye® Terminator v3.1 sequencing kit (Applied Biosystems)
- Phenol:Chloroform:Isoamyl Alcohol (25:24:1) pH 8
- 3 M Sodium Acetate pH 5.2

- Ethanol (70%, absolute)
- Benchtop centrifuge
- DH5α chemically competent *E. coli* cells

Method

(A) Cloning into the First Hairpin Site

Preparation of vector backbone

1. Digest 1 µg of pRFPRNAiC with 25 units each of *Nhe*I and *Mlu*I in a 100 µl reaction and incubate at 37°C for 2 h. It is a good idea to set up two single digests with 25 units of each enzyme in parallel as a control to confirm that both enzymes are digesting the vector equally well. After incubation run 5 µl of each reaction along with 50 ng of undigested plasmid on a 0.5% agarose gel. The remainder of the single digests may then be discarded. All three digests should produce a single band of linearised DNA. If more than one band is observed, the plasmid has not digested completely and the digest must be repeated with a fresh sample of plasmid DNA.
2. To dephosphorylate the digested plasmid ends add 2 units of calf intestinal alkaline phosphatase to the remaining digested DNA and incubate for 15 min at 37°C. This step is required to prevent the short fragment between the *Nhe*I and *Mlu*I sites reinserting into the vector.
3. To extract DNA from the reaction, add 100 µl 25:24:1 Phenol: Chloroform: Isoamyl Alcohol (pH 8) solution to digested plasmid and vortex thoroughly. Centrifuge at full speed for 1 min to separate the two liquid phases. Recover the upper (aqueous) phase with a pipette and transfer to a new microfuge tube, the lower phase may be discarded.
4. Precipitate digested DNA in 0.1 volumes of 3 M Sodium acetate (pH 5.2) and 2 volumes of absolute ethanol. Mix briefly by inverting and incubate on ice for 10 min. Centrifuge at full speed using a bench top centrifuge for 10 min, discard supernatant and wash pellet with 70% ethanol. Centrifuge again at full speed for 5 min, discard supernatant and air dry pellet. Resuspend the pellet in 10 µl dH_2O. This digested DNA is now ready for ligation and provides enough digested plasmid for 10 ligations.

Preparation of miRNA hairpin

1. To amplify the miRNA hairpin encoding sequence, carry out a polymerase chain reaction (PCR) using both gene specific primers and the universal primers for the first hairpin site.

   10 ng each gene specific primer
   100 ng each of U1F and U1R universal oligonucleotides
   5 µl 10× dNTP mix
   5 µl reaction buffer
   1 unit *Pfu* DNA polymerase
   Make volume to 50 µl with dH_2O
   PCR conditions:

94°C for 30 s
55°C for 30 s
72°C for 45 s
Repeat 40 times

In this reaction we use 10 times more universal primer than the gene specific primers to ensure that the reaction is biased towards generating a complete miRNA encoding sequence rather than the smaller product formed by annealing of only the gene specific primers. Separate the PCR products on a 2% agarose gel. The PCR amplification is not always clean and may contain more than one band. The 179 bp band (Fig. 26.1a) is the correct product and should be excised and the DNA extracted using a gel extraction kit such as the one manufactured by Qiagen. Whilst we use *Pfu* for amplification, other proof reading thermostable polymerases would suffice.

2. Digest extracted DNA overnight at 37°C with 25 units each of *Nhe*I and *Mlu*I in a 100 µl reaction.
3. Extract DNA using phenol:chloroform and then ethanol precipitate as detailed in the protocol for vector linearization however the DNA pellet is resuspended in 7 µl dH_2O. The PCR product is now ready for ligation into the linearised vector.

Ligation and sequence verification

1. Ligate the amplified hairpin into the linearized vector using the following reaction:

   7 µl amplified hairpin
   1 µl linearized vector (~50–100 ng)
   1 µl reaction buffer
   1 µl T4 DNA ligase
   Incubate at room temperature overnight
2. Transform 5 µl of the ligation reaction into competent *E. coli* (DH5α strain) and spread on LB-agar plates containing 50 µg/ml ampicillin.
3. Pick colonies into 2 ml LB broth containing 50 µg/ml ampicillin, incubate overnight at 37°C and extract plasmid DNA using a miniprep kit such as the one manufactured by Qiagen.
4. Digest 10 µl of the miniprep DNA with 10 units of *Kpn*I and *Bam*HI in a 30 µl reaction for 1 h at 37°C and resolve the DNA fragments on a 1% agarose gel. This digest releases the entire microRNA operon expressing cassette (MOEC) without the promoter. Clones with correct insert will release a 280 bp fragment whereas clones lacking an insert release a 130 bp fragment (Fig. 26.1b).
5. Sequence the clones containing an insert of the correct size using the sequencing primer:

   5′-ACAGTCACTGTGTTCTAAAAGAACTTG-3′

   This primer anneals to the 3′ end of the U6 promoter that drives expression of the microRNA operon expressing cassette. Set up sequencing reactions as follows:
   0.5–1 µg template DNA (5 µl of miniprep)

1 µl sequencing primer at 3.2 pmol/µl (1:35 dilution of 1 µg/µl stock solution)
4 µl Big Dye® terminator sequencing mix v3.1
Make volume to 10 µl with dH_2O
PCR conditions for sequencing are as follows:
96°C for 30 s
50°C for 15 s
60°C for 4 min
Repeat 45 times

6. To clean up the sequencing reactions, prepare the following master mix, which is enough to clean up 10 reactions:

30 µl 3 M sodium acetate pH 5.2
245 µl dH_2O
625 µl absolute ethanol
Vortex briefly

Add 90 µl of this solution to each reaction, vortex very briefly and leave at room temperature for 20 min. Centrifuge at full speed in a bench top centrifuge for 20 min, wash with 150 µl 70% ethanol, vortex very briefly and centrifuge again for 20 min and air dry pellets. The reactions are now ready to be run on a sequencing machine. It is essential to verify the correct sequence as errors made by the polymerase could alter the target specificity of the miRNA hairpins. The full sequence of the transcribed MOEC without an insert is shown in Fig. 26.1d.

(B) Cloning into the Second Hairpin Site

1. To clone miRNA hairpin sequences into the second site of pRFPRNAiC, follow the same steps as above. The PCR reaction should include gene specific and universal primers for the second site, the plasmid must be linearized with *Mlu*I and *Sph*I and the PCR products digested with the same enzymes.

2.2 In Ovo Electroporation of Chicken Embryos

This protocol focuses on electroporation of the chicken neural tube. Several other tissues including the limb and somites may be electroporated by following this protocol (Itasaki et al., 1999) with modifications to the site of DNA injection and adjusting the pulsing parameters as necessary.

Where several different RNAi vectors have been prepared against a single gene these can initially be mixed for electroporation. We have mixed up to three plasmids in this way and still achieved efficient silencing although we would ideally recommend using two plasmids at a time. Care should be taken to ensure that the DNA concentration used does not exceed 0.5 µg/µl. We have observed that using these RNAi vectors at a concentration of more than 0.5 µg/µl leads to excessive amounts of RFP in the electroporated side of the neural tube. This can cause cellular damage and cell death of the transfected cells and so can produce artefactual results. Therefore, it is

important to determine the concentration of RNAi vectors empirically depending on the tissue to be targeted. We have also noted that using high concentrations of RNAi vector (more than 0.5 µg/µl) can lead to very intense RFP fluorescence which can bleed through into other fluorescent channels when embryo sections are visualized under UV light e.g. for immunofluorescence analysis of gene knockdown. Generally a total DNA concentration of 0.15–0.25 µg/µl is sufficient to generate efficient gene knockdown in the neural tube, whilst avoiding toxic effects on the embryo. In fact we have observed that luciferase gene expression can be silenced reasonably efficiently with levels of the RNAi vector as low as 10 ng/µl (Das et al., 2006).

Once an RNAi knockdown has been observed the vectors should be electroporated individually to identify which ones are active. It is an important control to be able to demonstrate that two different vectors targeting different sequences can silence the target gene. This control minimizes the possibility that any phenotype observed is due to an off-target effect i.e. silencing of a gene other than the intended target.

Materials

- Midi-prep plasmid DNA (~0.25 µg/µl) containing Fast-green dye (1/200 w/v)
- Fertile chicken eggs
- Incubator at 38°C
- Stereomicroscope (e.g. Leica MZ6)
- Intracel TSS20 Ovodyne electroporator or similar
- Micromanipulator to hold electrodes
- Gold plated electrodes 5 mm, bent (Genetrodes, Genetronics)
- Mouth pipette and pulled glass micropipette
- Curved Forceps, springbow scissors, fine forceps
- Gentamycin solution (1 mg/ml) (Sigma)
- Optional: Indian ink diluted 1:20 in 1x PBS (phosphate buffered saline)
- 10 ml syringe with 21G needle, 1 ml syringe with 25G needle
- Parafilm
- Sellotape
- Ethanol (70%)
- Egg holder i.e. plastic cap from media bottle or polystyrene ring
- 90 mm Petri dish
- L-15 medium (Gibco)
- 24-Well tissue culture plate (Corning)
- 4% Paraformaldehyde (PFA) solution prepared in 1x PBS at pH 7.4

Method

1. Fertile chicken eggs are available from several suppliers, depending on location. Our laboratory use White Leghorn eggs supplied by Henry Stewart, U.K. Once delivered, eggs are stored in the cardboard egg cartons in which they are shipped, at 18°C in a humidified room until incubation begins.
2. Eggs are incubated at 38°C for 45 h until they reach Stage 10–12 according to (Hamburger and Hamilton, 1951). Eggs are incubated with the pointed side of the egg downwards in cardboard egg cartons. A programmable incubator

(our lab uses a Sanyo MIR-153 incubator) is used to ensure that eggs reach the correct stage at a convenient time of the day.

3. Glass micropipettes are prepared from borosilicate glass capillaries (we use type TW100-4 available from World Precision Instruments). Each capillary is pulled using a Fleming/Brown P-97 glass micropipette puller to generate two micropipettes with the pulled end of each capillary being around 10 mm long. Alternatively, it is possible to pull the capillaries over a Bunsen burner flame. Fine forceps are used to remove 1–2 mm from the end of the glass micropipette and the micropipette is placed into the holder of the mouth pipette. The mouth pipette is used to aspirate approx 1–2 µl of DNA solution into the glass micropipette. Fast Green dye is added to the DNA solution at a 1:200 dilution to aid visualization of DNA solution for injection into the chicken embryo. A clean micropipette should be used for each DNA sample.
4. The egg is transferred to an egg holder under the microscope (Fig. 26.2a), ensuring that the pointed side of the egg is downwards, and the top of the egg is washed with 70% ethanol to sterilize. A small hole (approx. 2 cm in diameter) is made in the top of the egg using curved forceps, to reveal the air pocket overlaying the embryo. The thick shell membrane that overlays the albumin of the egg is removed using fine forceps, ensuring that the vitelline membrane surrounding the embryo is not damaged (Fig. 26.2c1).
5. Optional: to visualize the embryo more clearly, 100–200 µl of Indian ink (1/20 dilution in PBS) may be injected under the embryo using a 1 ml syringe and 25G needle.
6. The glass micropipette is placed in the lumen of the neural tube midway between the head and tail of the embryo (Fig. 26.2c2) and DNA solution is transferred into the neural tube using the mouth pipette such that the DNA solution fills a large portion of the neural tube.
7. The electrodes are placed either side of the embryo approximately 1–2 mm away from the embryo ensuring that the electrodes do not overlap the heart field (as shown in Fig. 26.2b and c3). The electrodes are lowered onto the embryo so that they make good contact with the membranes surrounding the embryo and the anode is positioned slightly lower than the cathode to ensure the current passes through as much neural tissue as possible.
8. The electroporator is used to pulse the embryo with square pulses of a suitable current. The current employed varies according to the stage of the embryo and should be determined empirically to maximize electroporation efficiency whilst ensuring good viability of the embryos. Typically, we use six pulses of 27 V with each pulse being 50 ms in duration and with 950 ms between pulses. During the electroporation, the electrodes should be slowly lowered slightly further onto the embryo to ensure good electroporation along the dorsal-ventral axis of the neural tube.
9. The electrodes are slowly lifted off the embryo and retracted from the egg. One drop of Gentamycin solution (1 mg/ml) is placed onto the embryo using a plastic Pasteur pipette and then approximately 1 ml of albumin is aspirated using a 10 ml syringe to ensure that the embryo does not touch the surface of the egg when it is resealed.

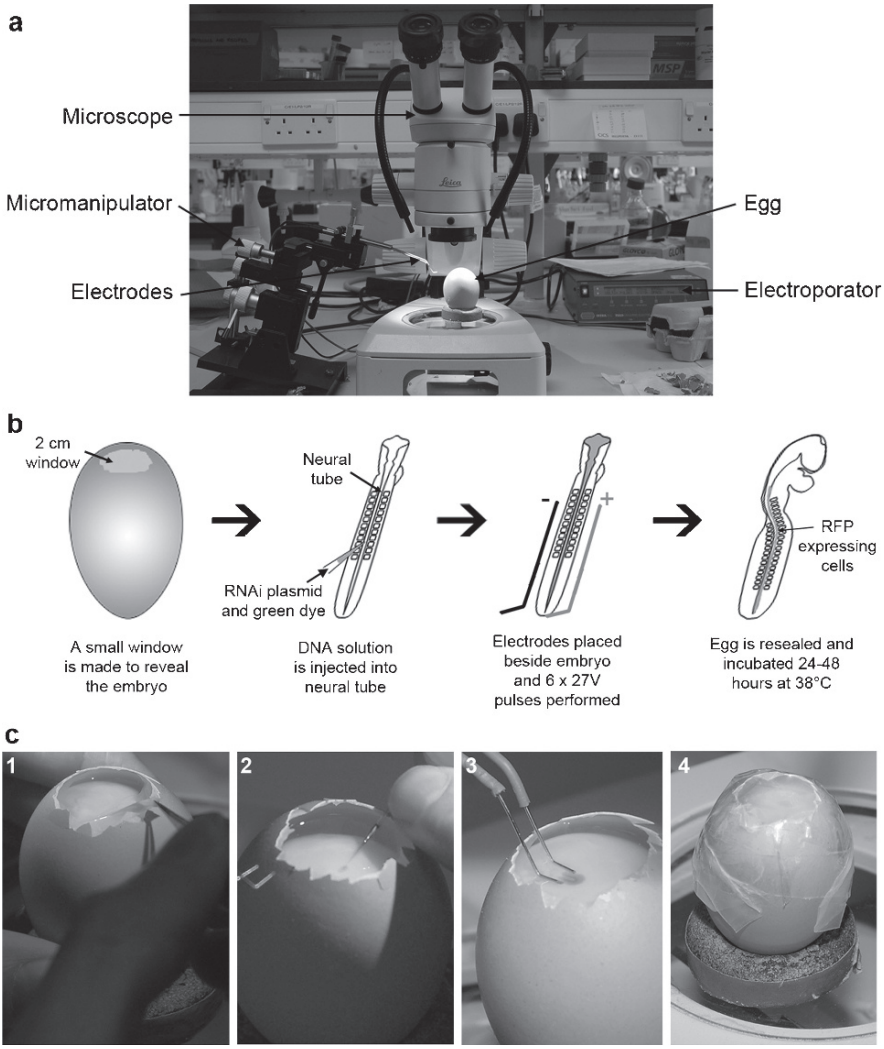

Fig. 26.2 Chick in ovo electroporation. (**a**) Photograph of electroporation apparatus set-up including key components. (**b**) Schematic procedure for chick electroporation showing principal stages. (**c**) Step-by-step photographs showing detail of the electroporation procedure. (**1**) Removal of shell membrane from a windowed egg; (**2**) injection of RNAi plasmid mixture into chicken neural tube; (**3**) placement of electrodes either side of embryo; (**4**) an egg resealed with parafilm and Sellotape, ready for incubation

10. Parafilm is used to reseal the egg and is secured in place with clear sticky tape such as Sellotape, ensuring that a large portion of the egg shell remains uncovered to allow air to permeate into the egg (Fig. 26.2c4).
11. The egg is returned to the incubator in a cardboard egg box to ensure that it remains upright and incubated at 38°C for a further 24–48 h. Typically, 24 h of

incubation is sufficient to see some knockdown of the target gene. The RNAi vectors used here remain active for at least 96 h of incubation so a longer incubation is possible if required.

12. Following incubation, the embryo is harvested by cutting away the seal on the top of the egg and then cutting around the embryo using fine spring scissors. The embryo is then transferred to a Petri dish containing ice-cold L-15 medium using a wide bore Pasteur pipette and the membranes surrounding the embryo are removed using fine forceps and/or small springbow scissors. This step is required to ensure efficient fixation of the embryo. The embryo is transferred into a 24-well plate containing ice-cold 4% paraformaldehyde/ PBS at pH 7.4 and embryos are fixed for 2 h at 4°C (for immunohistochemistry) or overnight at 4°C (for wholemount *in situ* hybridization).

2.3 Validation of Gene Silencing

2.3.1 *In Situ* Hybridisation

The best way to confirm that a gene has been silenced in the embryo is to carry out immunohistochemistry with an antibody specific for the target gene (Section 2.3.2). However, such antibodies are not always available. In these situations we advise confirming knockdown of the gene of interest using *in situ* hybridization with a probe generated from an EST or cDNA clone. The large collection of chicken ESTs generated by Boardman et al. (Boardman et al., 2002) are conveniently cloned into vectors with flanking T7 and T3 bacteriophage promoters which allow simple *in vitro* transcription of labelled antisense probes suitable for *in situ* hybridization.

Materials

- 4% PFA in PBS (pH7.4)
- 7 ml Bijou tube
- PBT (1x PBS + 0.1% Tween 20)
- Methanol
- Digoxygenin-labelled antisense riboprobe specific for the gene of interest (synthesized using an *in vitro* transcription kit such as the one supplied by Roche).
- Prehybridization solution (50% formamide, 5x SSC pH7.0 [20x SSC stock: 175.3 g NaCl and 88.2 g sodium citrate in 1 l distilled water], 2% blocking powder (Roche), 0.1% Triton X-100, 0.5% CHAPS (Sigma), 1 mg/ml Yeast tRNA, 5 mM EDTA, 50 µg/ml heparin).
- Wash Solution 1 (50% Formamide, 5x SSC pH4.5, 1% SDS)
- Wash Solution 2 (50% Formamide, 2x SSC pH4.5, 1% Tween 20)
- 1x TBST (1x TBS [10x TBS stock: 8 g NaCl, 0.2 g KCl, 25 ml 1 M Tris–HCl pH7.5 in 100 ml of sterile distilled water], 1% Tween 20)
- Block solution (10% heat inactivated goat serum (HINGS) in 1x TBST)
- Anti-digoxygenin F_{ab} antibody fragments conjugated to alkaline phosphatase (Roche)

- Glass embryo dishes coated with Repelcote (BDH)
- NTMT solution (0.1 M NaCl, 0.1 M Tris–HCl pH 9.5, 0.05 M Magnesium chloride, 1% Tween 20)
- NBT (Nitro blue tetrazolium chloride, 1.35 mg/ml final concentration (Roche)) and BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt, 0.175 mg/ml final concentration (Roche)) staining solution made up in NTMT

Method

NB. All washes are performed for 5 min at room temperature unless otherwise stated.

1. Fix embryos in fresh 4% PFA overnight at 4°C.
2. Wash twice in PBT for 5 min at 4°C and then take embryos through an ascending methanol/PBT series (25%, 50%, 75%, 100% methanol and wash embryos for 30 min at 4°C in each solution). Store embryos in 100% methanol at −20°C overnight.
3. Rehydrate embryos using descending methanol/PBT series (70%, 50%, 25% methanol) then wash twice in PBT, all washes are 30 min at 4°C. Add 1 ml prehybridization solution to embryos and incubate for 1–2 h at 70°C with very gentle shaking. Embryos can be stored in prehybridization solution at −20°C before the prehybridization step, if required.
4. Add 0.2–1 µg of DIG-labelled antisense riboprobe specific for the gene of interest to 10 µl prehybridization solution and warm in 37°C heating block for 5 min. Add this to embryos in prehybridization solution and incubate overnight at 70°C with very gentle shaking.
5. Wash twice with Wash Solution 1 for 30 min at 70°C then wash twice with Wash Solution 2 for 30 min at 70°C. Finally, wash three times with 1x TBST for 5 min at room temperature with gentle rocking/shaking.
6. Pre-block embryos with fresh Block solution for 60–90 mins at room temperature with gentle shaking. Remove this Block solution and replace with fresh Block solution containing 1:2000 of anti-Digoxygenin AP F_{ab} fragments, store overnight at 4°C with gentle rocking.
7. Wash three times with 1x TBST followed by five 1 h washes in 1x TBST at room temperature (all washes are performed with gentle rocking/shaking). Leave embryos washing overnight at 4°C.
8. Transfer embryos to an embryo dish and wash three times with NTMT for 10 min at room temperature. Replace this solution with NTMT containing the color reagents NBT and BCIP and keep embryos in the dark at room temperature without rocking or shaking.
9. When color has developed to desired extent, wash twice with PBT. Embryos can then be photographed under a dissecting microscope to visualize the effects of any gene knockdown (Fig. 26.3b).
10. The embryos can be washed overnight in 30% sucrose at 4°C and then mounted for cryosectioning (as detailed in Section 2.3.2). Once sectioned, the *in situ* staining can be visualized in cross-section and immunohistochemistry can be performed with an anti-RFP antibody to determine which cells have received the RNAi knockdown construct (Fig. 26.3c).

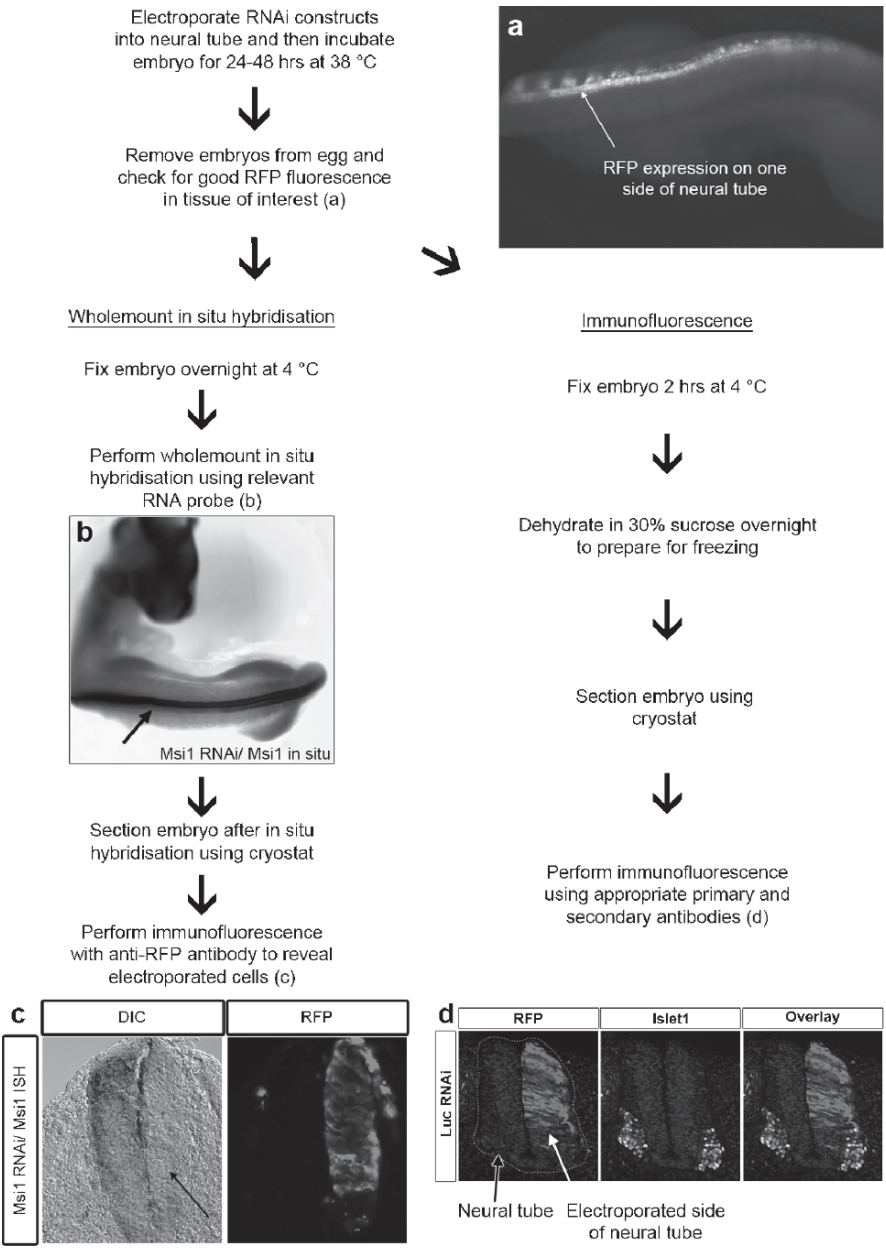

Fig. 26.3 Analysis of results following RNAi. A flow-chart describes the principal stages in analysis of the knockdown using *in situ* hybridization or immunohistochemistry where an appropriate antibody to the target protein is available. (**a**) RFP fluorescence is observed in one side of the neural tube only. (**b**) An embryo after wholemount *in situ* hybridization with a Musashi-1 (*Msi1*) antisense riboprobe. Expression is observed in the neural tube of the embryo with a loss of expression observed in the electroporated region of the neural tube (*arrowed*).

2.3.2 Embryo Sectioning and Analysis with Antibodies

Materials

- 30% sucrose (w/v) in 0.2 M Phosphate buffer pH 7.4 [21.8 g Na_2HPO_4, 6.4 g NaH_2PO_4 in 1 l distilled water]
- Epi-fluorescence dissecting microscope
- OCT mounting medium (VWR)
- Dry ice pellets
- Cryostat and specimen holders ("chucks")
- Superfrost Plus slides (VWR)
- Heat inactivated goat serum (HINGS) (Sigma) – prepare by treating goat serum at 60°C for 20 min
- 20% Triton X-100 (Sigma)
- Primary antibody specific for protein of interest
- Relevant secondary antibody conjugated to fluorescent dye e.g. AlexaFluor488 (Invitrogen)
- DAPI solution or TOPRO-3 solution to counterstain cell nuclei (Invitrogen)
- Glass coverslips (22 × 64 mm No.1)
- Vectashield mounting medium (Vector labs)
- Light-tight box for hybridization
- Epi-fluorescence compound microscope or confocal microscope for visualization

Method

1. After fixing electroporated embryos in 4% PFA/PBS for 2 h at 4°C, wash embryos three times for 5 min with ice-cold 1x PBS. Replace the PBS solution with 30% sucrose solution and store embryos overnight at 4°C to dehydrate and prepare the tissue for freezing.
2. Transfer embryos individually to a small Petri dish and examine using fluorescence microscope to dissect out the electroporated region of the embryo (marked by expression of Red Fluorescent Protein (RFP) – Fig. 26.3a). Transfer this segment of the embryo into a small dish containing a small amount of OCT mounting medium.
3. Chill specimen holder for cryostat in an ice box containing dry ice pellets. Add some room temperature OCT medium to the surface of the specimen holder and transfer the embryo segment into the medium. Quickly orientate the embryo segment such that the required sectioning plane is parallel to the top

Fig. 26.3 (continued) (**c**) Immunofluorescence can be performed on cryosectioned embryos after *in situ* hybridization to allow visualization of RFP expression in the neural tube. RFP expression is observed on the side of the neural tube lacking *Msi1* expression (*arrow*) demonstrating a knockdown of *Msi1* mRNA by the *Msi1* RNAi vectors. (**d**) Analysis of phenotype following RNAi using immunofluorescence. Cryosections of neural tubes targeted by luciferase RNAi show RFP expression on one side of the neural tube only (*first panel*). Antibody staining with an antibody to Islet-1 shows no change in expression on the electroporated side of the neural tube compared to the unelectroporated side (*second and third panels*)

of the specimen holder and monitor the orientation of the sample to ensure it does not move whilst the OCT freezes (turns opaque white), this takes around 30–60 s.

4. Mounted specimens can be stored at −80°C for several weeks or can be sectioned immediately using a cryostat.
5. Section the embryo at a thickness of 15–20 µm and collect the sections on Superfrost Plus slides. When sectioning an embryo after wholemount *in situ* hybridization, it may be necessary to increase the section thickness to 25–30 µm in order to visualize weakly stained tissues. Leave slides at room temperature in the dark for 60 min to allow sections to adhere to the slides properly.
6. Place the slides horizontally in a humidified, light-tight box and lay the slides on top of sections of plastic pipette to ensure wash solution does not seep off of the slides by capillary action. Wash the slides horizontally with 500 µl–1 ml of 1x PBS three times for 5 min at room temperature in the dark.
7. Incubate slides with 500 µl PBS2 + solution (1x PBS, 1% HINGS, 0.1% Triton X-100) containing an appropriate dilution of primary antibody, store overnight at 4°C.
8. Wash slides three times for 5 min with 1x PBS and then incubate slides 30 min at room temperature in 500 µl PBS2 + containing an appropriate dilution of a relevant secondary antibody conjugated to a fluorescent dye and 1/10,000 dilution of DAPI solution or 1/2,000 dilution of TOPRO-3 solution to counterstain nuclei.
9. Wash slides three times for 5 min with 1x PBS at room temperature and then coverslip the slides using Vectashield mounting medium.
10. Visualize slides using a fluorescence microscope to determine the effect of the gene knockdown (Fig. 26.3d).

3 Conclusion

The protocols described in this chapter allow the expression of a particular gene of interest to be knocked down by RNAi in the chicken neural tube. The electroporation conditions may be varied to target different regions of the neural tube or other tissues. The knockdown can be validated by examining the expression of the target gene by *in situ* hybridization or immunohistochemistry. Once knockdown of the target gene is confirmed, further standard molecular techniques can be used to study the downstream effects of the knockdown. These techniques might include expression analysis of downstream target genes/proteins or further phenotypic analysis.

It may be desirable to rescue the loss of expression of the target gene. This can be achieved by co-electroporation of a cDNA clone of the target gene which is resistant to RNAi with the RNAi vectors used. One method to achieve this is to use a mouse or human cDNA clone which has a different sequence in the regions corresponding to the siRNA targets and is, therefore, not targeted by the RNAi vectors. If such a clone is not available, it is possible to introduce silent mutations

(i.e. in the third base of each codon) into the cDNA which render it resistant to RNAi with the vectors in question. Such mutations are most effective when around 3 bases are changed in the "seed" portion of the antisense siRNA strand. This corresponds to the first eight bases from the 5′ end of the antisense siRNA strand (i.e. the last eight bases of the sense siRNA strand).

The vectors detailed here also allow two genes to be targeted from the same vector which can be used to overcome potential functional redundancy issues where several gene family members are expressed in the same tissue. Therefore, these vectors and the protocols detailed here allow any gene of interest to be inexpensively and robustly knocked down by RNAi in order to examine its role in neural tube (or other tissue) development.

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Part II
Sonoporation

Chapter 27
Sonoporation in Developmental Biology

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1 Introduction

Recent, molecular biology techniques have accomplished a large contribution to developmental biology. Especially, gene transduction techniques are indispensable to study the roles of regulatory genes underlying embryogenesis.

Replication-competent retroviruses, transfection with lipofection and an in ovo electroporation have been established as gene transduction techniques for embryos. (Yamada et al., 1997; Muramatsu et al., 1998; Fukuda et al., 2000; Iba, 2000; Nakamura et al., 2000). Particularly, in ovo electroporation for chick embryos has been recognized as a powerful method to efficiently induce exogenous genes into target cells or tissues, and it has been widely utilized to investigate their functions during embryonic development. Because the electric current tend to affect mainly the epithelial cells of the embryo, in ovo electroporation is suitable for the studies of neurogenesis and neuronal differentiation. In fact, large number of information relating to neuronal development has been brought from the studies using in ovo electroporation (Okafuji et al., 1999; Nakamura and Funahashi, 2001).

However, gene transduction using in ovo electroporation for mesenchymal tissues, e.g., somite, limb mesenchyme generally show lower efficiency. Hence, it is necessary to design appropriate electrodes to set up conditions of the electric pulses (voltage, pulse length, number of pulses) for each tissue in order to avoid tissue damages for embryos.

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The ultrasound has the character of passing through the tissues easily. By utilizing this physical character, attempts to introduce the materials (including genes) into the cells by ultrasound exposure have been performed (Tachibana and Tachibana, 1995; Kim et al., 1996; Wasan et al., 1996; Newman et al., 2001). It has been known that ultrasound itself can transiently increase cell membrane permeability for various agents. This phenomenon has been suggested that acoustic cavitation by ultrasound exposure likely induces such cell membrane permeability (Koch et al., 2000). Recent studies using high-speed camera recording demonstrated the behavior of cavitation bubbles (Xu et al., 2007). Such acoustically driven cavitation bubbles show complex physical movement and the collapse of cavitation bubble generates small holes on cell membrane (Taniyama et al., 2002). It has been suggested that substances, e.g., DNA, pass cell membrane through these transient holes intracellularly. Normally, High ultrasound intensities are required to create cavitation within tissues. Hence, Such exposure of high ultrasound intensities often causes temperature increase and mechanical damages to the tissues. However, the presence of microbubbles can significantly reduce the required level of ultrasound intensities for acoustic cavitation production upon ultrasound exposure in target tissues (Kim et al., 1996; Bao et al., 1997; Tachibana and Tachibana, 2001). Therefore, gene transduction techniques using ultrasound exposure are generally performed together with microbubble reagent. This method is called sonoporation and has been mainly studied for the gene delivery system. We found out that sonoporation is also useful as gene transduction method for embryos including chick or mouse tissues (Ohta et al., 2003, 2007a). As described above, gene transduction utilizing ultrasound exposure efficiently facilitates to transduce genes into the mesenchymal cells. In this chapter, we describe the procedure of sonoporation and our current findings by using the methods.

2 Procedure

The procedure of sonoporation is composed of several steps including DNA-microbubble mixture preparation, injection into target tissue(s) and exposure to the ultrasound.

2.1 Preparation of DNA-Microbubble Mixture

Ultrasound contrast agents are gas-filled microbubbles that are composed by lipids or proteins. Efficiency of gene transduction is sufficiently increased in presence of such agents. Here, we describe one of the typical microbubble solutions, Optison (Nycomed-Amersham or NEPA GENE) as an example. Before using the Optison, it is necessary to shake gently enough until the white part of the solution will be mixed completely in the reagent vial. After such mixture, the vial includes homogeneous white solution (Fig. 27.1a, b). The preparation of DNA-microbubble mixture was described below (Fig. 27.1c).

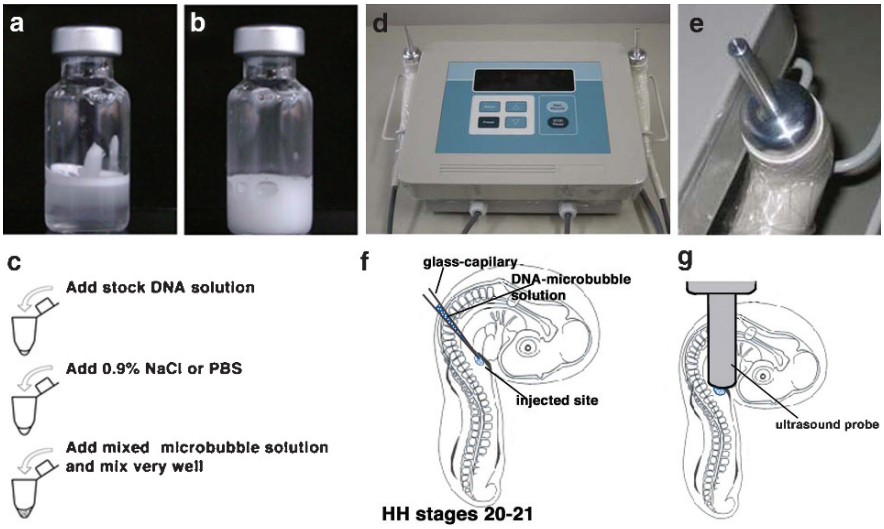

Fig. 27.1 (**a**) Microbubbles flow up to the top of solution while preserving it in refrigerator. (**b**) Gently inverting of a vial is very necessary to homogenize microbubble solution before using Optison (do not use voltex). (**c**) The procedure of preparation of microbubble-DNA mixture. (**d**) Sonitron 2000N. (**e**) High magnification of ultrasound probe. (**f**) The scheme of injection of microbubble-DNA mixture into chick limb bud. (**g**) The scheme of ultrasound exposure. Ultrasound is irradiated using ultrasound probe after the injection of microbubble-DNA mixture

1. Add 5 µl of stock DNA solution (e.g., pEGFP 1.0–2.0 µl/µg) into 0.5 ml tube.
2. Add 15 µl of 0.9% NaCl or PBS into the tube.
3. After shaking gently the vial of Optison until the solution becomes homogeneous, add 10ul microbubble solution into the tube and mix them well and keep the tube on ice.

2.2 Procedure of Sonoporation (e.g., Gene Transduction for Chick Limb Bud)

There are several types of sonoporation machines such as Sonitron 1000N or 2000N (Fig. 27.1d; Richmar, USA). They are equipped with either one or two probe connections for ultrasound emission (Fig. 27.1e). The condition of gene transduction is defined by three parameters, namely ultrasound intensity (W/cm^2), duty cycle (%) which shows the rate of pulse wave irradiation, and exposure time (seconds). In our experiments, it was observed that gene transduction in condition of 2.0 W/cm^2, duty cycle 20% and 60 s generally resulted in a highly efficient gene transduction into embryonic mesenchymal cells.

1. Incubate fertilized chick eggs until the desired stage for manipulation.
2. The egg shells should be sterilized with 70% alcohol and opened with a small hole at the blunt end of the eggs. Suck 4–5 ml of thin albumen by a 18G needle with a 5 ml syringe.

3. Cut the shell with straight fine scissors to open a window for operation.
4. To visualize chick embryos, inject the diluted Black ink by a 26G needle on a 1 ml syringe under the blastderm.
5. Using fine forceps, cut the vitelline membrane adjacent to the chick limb bud. Drop a few drops of Ringer solution, if required.
6. Before filling DNA-microbubble mixture into glass-needle, mix DNA-microbubble mixture very well by Pipetman. Suck 1–2 µl of DNA-microbubble mixture by Pipetman and spot it on the 35 mm Petri dish. Fill 0.25–0.5 µl of DNA-microbubble mixture into glass-needle by sucking the spotted DNA-microbubble mixture on the dish. It is better to check that microbubbles are assuredly included in the sucked DNA-microbubble mixture.
7. Inject DNA-microbubble mixture into limb bud mesenchyme (Fig. 27.1f). Since DNA-microbubble mixture tends to be spread from the injected site, it is necessary to devise the method of injection depending on each experiment.
8. Expose ultrasound for the injected limb bud region by gently contacting the probe to the injected region (Fig. 27.1g).
9. After ultrasound exposure, cover the window with vinyl-tape and return the egg into the incubator.

3 Application and Results

3.1 Gene Transduction by Sonoporation

In pEGFP transduction by sonoporation, low level of GFP expression was detected at 3 h after sonoporation. By 6 h, the sonoporated embryos displayed significant level of GFP expression and its expression reached to the maximum level after 12 h (Fig. 27.2a). Although GFP expression gradually decreased at 24 h after sonoporation, GFP positive cells were still observed after 48 h. The survival rate of the operated embryos was more than 90% during the period of 24–48 h after sonoporation. All of the surviving chick embryos displayed GFP expression. Approximately 60% of the operated embryos reached to the stage 35–36 without showing significant abnormalities.

In order to examine whether the genes were efficiently transduced into the mesenchymal cells, β-galactosidase expression vector was introduced into the limb bud mesenchyme at HH stage 20–21. β-Galactosidase staining was performed at 12 hafter in ovo sonoporation. β-Galactosidase positive cells were prominently observed at the injected region. In addition, histological sections revealed that almost all positive cells were localized in the mesenchymal region (Fig. 27.2b). Thus, these results show that sonoporation is suitable for gene transduction into embryonic mesenchymal tissues.

Sonoporation is also applicable to mouse tissues (S. Ohta et al., 2003, unpublished data). Mouse external genitalia is mesenchymal tissues covered by ectodermal cells,

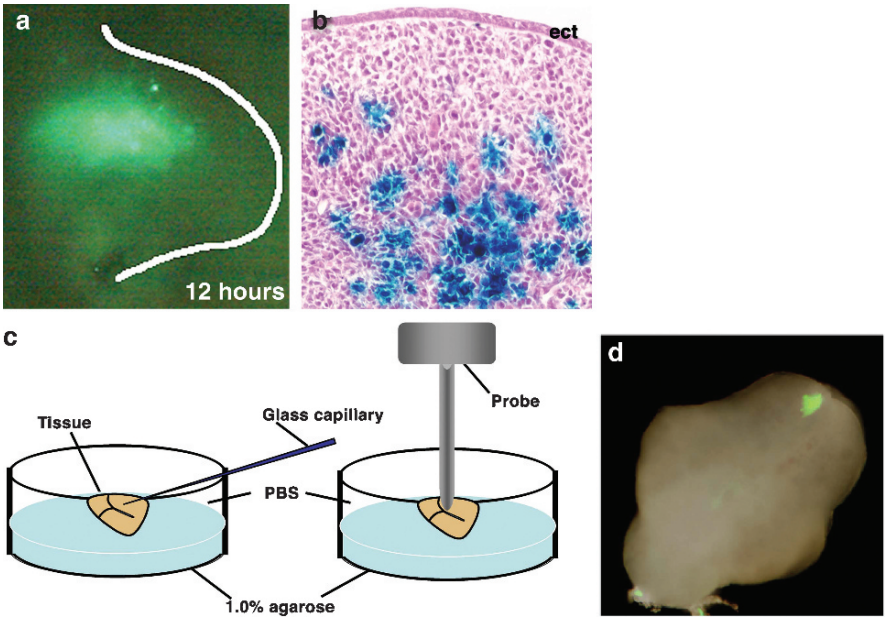

Fig. 27.2 (**a**) GFP expression was observed in chick limb bud at 12 h after sonoporation. (**b**) LacZ positive cells were mainly observed at the limb bud mesenchyme. Ect: Limb bud ectoderm. (**c**) The procedure of sonoporation for mouse tissues. (**d**) GFP expression was observed in the mesenchyme of mouse external genitalia at 12 h after sonoporation

and the organ culture of mouse external genitalia has been developed (Haraguchi et al., 2000). Thus, to examine whether sonoporation is useful for mouse tissues, mouse external genitalia was considered as one of the good target tissues.

1. Prepare 1.0% agarose/PBS plates with 35 mm plastic dishes.
2. Pour PBS on top of the agarose after it becomes solid.
3. Put the tissues on a 1.0% agarose/PBS plate.
4. Inject DNA-microbubbles mixture into the mesenchymal region (Fig. 27.2c).
5. Expose ultrasound (Fig. 27.2c).

Gene transduction for mouse external genitalia was performed with same condition described above. These sonoporated external genitalia were cultured with appropriate medium during 12–24 h. GFP expression was observed at the mesenchymal region of external genitalia (Fig. 27.2d).

3.2 Approach for Molecular Mechanism of Cessation of Gastrulation

Recently, We revealed one of the molecular mechanisms during cessation of gastrulation by utilizing of Sonoporation (Ohta et al., 2007b).

The epiblasts migrate toward the primitive streak and ingress through the primitive groove in the gastrula stage embryo (Lawson and Schoenwolf, 2001; Chuai et al., 2006). Subsequently, the ingressing epiblasts undergo Epithelial-Mesenchymal Transition (EMT) and differentiate into the definitive endoderm and mesoderm during gastrulation (Shook and Keller, 2003). However, the molecular mechanisms at the end of gastrulation have not been elucidated so far. The primitive streak is gradually regressed and its remnant contributes to the Ventral Ectodermal Ridge (VER) which is the thickened ectodermal tissue located at the tailbud ventro-distally, following embryogenesis (Schoenwolf, 1981; Tam and Beddington, 1987; Catala et al., 1995; Wilson and Beddington, 1996). According to our fate map analyses, chick VER undergoes EMT and displayed gastrulation-like ingressive cell movement until HH stage 20–21 (Ohta et al., 2007b). We hypothesized that EMT in the chick VER is suppressed by some regulatory factor(s), thus resulting in the cessation of gastrulation-like ingressive cell movement from the VER at the end of gastrulation.

Several bone morphogenetic protein (*Bmp*) genes (e.g., *Bmp2*, *Bmp4*, *Bmp7*) are expressed adjacent to the primitive streak, and BMP signaling has been shown to be essential for mesoderm formation from the gastrula to the somitogenesis stage (Mishina et al., 1995; Ohta et al., 2004; Komatsu et al., 2007). In addition, BMP signaling can induce EMT during the formation of neural crest (Liem et al., 1995; Liu and Jessell, 1998). Recently, the binding site of SMAD has been identified in the promoter region of *Snail*/*Slug* (Sakai et al., 2005). This indicates that BMP signaling is involved in the regulation of EMT during gastrulation. Thus, It was thought that Bmp(s) and its antagonists are candidates to regulate the cassation of gastrulation. *cBmp4* and *cBmp7* were expressed in or adjacent to the chick VER. *cNoggin* was prominently expressed in the ventro-lateral region rather than in the midline of the mesenchymal region adjacent to the chick VER. Accompanying the elongation of the tailbud caudally, *cNoggin* expression gradually expanded to the entire ventral mesenchyme adjacent to the chick VER. These data suggested that EMT in the chick VER is induced by BMP signaling and it is thus suppressed through the inhibition of BMP signaling by temporal/spatial *cNoggin* expression at the end of gastrulation.

To elucidate the roles of *cNoggin* during cessation of gastrulation, Sonoporation was performed to introduce *Noggin* expression vector into the mesenchymal region adjacent to the chick VER (Fig. 27.3a–c). Next, *Noggin*-overexpressing embryos were examined whether EMT in the chick VER is inhibited. The control embryos showed no significant abnormalities, while epithelial cells accumulated in the midline of ventral ectoderm of *Noggin*-overexpressing embryos. pSmad signals were localized in the nucleus of the ectodermal cells including the control VER or the juxtaposed mesoderm. However, the number of pSmad positive cells was remarkably decreased in the ventral ectoderm of *Noggin*-overexpressing embryos. Control embryos showed basal membrane degradation underlying the chick VER, and *cSlug* was expressed adjacent to the site of the basal membrane degradation. In contrast, *Noggin*-overexpressing chick embryos formed a basal membrane surrounding abnormally accumulating ectodermal cells in the midline of the ventral ectoderm with remarkably decreased *cSlug* expression. These results indicate that EMT in

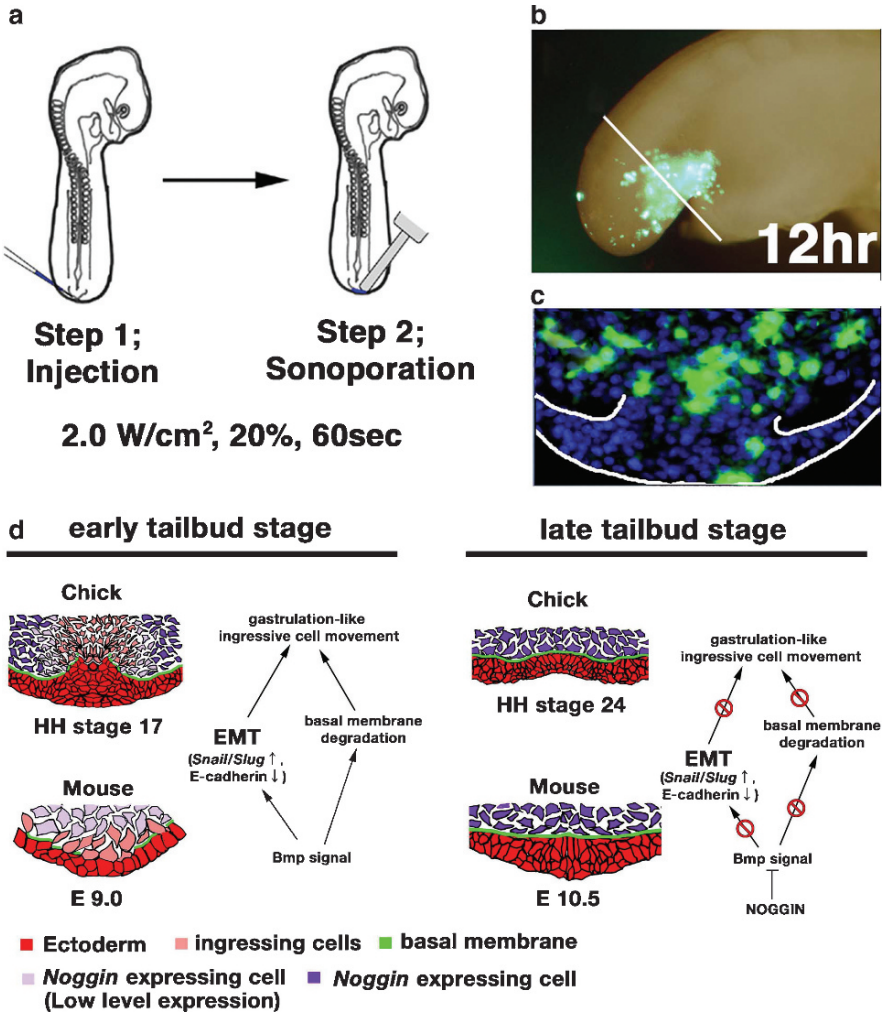

Fig. 27.3 (**a**) A schematic drawing of the sonoporation procedure. Microbubble-DNA mixture was injected into chick tail bud region (step 1). Ultrasound was irradiated at the injected region. (**b**) GFP expression at 12 h after Sonoporation. (**c**) The sections within the white line are shown in **b**. (**d**) Schematic diagram showing the cessation of gastrulation. During the early tailbud stage of chick embryos, *cNoggin* expression is initiated at the ventro-lateral region of the TVM (*purple indicated the Noggin-expressing cells*). BMP signaling could induce EMT with basal membrane degradation (*green indicates the basal membrane*). The chick VER shows gastrulation-like ingressive cell movement (*pink indicates the ingressing cells*). In the late tailbud stage of the chick embryos, *cNoggin* expression extends to the entire TVM (*purple indicated the Noggin-expressing cells*) and inhibits BMP signaling, which results in the suppression of EMT and basal membrane formation. In the early tailbud stage of mouse embryos, *mNoggin* expression in the TVM is low (*light purple*). The ectoderm derived from the late primitive streak shows ingressive cell movement with basal membrane degradation (*pink indicates the ingressing cells and green indicates the basal membrane*). In the late tailbud stage of mouse embryos, *mNoggin* is prominently expressed in the entire TVM (*purple indicated the Noggin-expressing cells*) and inhibits BMP signaling, which results in the suppression of EMT and basal membrane formation (*See Color Plates*)

the chick VER was inhibited by *Noggin* overexpression, suggesting that decreased BMP signaling adjacent to the chick VER leads to the arrest of gastrulation-like ingressive cell movement though suppressing EMT.

Noggin expression was also observed underlying the mouse VER. This suggests that mechanism of cassation of gastrulation is conserved among species. To examine the functions of *mNoggin* in the regulation of EMT of the mouse VER, analyses of Noggin mutant mice was performed (Brunet et al., 1998; McMahon et al., 1998; Bachiller et al., 2000; Suzuki et al., 2003). *Noggin* mutant mice displayed the absence of apparent ectodermal cells corresponding to the prospective mouse VER region. The loss of laminin and E-cadherin expression were observed in such regions of *Noggin* mutant mice. Furthermore, an increase of *mSnail* expression was observed at the ventral region corresponding to the loss of E-cadherin expression. These results suggested that *Noggin* mutant mice exhibited the aberrant EMT in response to the increased BMP signaling and that *mNoggin* expression is required to suppress EMT in the mouse VER. These results provided a framework to understand the mechanisms during the cessation of gastrulation. These findings suggest that the inhibition of BMP signaling by temporal/spatial *Noggin* expression adjacent to the VER could suppress EMT concomitant with the basal membrane formation, thus resulting in the cessation of gastrulation-like ingressive cell movement from the VER at the end of gastrulation (Fig. 27.3d).

Proper regulation of the embryonic ventral mesenchymal cell supply and cell migration has been poorly understood in relation with caudal embryonic anomalies. These results will also provide essential information to elucidate the mechanisms of caudal embryonic malformations, such as caudal regression syndromes and mermaid syndromes (OMIM 600145). Thus, the above experimental system also suggested that Sonoporation technology for the embryos is one of the efficient molecular tools to analyze embryogenesis. Sonoporation can be considered as one of the appropriate and compensatory tools for transducing genes for embryos. Cessation of gastrulation has been poorly analyzed partly due to the technical difficulties of handling early staged chick embryos. Incorporating new technologies will open a way to visualize the buried tomography of embryonic development.

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