Chapter 13
Electroporation into Cultured Mammalian Embryos

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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
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 O₂/CO₂/N₂ gas; where the percentage of O₂ 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 24h. The embryo can be cultured in this system for up to 48–72h 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 homebox, 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
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. 24h 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 \textit{fabp7} in the developing cortex, we designed several types of \textit{Fabp7}-specific siRNA, expressed using \textit{pSUPER} basic vector (oligoengine, Seattle, WA). These vectors were introduced into the developing cortical primordium by electroporation. To study the effect of \textit{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 \textit{Fabp7}-siRNA remarkably reduced the number of BrdU-incorporated cells in the developing cortex. Furthermore, we noticed that \textit{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 \textit{Fabp7} expression promotes premature cell cycle exit and neurogenesis, and that \textit{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 \textit{in vivo} assay system allowing such studies on stage and tissue-specific transcriptional regulation. Specifically, we demonstrated that, although Pax6 activates the expression of \textit{Fabp7}, no strong induction of \textit{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 \textit{Fabp7} using this technique (Numayama-Tsuruta and Osumi, in preparation). Complementary approaches such as ChIP on chip or \textit{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-specific 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, combinatorial 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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