doi:10.1111/j.1741-4520.2008.00203.x

NOTE

Mouse *exo utero* development system: Protocol and troubleshooting

Makiko Yamada[1], Toshihisa Hatta[2], and Hiroki Otani[1]

[1]Department of Developmental Biology, Faculty of Medicine, Shimane University, Izumo, and [2]Department of Molecular and Cell Structural Science, Kanazawa Medical University, Uchinada, Japan

ABSTRACT **The *exo utero* development system allows us to manipulate or operate on live embryos of mice or rats at mid- to late gestation stages, from late organogenetic to histogenetic periods, and keep them alive *in situ* until the analysis of their effects at a desired time point. We can examine the effects of injecting bioactive molecules or cells into targeted parts of a live embryo, destroying specific embryonic regions, or performing fetal surgery. This system is far simpler and more time- and cost-effective for *in vivo* functional analyses than establishing genetically modified mouse lines and provides a fine-tuned experiemental design for developmental scientists. To promote use of the mouse *exo utero* development system, we elaborate on the technical procedures, discuss critical points for trouble-shooting the system, and illustrate some apparatuses essential for fetal microinjection.**

Key Words: embryo, *exo utero* development, fetus, microinjection, mouse

INTRODUCTION

We previously reviewed the *exo utero* development system in the present journal, *Congenital Anomalies* (Hatta *et al.* 2004), and have since received a number of detailed technical inquiries about the system. To promote use of the mouse *exo utero* development system in the teratology societies and related fields, we here elaborate on the technical procedures, discuss critical points for troubleshooting the system, and illustrate some apparatuses essential for fetal microinjection. This is a part of a complete protocol, which can be accessed on the Japanese Teratology Society website (http://jts.umin.jp/resources/exoutero.htm).

The *exo utero* development system was introduced by Muneoka *et al.* (1986). This experimental system allows researchers to manipulate or operate on mid-to-late-gestation live embryos of mice or rats and keep them alive *in situ* until the analysis of their effects at a desired time point either pre- or postnatally. For example, we can examine the effects of injecting bioactive molecules or cells into targeted parts of a live embryo (Hatta *et al.* 1994; Zhang *et al.* 1998; Hatta *et al.* 2002), transferring DNA into cells in a limited area (Takiguchi-Hayashi *et al.* 2004), destroying specific embryonic regions (Naruse & Kameyama 1990; Naruse & Keino 1993; Naruse *et al.* 1996), or performing fetal surgery (Kihara *et al.* 1998; Habib *et al.* 2005). In principle, the *exo utero* system enables us to make a time- and region-specific intervention into developmental phenomena simply by allowing us to choose the desired time and region for manipulation. This system is far simpler and more time- and cost-effective for *in vivo* functional analyses than establishing genetically modified mouse lines.

Gene delivery systems have also been established for *in vivo* gene function analyses without genetic modification of mouse lines, such as the *in utero* electroporation technique for mouse embryonic brains (Saito & Nakatsuji 2001; Tabata & Nakajima 2001; Shimogori & Ogawa 2008). In these systems, DNA or small interfering RNA constructs are efficiently transferred into the embryonic brain spatio-temporally for gain-of-function or loss-of-function analyses. These gene-delivery techniques can be combined with the *exo utero* system to design fine-tuned *in vivo* experiments whose effects are controlled more focally and precisely than in experiments using the *in utero* method. Compared to the *in utero* method, a merit of the *exo utero* method for embryo manipulation is its clear visualization of the fine details of embryos, which makes it easier to locate the organs for manipulation. In contrast, because the *exo utero* embryos are not clearly visible before E11.5 due to their thick embryonic membranes (as will be discussed later), use of the *exo utero* system is limited mainly to the mid-to-late gestational period (Hatta *et al.* 2004). Because brain histogenesis begins after the mid-gestational stage, this *exo utero* technique has been utilized in developmental neuroscience. However, the *exo utero* system is a useful tool not only for analyses of the developing nervous system, but also for analyses of almost all organ systems during the histogenetic period. Over the past two decades, the concept of developmental origins of health and disease (DOHaD) has gained importance in the medical sciences. Based on the results of several human and animal studies, it is hypothesized that chronic diseases, such as cardiovascular disease and type 2 diabetes originate from adaptive changes in the epigenetic control of metabolism and organ histogenesis during fetal development (Gillman 2005; Mcmillen & Robinson 2005; Otani 2008). To continue this line of research, we now need to determine the mechanisms of these changes during organ histogenesis, including the molecules actively involved, which could lead to new methods for the prevention and treatment of many illnesses. To this end, we can use the *exo utero* development system to screen the functions of various proteins by injecting them into fetuses and following up their consequences later in life.

ANIMAL PREPARATION

Mating

Timed pregnancies are required for a time-specific intervention into the embryos. In the setting of natural mating, estrus female mice are chosen by their external genitalia, which show red-colored swelling. Vaginal smears can be used to identify the stage of the estrus cycle more precisely (Bronson *et al.* 1966). An estrus female is housed overnight with a potent male.

Checking for vaginal plugs

When successful mating is confirmed by the presence of a vaginal plug on the following morning, noon of the same day is designated

Correspondence: Hiroki Otani, MD, PhD, Department of Developmental Biology, Faculty of Medicine, Shimane University, Izumo 693-8501, Japan. Email: hotani@med.shimane-u.ac.jp

Received August 12, 2008; revised and accepted August 18, 2008.

as embryonic day 0.5 (E0.5). This definition is based on the assumption that the copulation generally takes place at midnight. To determine the timing of pregnancies more precisely, the vaginal plugs can be checked at several-hour intervals. After plugs are observed, each female mouse is isolated. The superovulation technique usually increases the success rate of mating, but it also increases the number of implanted embryos, and culling the large number of embryos during the operation (as described in section 3.2) takes a long time. The superovulation technique is therefore not recommended.

OPERATIVE PROCEDURES

Anesthesia

The pregnant mouse is anesthetized with sodium pentobarbital (40–50 mg/kg body weight) intraperitoneally. If the induction of anesthesia is delayed for more than 10 min after injection, possible misadministration of pentobarbital into the intestines or uterus should be considered. A serious adverse effect of pentobarbital is respiratory depression, which requires close monitoring. Anesthesia increases secretion from the salivary glands or upper airways, which should be gently sponged from the oral cavity to prevent suffocation. Pentobarbital anesthesia is usually effective for 1 h.

Relaxation of the myometrium

Ritodorin hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), 1.4 mg/kg dissolved in 0.9% saline, is administered intraperitoneally around 5 min after pentobarbital administration, which relaxes the myometrium. A relaxed myometrium is easier to cut through; this is important because if the myometrium is tense, the fetal membrane can be injured by the scissors due to the tight space between the contracted muscle wall and fetal membrane.

Sterilization of instruments

Forceps, scissors and an aluminum or stainless plate are disinfected with 70% ethanol. A 3–4 cm square of gauze is prepared and autoclaved beforehand.

Abdominal incision

We usually perform this operation at room temperature without use of an animal heating plate because heating can increase insensible water loss from the mouse. After the hair over the abdomen is shaved, the mouse is placed in the supine position on an aluminum or stainless plate with its limbs and tail held by plastic surgical tape. The abdomen is wiped with 70% ethanol. A midline skin incision is made from the level of the upper border of the urinary bladder to the level of the lower border of the subxiphoid process. The skin is detached from the abdominal wall to secure a margin for suture. One should be careful not to injure the perforating abdominal wall arteries that are supplying the skin. Finally, the abdominal wall is opened along the linea alba.

Incision of the myometrial wall

One horn of the uterus is pulled out from the abdomen using blunt-end forceps. The ritodorin-treated myometrium becomes loose and soft, therefore, it can be easily grasped or incised. A small incision is made at the opposite side of the vascular (placental) border of the uterus, and then a longitudinal incision is made with scissors along the antivascular border tracing to the root of the horn (Fig. 1). Scissors with slightly dull points are best for handling the uterus to prevent accidental perforation of the embryonic membrane. During the procedures, all of the embryos except for the one being manipulated are covered with a piece of gauze soaked in normal saline. One should be careful not to injure the intestines during the operation. Bleeding from the cut end of the uterus wall is usually trivial.

EMBRYO MANIPULATIONS

Prevention of exsiccation

Embryos enveloped by an embryonic membrane should be covered with sterile normal saline-soaked gauze to protect them from loss of surface fluids. If the membrane dries up, its transparency will be lost, making it difficult to manipulate the embryos.

Removal of embryos

An ICR (Jcl: ICR, CLEA Japan, Tokyo, Japan) mouse mother usually conceives 10 to 15 embryos by natural mating. Five to seven embryos implant to one side of the uterus horn. In order to increase the viability rate, three embryos should be left on either side of the uterus, and all of the other embryos should be dissected out and used as controls at the time of injection. If one attempts to leave all

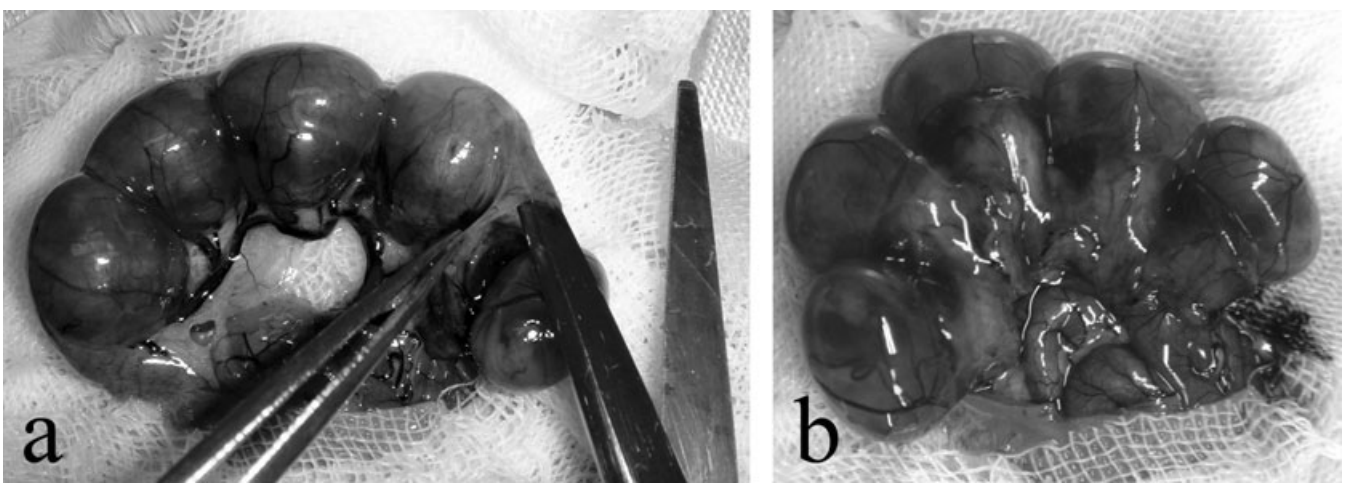

Fig. 1 (a) Longitudinal incision along the antivascular border of the uterine wall. (b) Exposed E13.5 embryos within the embryonic membrane.

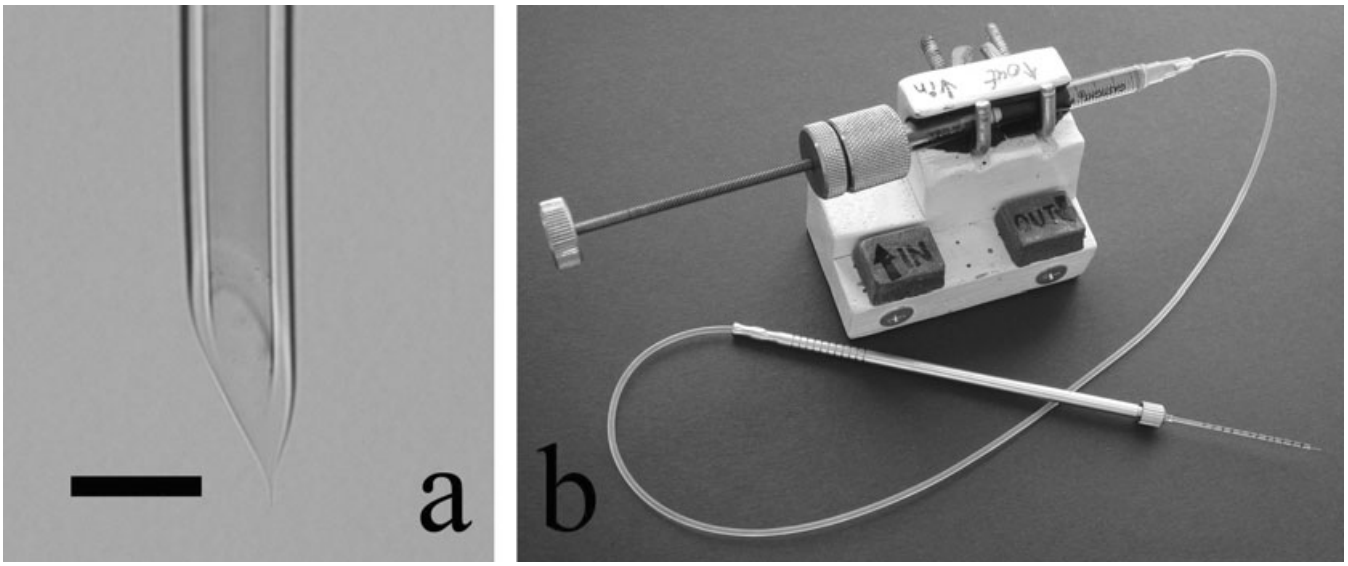

Fig. 2 (a) Apex of a glass micropipette (bar, 50 μm). (b) Microinjection apparatus. A micropipette is connected to a polyethylene tube which is attached to a 0.5 mL threaded plunger Hamilton gastight syringe #1750 (Hamilton, Reno, NV, USA), then filled with mineral oil.

embryos in the uterus, the embryos will have low viability (Toshihisa Hatta *et al.*, unpublished data). In addition, care should be taken to ensure that the remaining embryos are not too close to each other.

When untreated embryos are removed from the uterus, the embryonic membrane is cut through and the umbilical cord is cut. Bleeding from the cut-end of the umbilical cord from the placental side is trivial and stops quickly. The placenta is left as it is exposed.

Care should be taken not to touch the placenta at any stage of the operative procedure. Damage to the placenta should be minimized because it causes bleeding, which leads to unfavorable results. In severe cases of placental bleeding, the mother can die of hemorrhagic shock. In cases of more minimal bleeding, the bleeding can cause adhesion of the surrounding tissues, which leads to low viability of the embryos. Adhesive torsion of uterine arteries can lead to an inadequate blood supply from the placenta, and thereby to embryo death. To ensure that the embryos survive in this *exo utero* system, it is of utmost importance to protect the embryos from adhesion. If there is no adhesion, one can manipulate embryos more than twice during *exo utero* development. Prophylaxis for adhesion will be discussed later.

Observation of targeting embryos

To observe the live embryos clearly, a proper lighting apparatus with a freely adjustable light axis is required. Incandescent light is not suitable because it transmits heat that can instantly dry the fetal membrane. An operation microscope with a long working distance is preferred for performing the microsurgical procedures. We use a microscope with foot-operated controls for adjusting the focus and magnification (Olympus OME; Olympus Optical, Tokyo, Japan). With the aid of a stereoscopic microscope or an operation microscope and a lighting apparatus, intact embryos can be illuminated in detail (Fig. 1). At stages earlier than E11.5, it is difficult to observe embryos because the decidua around the embryonic membrane is too thick to see through. Stripping off the decidua often endangers the embryos because in these early stages the decidua plays an important role in nourishing embryos instead of the poorly developed placenta. Moreover, incision of the membrana decidua causes severe bleeding and may lead to embryonic death. Thus, embryos before E11.5 are too young to be manipulated in *exo utero* experiments.

Microinjection

The most critical point in terms of a successful microinjection leading to embryos with high viability is the use of sharp-tipped micropipettes. With a blunt-end pipette, the fetal membrane can be torn due to the application of extra force at the injection point, causing amniotic fluid to leak out. Fetal deaths are often attributable to injuries of the membrane or placenta rather than the manipulation procedure itself.

The tip of the glass micropipette should be ground to a sharp point by a pipette grinder (MCG-II; Chatani Limited, Tokyo, Japan) covered with diamond paste (Metadi II, Buehler, Lake Bluff, IL, USA). A needle-like point is then formed at the top of the apex by a micro-forge (MF-79; Narishige Scientific Instrument Lab., Tokyo, Japan). A pipette tip with an outer diameter of 20 μm is sufficient for solution injection. If suspended cell clusters are to be injected, the outer diameter size should be larger (e.g. 50 to 100 μm; Fig. 2).

The glass pipette is connected to a polyethylene tube, which is attached to a 0.5 mL threaded plunger Hamilton gastight syringe #1750 (Hamilton, Reno, NV, USA), then filled with mineral oil (Fig. 2). Prior to use, any air dissolved in the oil should be removed by means of a vacuum pump with a closed chamber. No air bubbles should be present in the syringe, tube or pipette. In order to easily identify the target location at the time of injecting and harvesting, we mix carbon ink or fast green ink with the injection solution (Fig. 3).

Other surgical techniques

There are several surgical techniques. Resection of certain parts of the fetal organs is one of them. Naruse and Kameyama (1990) performed an experimental fetal surgery on genetic polydactyly mice: the authors combined the *exo utero* system with argon laser irradiation to the extra digits of the polydactyly mice. In this *exo*

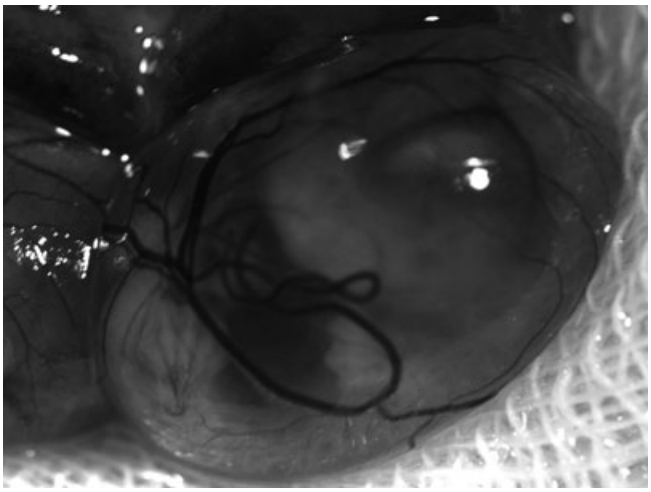

Fig. 3 Microinjection of carbon ink into the left lateral ventricle at E13.5. The ventricle is delineated as a black area due to injected ink.

utero system, argon laser irradiation yielded embryos with high viability because little bleeding occurred in the area irradiated.

Suturing is another surgical technique. Habib *et al.* (2005) sutured the E15.5 mouse embryo's mandible and maxilla through the fetal membrane by an 8-0 nylon thread: the authors restrained the jaw movement during the fetal period to investigate the effects of mechanical factors on the development of joint cartilage. One of the adverse effects of suturing is the loss of amniotic fluid from the suturing point or the inadvertent tearing of tissue from the suturing point. Avitene (Davol, Woburn, MA, USA), a microfibrillar collagen hemostat, can be placed over cracks in the membrane to seal them. However, care should be taken not to use too much Avitene because it causes adhesion in the peritoneal cavity.

Returning embryos to the peritoneal cavity

When manipulated embryos are being placed back into the abdomen, they should not be grabbed directly with the forceps. Instead, they should be moved by pushing or pulling or pushing the gauze wrapping them. The uterus is left unsutured. The embryos in the intact fetal membrane continue to develop inside the peritoneal cavity but outside the uterine cavity. This is why the technique is designated as *exo utero* development.

ABDOMINAL CLOSURE AND RECOVERY

Abdominal closure

Before closing the abdomen, copious peritoneal lavage with warm (37°C) 0.9% saline is effective for washing out blood and protecting the organs and embryos from adhesion. Another way to prevent adhesion is to apply sterile Hank's solution (1.0–1.5 mL) before closure. This also effectively prevents adhesion between the organs and the embryos. The abdominal wall is closed by continuous suturing, and the skin is closed by autosuture (MikRon 9 mm Autoclip Applier; BD, Sparks, MD, USA).

Investigators inexperienced in the procedure should keep a record of how long the operation takes. For the experienced practitioner, it should take 30 to 35 min to perform an operation that includes, for example, the injection of solution into the lateral ventricle of six embryos. Operation for more than 1 h debilitates the animal and delays recovery from anesthesia.

Recovery

After skin closure, the mouse is placed on a heating plate at 37°C (e.g. a slide-drying hotplate) for rewarming. When the mouse wakes up from anesthesia, it is then put back in a clean cage.

POSTNATAL ANALYSIS

The *exo utero* development system can also allow us to study postnatal effects of prenatal manipulations. One of the advantages of this *exo utero* system is that the course of postnatal development and the aging process can be traced, which is impossible in the settings of organ cultures or whole embryo cultures. In terms of DOHaD, the system also provides an ideal experimental model to analyze the later consequences which originate from developmental programming during histogenesis. To accomplish this, the techniques described below are required.

Harvesting and resuscitation of newborns

At E18.5, the mother is euthanized and the embryos are removed from the abdomen. The fetal membrane is removed and the amniotic fluid is wiped off. This can be done by rolling the newborns gently on soft tissue paper. At first, the newborns will not be able to breathe by themselves. The operator must facilitate the initiation of breathing by swabbing the oral cavity with a string of twisted tissue paper in order to absorb amniotic fluid in the mouth or pharynx. As soon as a newborn starts to breathe, its body will turn a bright pink color, indicating successful resuscitation. Protracted cyanosis of the newborns will result in a poor prognosis.

Foster parenting

Resuscitated newborns need a foster mother immediately. The foster mother should be an experienced female with her own newborn litter (the date of birth should be within 2 days). The standard method of foster parenting has been described in detail previously (Silver 1995). To distinguish the pups to be fostered from the foster mother's own litter, we mark them with a subcutaneous carbon ink injection. To prevent the new litter from being killed by their foster mother, we temporarily put them into a bag containing nesting material from the foster mother's cage. We then mingle the pups with the material gently, which transfers the mother's scent to the newborn litter. After this procedure, the pups are put into the foster mother's cage. There seems to be some difference among mouse strains concerning the capability of foster nursing, with ICR mice being particularly suitable for retention of the litter.

CONCLUSION

We have elaborated on the technical procedures of the *exo utero* development system. This system is a useful method for *in vivo* functional analyses from late organogenetic to histogenetic periods. It is far simpler and more time- and cost-effective than establishing genetically modified mouse lines and provides a fine-tuned experimental design for developmental scientists.

REFERENCES

Bronson FH, Dagg CP, Snell GD (1966) *Biology of the Laboratory Mouse*, 2nd edn. Dover Publications, Inc., New York. [Cited December 2007.] Available from URL: http://www.informatics.jax.org/greenbook/index.shtml

Gillman MW (2005) Developmental origins of health and disease. *N Engl J Med* **353**: 1848–1851.

Habib H, Hatta T, Udagawa J, Zhang H, Yoshimura Y, Otani H (2005) Fetal jaw movement affects condylar cartilage development. *J Dent Res* **84**: 474–479.

Hatta T, Matsumoto A, Otani H (2004) Application of the mouse *exo utero* development system in the study of developmental biology and teratology. *Congenit Anom Kyoto* **44**: 2–8.

Hatta T, Moriyama K, Nakashima K, Taga T, Otani H (2002) The role of gp130 in cerebral cortical development: *In vivo* functional analysis in a mouse *exo utero* system. *J Neurosci* **22**: 5516–5524.

Hatta T, Tanaka O, Otani H (1994) Contribution of RGD sequence to neuronal migration in developing cerebral cortex. *Neuroreport* **5**: 2261–2264.

Kihara I, Hashimoto R, Otani H (1998) Effects of fetal movement on the development of the rat joint. *Congenit Anom Kyoto* **38**: 259–270.

McMillen IC, Robinson JS (2005) Developmental origins of the metabolic syndrome: Prediction, plasticity, and programming. *Physiol Rev* **85**: 571–633.

Muneoka K, Wanek N, Bryant SV (1986) Mouse embryos develop normally exo utero. *J Exp Zool* **239**: 289–293.

Naruse I, Kameyama Y (1990) Fetal laser surgery in genetic polydactyly mice. *Teratology* **41**: 731–735.

Naruse I, Keino H (1993) Induction of agenesis of the corpus callosum by destruction of anlage of the olfactory bulb using fetal laser surgery *exo utero* in mice. *Brain Res Dev Brain Res* **71**: 69–74.

Naruse I, Keino H, Taniguchi M (1996) Fetal laser surgery exo utero in mice. *Congenit Anom Kyoto* **36**: 107–113.

Otani H (2008) Development of the brain as an integral part of the harmonized systemic histogenesis. *J Brain Sci* (in press).

Saito T, Nakatsuji N (2001) Efficient gene transfer into the embryonic mouse brain using *in vivo* electroporation. *Dev Biol* **240**: 237–246.

Shimogori T, Ogawa M (2008) Gene application with *in utero* electroporation in mouse embryonic brain. *Develop Growth Differ* **50**: 499–506.

Silver LM (1995) *Mouse Genetics: Concepts and Applications*. Oxford University Press, Oxford. [Cited November 2001.] Available from URL: http://www.informatics.jax.org/silverbook/index.shtml

Tabata H, Nakajima K (2001) Efficient *in utero* gene transfer system to the developing mouse brain using electroporation: Visualization of neuronal migration in the developing cortex. *Neuroscience* **103**: 865–872.

Takiguchi-Hayashi K, Sekiguchi M, Ashigaki S *et al*. (2004) Generation of reelin-positive maginal zone cells from the caudomedial wall of telencephalic vesicles. *J Neurosci* **24**: 2286–2295.

Zhang H, Hatta T, Udagawa J, Moriyama K, Hashimoto R, Otani H (1998) Induction of ectopic corticotropic tumor in mouse embryos by *exo utero* cell transplantation and its effects on the fetal adrenal gland. *Endocrinology* **139**: 3306–3315.