World Journal of Surgical Procedures

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DOI: 10.5412/wjsp.v5.i2.198

World J Surg Proced 2015 July 28; 5(2): 198-207
ISSN 2219-2832 (online)

REVIEW

In utero and *exo utero* fetal surgery on histogenesis of organs in animals

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Author contributions: Jahan E conceived, designed and drafted the paper; Rafiq AM drafted the references; Otani H ideated, guided and reviewed the paper.

Conflict-of-interest statement: There is no conflict of interest.

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Received: September 27, 2014
Peer-review started: September 28, 2014
First decision: December 17, 2014
Revised: February 22, 2015
Accepted: March 16, 2015
Article in press: March 18, 2015
Published online: July 28, 2015

Abstract

Until recently, fetal surgery was only used for fetuses with very poor prognosis who were likely to die without intervention. With advances in imaging, endoscopic techniques, anesthesia and novel interventions, fetal surgery is becoming a realistic option for conditions with less severe prognoses, where the aim is now to improve quality of life rather than simply allow survival. Until forty years ago, the uterus shielded the fetus from observation and therapy. Rapid changes in the diagnosis and treatment of human fetal anatomical abnormalities are due to improved fetal imaging studies, fetal sampling techniques (*e.g.*, amniocentesis and chorionic villus sampling), and a better understanding of fetal pathophysiology derived from laboratory animals. Fetal therapy is the logical culmination of progress in fetal diagnosis. In other words, the fetus is now a patient. Now-a-days, *in utero* (*IU*) and *exo utero* (*EU*) surgical methods are popular for experimental analyses of the histogenesis of organ development. Using these surgical methods, developmental anomalies can be created and then repaired. By applying microinjection and/or fetal surgery with these methods, models of developmental anomalies such as neural tube defects, temporomandibular joint defects, hip joint defects, digit amputation, limb and digit development and regeneration, and tooth germ transplantation in the jaw could be created and later observed. After observing different types of anomalies, novel *IU* and *EU* surgical techniques would be the best approach for repairing or treating those anomalies or diseases. This review will focus on the rationale for the *IU* and *EU* creation of animal models of different organ defects or anomalies and their repair, based on analyses of organ histogenesis and pathologic observations. It will also focus in detail on the surgical techniques of both *IU* and *EU* methods.

Key words: Myelomeningocele; Microinjection; Rodent; Sheep; Neural tube defect; Temporomandibular joint; Fetal surgery; *In utero*; *Exo utero*

Core tip: Fetal surgery in animal models has become a promising technique for analyses of organ histogenesis

and organogenesis. Using unique *in utero* (*IU*) and *exo utero* (*EU*) methods, developmental anomalies could be created and repaired during the prenatal period. Here, we review the *IU* and *EU* surgical techniques, focusing on methods and outcomes in various experimental animals.

Jahan E, Rafiq AM, Otani H. *In utero* and *exo utero* fetal surgery on histogenesis of organs in animals. *World J Surg Proced* 2015; 5(2): 198-207 Available from: URL: http://www.wjgnet.com/2219-2832/full/v5/i2/198.htm DOI: http://dx.doi.org/10.5412/wjsp.v5.i2.198

INTRODUCTION

Fetal surgery has a potential role in managing structural anomalies, where antenatal intervention might theoretically result in an improved outcome for the baby. Many anomalies do not meet these criteria and are likely to remain best managed after birth.

The first attempted intrauterine surgical intervention was a transfusion for Rh incompatibility in 1961. In the 1980s, the developmental pathophysiology of potentially correctable anatomical malformations was studied in animal models. Serial observations, using advances in imaging techniques, helped elucidate the natural history of certain anomalies in human fetuses. Novel obstetric therapies, endoscopic techniques and instruments now make it possible to correct some structural anomalies *in utero (IU)*.

The fundamentals of fetal surgery[1,2] are to (1) understand the natural history of the untreated anomaly *IU*; (2) have a sound pathophysiological rationale for prenatal treatment; (3) demonstrate the safety and efficacy of the fetal procedure in an animal model; and (4) define inclusion and exclusion selection criteria for treatment.

Until recently, only fetuses with a poor prognosis and a life-threatening anomaly were considered for prenatal intervention. Advances in techniques and a better understanding of the natural history of the anomalies have allowed intervention for non-life-threatening conditions, where outcome might be substantially improved. Life-threatening defects include myelomeningocele (MMC), congenital diaphragmatic hernia (CDH), airway obstruction, aqueductal stenosis, twin-to-twin transfusion syndrome, cleft lip and palate, and metabolic and cellular defects. Upadhyaya reviewed how to correct these types of defects[3]. Over the past two decades, the concept of developmental origins of health and disease 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[4-6].

The *exo utero* (*EU*) developmental system was introduced by Muneoka *et al*[7]. This experimental system allows researchers to manipulate or operate on mid-to-late-gestation live mouse or rat embryos and to keep them alive *in situ* until the analysis of their effects at a desired pre- or postnatal time point. The *EU* system enables 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 altered mouse and rat lines. Compared to the *IU* method, one merit of the *EU* method for embryo manipulation is its clear visualization of the fine details of embryos, making it easier to locate the organs for manipulation. In contrast, because *EU* embryos are not clearly visible before embryonic day (E) 11.5 in mice due to their thick embryonic membranes, use of the *EU* system is mainly limited to the mid-to-late gestational period[8]. However, the *EU* system is a useful method not only for analyses of the developing nervous system but also for investigations of almost all organ systems during the histogenetic period[6,8].

For many genetic disorders, early onset and irreparable tissue and organ damage necessitate innovative methods that allow therapeutic intervention early in development, if a full cure is to be realized. The studies outlined in this review focused on *IU* and *EU* surgery for intervention during organ histogenesis using a variety of animals, including large mammals such as sheep, pigs and primates, and small mammals such as mice and rats. Larger mammals, such as sheep and monkeys, carry on average one embryo per pregnancy and typically tolerate surgical manipulations well, but are more expensive and have longer gestations (145 and 160-180 d, respectively) as well as higher ethical limitations. These factors reduce the number of experiments that can be performed in a given time frame. Most small experimental animals are multiparous, allowing for experimentation on large numbers of embryos, ranging from 3 to 10 embryos per pregnancy and shorter gestational periods of 3-4 wk. Drawbacks include difficulties with the manipulation of the uterus and the subsequent survival of the embryo. To this end, we can use the *IU* and *EU* development systems to screen the functions of various proteins/cells by injecting them into embryos, or to perform fetal surgery and follow up on consequences later in life. Here, we review procedures for mammalian embryo surgery both *IU* and *EU* and highlight technical innovations that have been published using this approach.

GENERAL PREPARATION FOR *IU* AND *EU* SURGERY

Here, we describe in detail *IU* surgical procedures in rodents and briefly describe these in other animals such as sheep, pigs and primates. We will only describe the *EU* surgical procedure in rodents, as thus far no experimental works or reports have been published applying this

method in other mammals. Preparation of pregnant mice or rats and abdominal surgery for *IU* and *EU* surgery are identical, to some extent. Similar procedures in rodents are described below, and later, we separately describe the procedural differences between *IU* and *EU* surgery.

Preparation for IU and EU surgery in rodents

The two generally used approaches are *IU* or *EU* surgery. Both are demanding procedures that require some level of expertise. The post-implantation embryo is encased in its extraembryonic membranes (amnion and yolk sac) within the tubular uterus. The embryo can be accessed by injection, passing through the layers of the uterine wall (perimetrium, myometrium, and endometrium) and the extraembryonic membranes. Intrauterine embryo injections can be successfully carried out on mouse embryonic stages as early as E8[9,10]. For direct surgery on the embryo, *IU* studies require opening and closing the uterus and extraembryonic membranes. This approach is restricted to late embryonic/fetal stages (E14.5 and later) because early embryos are too fragile to survive the postsurgical forces resulting from the contracting uterus. *EU* surgery is based on the finding that embryonic development is not perturbed when the uterine tube is opened but not sutured closed[7]. The embryos remain attached to the open uterus *via* the placentae and develop suspended within the abdominal cavity of the female. When embryos are exposed in this manner, it is possible to perform various embryo surgeries at early embryonic stages. Injection experiments using *EU* surgery have been carried out on stages as early as E8.5[11], and direct surgery on the embryo can be carried out on E11.5 embryos and older[12]. While technically demanding, direct manipulation of the rodent embryo is possible and, in combination with other experimental approaches, provides another avenue for experimental studies of mammalian development.

Preparation of animals and required instruments before surgery were described in detail by Yamada *et al*[13].

Anesthesia: Several different approaches to anesthesia have been used for studies on embryonic and fetal rodents, as reviewed in Ngo-Muller and Muneoka[14]. In all cases, the anesthetic target is the pregnant female and not the embryo/fetus, although the embryo/fetus is exposed to maternal levels of the drug. Anesthesia with ketamine/xylazine (K/X) or pentobarbital induces prolonged anesthesia (30-45 min with K/X; > 45 min with pentobarbital) and is administered by intraperitoneal (*i.p.*) injection. For mice, K/X is administered at a dose of 100 mg/kg of ketamine and 10 mg/kg xylazine (80 mg/kg ketamine and 8 mg/kg xylazine for rats). Reversal of K/X anesthesia can be obtained by injecting the antagonist yohimbine (1.0 mg/kg, *s.c.*) when surgery has been completed[14]. Alternatively, the pregnant female mouse/rat is also anesthetized with sodium pentobarbital (Nembutol) (50 mg/kg body weight *i.p.*)[8,13]. Recently, a combination of anesthetics (Medetomidine/Midazolam/Butorphanol) in solution is widely used. This combination is prepared with 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol (M/M/B: 0.3/4/5)[15]. The induction time of M/M/B was identical to the induction time of K/X. The emergence time of M/M/B was the similar to that of K/X. The anesthetic time of M/M/B, however, was longer than the anesthetic time of K/X[15].

Abdominal incision: A sterilized operating aluminum or stainless steel plate is used during operation. Operating field (abdominal skin) of the pregnant dam should be wiped by 70% ethanol after removal of the hair, and the mouse/rat is placed in a supine position on the operating plate. To open the abdomen, an initial large midline incision of the belly skin is made with microdissection scissors. Blunt forceps should be used to handle the skin. A second incision is made along the linea alba to open the abdomen. With the abdomen open, the uterine horns can be found in the lateral regions of the abdominal cavity and simply pulled out onto sterile damp gauze placed on the ventral surface.

IU SURGERY

Mammalian development has been best characterized using rodent (mouse, rat) models. Direct intervention of the post-implantation mouse/rat embryo *IU* represents one of several experimental methods that can be used to probe mammalian embryogenesis. Here, we will elaborately describe the surgical technique in the mouse/rat and also briefly describe it in other animal models.

Rodents

Most studies using *IU* manipulation were performed on mouse embryos, though a few studies have been applied to rat embryos[2,16-18]. *IU* surgery requires that the abdomen be opened to access the uterus. After the surgical procedure, the abdomen is closed and the animal is allowed to recover.

Microinjection

IU manipulations generally involve injections into the embryo that must pass through the uterine wall and the extraembryonic membranes (yolk sac and amnion). The injection should avoid any blood vessels. Embryo manipulation is best performed using a stereo zoom surgical microscope. Injections generally utilize glass needles made from micropipettes of varying size. The making procedure was described in detail by Yamada *et al*[13]. Injection studies include the use of markers, such as carbon particles for establishing fate maps[19] or lipophilic tracers such as DiI (CellTracker; Molecular Probes) to characterize cell migration patterns[10,20-22]. Injection of virus has been used to study cell lineage[23] and the targeted effect of a specific virus on develop-

ment[24,25]. Targeted injection of purified growth factors or signal transduction antagonists directly into the embryo has been used to study signaling during normal and abnormal development[26-28]. Electroporation has been applied to inject plasmids encoding genes for functional studies and/or marker genes for cell labeling studies[29-36], plasmids encoding short hairpin RNA for RNA interference[2,16-18,37], and dual-fluorescence reporter/sensor plasmids for single-cell detection of microRNAs[38].

Recent studies demonstrate that cell transplantation (CT) at progressively earlier embryonic stages resulted in higher levels of chimerism[39]. Clinically relevant studies include the rescue of a genetic mouse model of autosomal recessive osteopetrosis, a human disorder associated with defective osteoclasts, with allogenic fetal liver CT[40], and the rescue of a mouse model of osteogenesis imperfecta with transplantation of adult bone marrow cells[41].

Fetal surgery

Open spina bifida, or MMC, the most common type of neural tube defect (NTD), is defined as a protrusion of the spinal cord and/or meninges through a defect in the vertebral arches. Creating the ideal animal model to study the effects of intrauterine surgery requires that the mechanisms of aberrant primary neurulation, resulting in an open NTD and associated nervous system anomalies, be reproduced. To create the NTD lesion fetus and repair experiments by Heffez, two studies utilized this animal model[42,43]. In the first study[42], pregnant rats at day 18 of a 22-d gestation were anesthetized, and the surgery was performed using an operating microscope. A single horn of the bifid uterus was exteriorized through a midline abdominal incision. Only the fetus being treated was mobilized. Following the opening of the uterus and amniotic membrane, a 2- to 3-level laminectomy was done, and the dura was opened. This group of fetal rats was returned to the uterus with the lesion. In a second study, identical surgical techniques were used by the same authors to lesion fetal rats, and a second group received a repair treatment prior to return to the uterus[42,43]. The rat model utilized two strategies to repair the spinal defect at embryonic day 18[42]. The open spinal cord was either repaired immediately with a nonocclusive peritoneal cover from the mother, or was re-exposed the following day and underwent a primary skin closure. Control embryos did not recover any function and had significant degradation of the spinal cord. The embryos that were repaired by primary skin closure, even after a 24-h delay, demonstrated better outcome than the embryos with closure using peritoneum. The results of this study point to the harmful effects of amniotic fluid, due to the worse outcome after a nonocclusive barrier (peritoneum) was used instead of skin. Stiefel studied the curly tail mouse model of exposed lumbosacral spina bifida and revealed the progressive deterioration of neuroanatomic appearance and neurologic function with increasing gestational age[44]. Danzer developed a retinoic acid-induced MMC model in fetal rats, and histopathology confirmed the entire spectrum of severity observed in human MMC, as well as features of the Arnold-Chiari malformation[45,46]. While these studies support the principle of improved neurologic function with *IU* coverage of the spinal cord, a large animal model with lengthy periods of time *IU* after surgical manipulation is needed before the extrapolation of these findings to humans.

Sheep

Sheep are much easier to breed and maintain and are a well-established animal model of human fetal physiology. Sheep have a consistent gestation period of 145 d, and the development of the fetus and its immune system is very similar to that of humans. Fetal sheep have been used widely to study mammalian fetal physiology, and the results obtained with this model have been directly applicable to the understanding of human fetal growth and development[47]. The first attempt at *IU* gene therapy in the sheep[48] utilized a stem CT based method, in which peripheral blood was collected from 110-d-old fetal sheep by exchange transfusion. Once its full clinical potential has been realized, hematopoietic stem cell-based gene therapy promises to cure a wide array of both inborn and acquired diseases. Both hematopoietic cells and non-hematopoietic cells within the liver and lung are transduced following the direct injection of murine retroviral vector supernatants into the peritoneal cavity of pre-immune fetal sheep, suggesting that the developmental stage of each organ at the time of injection may determine its susceptibility to *IU* gene transfer[49]. Using pregnant sheep, David *et al*[50] have adapted ultrasound-guided injection techniques from fetal medicine practice and established new methods to deliver gene therapy to fetal sheep, including intratracheal injection to target the distal respiratory epithelium[51], intragastric injection to target the intestinal mucosa[52], and fetoscopic techniques including the placement of an intratracheal balloon at the time of vector installation to enhance pulmonary epithelial transduction[53]. The combination of ultrasound guidance and fetoscopic techniques was described in detail[1].

Sheep models have also been used to study the embryopathy and pathophysiology of neurological deterioration in NTD. For NTD treatment, spina bifida lesions were created in fetal sheep by *IU* surgery techniques (reviewed in[54]). The model that most closely simulated the human disease and most clearly demonstrated the feasibility of fetal MMC surgery was the fetal lamb model of MMC introduced by Meuli *et al*[55]. Pregnant sheep were placed under general halothane oxygen anesthesia. The fetuses were then exteriorized through an infra-umbilical midline laparotomy, followed by hysterotomy to expose the backs of the fetuses. A MMC lesion was made using low-power loupe magnification with microsurgical instruments at 75 d. The fetuses with the open spinal defect were then returned to the uterus,

and the amniotic fluid volume was restored with warm sterile saline. The sheep fetuses that underwent repair of the spina bifida defect were lesioned, and the defect was then closed using a latissimus dorsi muscle flap at 100 d of gestation[55,56]. The fetal sheep MMC model was the first large animal model to demonstrate that a spinal cord lesion could be created *IU* and covered at a later time point, with preservation of neurologic function. Unlike previous animal models, this sheep model more closely resembled that of human MMC in the duration of the exposure of the cord to the environment, clinical examination, and histology.

Pigs

IU cell transplantation (IUCT) and potential tolerization are based on the immunologic immaturity of the early developing fetus, leading to the possibility of donor or species specific tolerance to xenogeneic cells. Fisher's group established an IUCT procedure by which piglets are stably engrafted with human hepatocytes during early gestation and explored the possibility of producing a state of hyporesponsiveness in pigs to human hepatocytes by transplanting human hepatocytes into fetal pig livers[57]. Briefly, at gestational day 40, all gilts underwent general anesthesia and lower midline laparotomy. Both uterine horns were exposed. All fetuses in the right uterine horn received direct intrahepatic injection under ultrasound guidance using a 1.5 inch 25 gauge needle.

Furthermore, to determine whether cells could transfer between porcine littermates, McConico[58] performed IUCT. Briefly, at 40-43 d gestation, pregnant pigs/swine were anaesthetised with intra-muscular (*i.m.*) injections of telazol (5 mg/kg), xylazine (2 mg/kg) and glycopyrolate (0.06 mg/kg). Anaesthesia was maintained with inhaled isoflurane (3%-5%). A paramedian incision was made along the dorsolateral margin of the mammary glands, with the pig in lateral recumbency. One horn of the uterus, containing four to eight fetal swine, was then exposed. Guided by ultrasound, 50 million T cell-depleted umbilical cord blood cells were injected into the peritoneum of three to four fetal swine per litter[58].

If an intrauterine event has occurred, then intrauterine interventions, such as surgical repair, might prevent progressive neurological deterioration. Animal models of spina bifida or NTD repair *IU* have been designed by Heffez[42] and reviewed by George[54]. Surgical manipulation of pregnant Hanford mini-pig sows began with sedation *via* intramuscular administration of ketamine and acepromazine. The sows were intubated, ventilated and anesthetized with isoflurane. The fetal pigs were operated on at day 80-85 of the 114-d gestation period. Surgery was performed with an operating microscope. One horn of the uterus was exteriorized. The fetus underwent a two-level laminectomy with opening of the dura. In one group, fetal pigs received repair treatment following lesioning before being returned to the uterus. In the second group, fetal pigs were returned to the uterus with an open wound. The abdominal wall of the sow was closed in two layers[42,54].

Rhesus monkeys

Several animal models of MMC have been developed to test the hypothesis that *IU* intervention can prevent further spinal cord damage and the consequent neurological deficits. Primate (Macaca mulatta) was the first model, developed by Michejda, in which a fetal L3–L5 laminectomy was done late in gestation[59]. Surgical methodologies employed on pregnant rhesus monkeys began with induction of general halothane-oxygen anesthesia. The lumbosacral region of the fetuses was exteriorized *via* hysterotomy. A vertebral opening *via* a lumbar laminectomy in the L3-L5 region was created, and the spinal cord was exposed following the opening of the dura over the spinal cord. The exact techniques, magnification and precise instrumentation were not described in the methodology[59]. A total of 8 fetuses at gestational day 110-125 were manipulated, with full gestational term at approximately day 160-180[60]. The unrepaired embryos showed cystic MMC-like lesions at birth and had neurological deficits. A similar group of monkeys underwent immediate repair of the laminectomy *IU* using allogeneic bone paste to reconstruct the resected dorsal arches. These fetuses, repaired *IU,* were neurologically normal at birth. Unfortunately, the experiment did not include an initial procedure for creation of the defect with a period of exposure to the uterine environment prior to closure.

EU SURGERY

The rodents' *EU* development system is useful for analyzing the roles of molecules or interactions between tissues in the histogenesis of organs from mid to late gestational period. Previously published technical reviews on *EU* surgery are of value to the new investigator, and this surgical treatment has been only performed in rodents (mouse/rat)[8,13,14,61]. The general operation involves making a longitudinal incision along the entire length of the uterus, so that the embryos remain attached to the uterus but are not contained within the uterine cavity. The exposed uterus is returned into the abdominal cavity, where development continues *EU*. In the original study, embryos from E9.5 to E13.5 were found to develop normally to term[7]. In a subsequent study by Serbedzija *et al*[11], *EU* survival of embryos that received injections into the amnionic cavity as early as E8.5 was reported. Early stage embryos are surrounded by a layer of decidual tissue that obscures the visualization of the embryo. Removal of this layer compromises embryo survival. In general, our experience is that the survival rate of mothers is 100%. That of manipulated embryos increases with later stages and with less invasive manipulations, and can reach 100% in cases without invasive manipulation.

Both the *IU* and *EU* surgical procedures were

identical, up to the abdominal incision before the uterine wall was cut. Yamada *et al*[13] described in detail how to relax and cut the myometrial wall, clearly observing the targeting live embryos and how to replace the manipulated embryos into the abdomen. Here, we briefly describe the procedure about how to manipulate the live embryos.

Embryo manipulations

The embryos were enveloped by very thin and transparent amniotic membrane. The amniotic membrane must be kept wet and covered by sterile gauze soaked with sterile saline, otherwise it will become dry and lose its translucency which causes difficulties. *EU* surgery is a lengthier procedure than *IU* manipulation, and not all embryos are manipulated in a single female. In cases where embryo surgery is compromising embryo survival, removing all unoperated embryos can dramatically improve the survival of operated embryos[62]. Two different techniques have been reported for removing embryos from the uterine horn during *EU* surgery.

To increase the viability rate, we have routinely left three embryos on both side of the uterus taking special care for bleeding and adhesion as Yamada *et al*[13] described in detail. Ngo-Muller and Muneoka[14] reported that they removed all but four embryos, leaving two embryos in each horn in positions toward the ovarian end of the uterus. Embryos and placentae are removed by placing a dry cotton-tipped applicator at the placental-uterine junction and gently rolling it across the placenta. This procedure separates the placenta from the uterus and causes a small amount of bleeding from the uterus. Bleeding is controlled by applying direct pressure with the cotton-tipped applicator at the former placental attachment site.

Once embryos are removed and any bleeding is controlled, the abdominal cavity is flushed with saline to remove any tissue debris that might induce a postsurgical fibrotic response. After the abdominal cavity is flushed, it is filled with sterile saline. The embryos are maintained submerged in saline during and after the operation. For older stage embryos, it may not be necessary to keep the embryos submerged. The various types of manipulations that have been accomplished using the *EU* approach are summarized below.

Microinjection

The use of sharp-tipped micropipettes is the most critical for a successful microinjection, since tear of the fetal membrane causes leakage of amniotic fluid. Fetal deaths are often attributable to damages of the embryonic membrane or placenta. Injections generally utilize glass needles made from micropipettes of varying size. Yamada *et al*[13] described how to make glass micropipettes with a beveled point using a microforge. The micropipette is connected to an automated hydrolic (mineral oil) microinjection system (*e.g.*, UltraMicro Pump, WPI Inc.) fitted with a Hamilton-type syringe that allows precise control over injection volume. It is often useful to co-inject a vital dye (*e.g.*, 0.05% Nile blue sulfate or 1% Fast Green) to monitor the injection procedure. Targeted injection of purified growth factors or signal transduction antagonists directly into the embryo has been used to study signaling during normal and abnormal development[26-28].

Cells have been introduced into the embryo by targeted injection for use as *in vivo* reporters, or to characterize the behavior of stem cells in the embryonic and adult environment. Fibroblasts introduced into the embryonic mouse limb proliferate and differentiate in a position-dependent manner[63,64]. The injection of cells that secrete high levels of specific hormones has been used to experimentally perturb embryogenesis[65-67]. Targeted injection of genetically labeled liver stem cells into the embryonic liver results in chimeric livers that persist to adult stages and can be used for both the investigation of liver development and regeneration[62].

Embryonic surgery

In many instances, experimental design calls for direct surgery on the embryo. For early stage embryos, such studies are best performed using the *EU* approach, because it eliminates the need to incise and suture the uterus and avoids postsurgical complications arising from uterine contractions. Clean visualization is the most critical and important factor for embryo manipulation/surgery, thus *EU* is the better option compared to the *IU* surgical procedure. Mechanical strain plays an important role during tissue morphogenesis, and many developmental processes depend on external and internal mechanical forces[68]. In our laboratory, we performed fetal joint movement restriction by surgical techniques using this *EU* method and observed how developmental processes were related to prenatal mechanical forces.

Hip joint movement restriction: Congenital dislocation of the hip (CDH) is one of the most common congenital skeletal deformities. The prevalent type, which constitutes up to 98% of CDH cases, is exhibited at birth by a dysplasia of the hip consisting of a flat acetabular roof and an underdeveloped proximal end of the femur, relatively minor anomalies that predispose to dislocation[69]. In our laboratory, Hashimoto and Kihara created a CDH model[70,71] to clarify its etiology and to develop prevention and treatment therapies. For these purpose, at E16.5 the hind limb of the rat embryos' one side was sutured with 9-0 thread for ophthalmic surgery at the knee joint or more distally to the amniotic membrane, whereas the other side was left unoperated. The hind limbs were tied *in situ* and were not forced into any specific abnormal positions[70,71].

TMJ movement restriction: To observe the proper development of the mandibular condylar cartilage, articular disc and temporalis muscle as related to mec-

hanical forces, we restrained jaw movement by this *EU* surgery technique. In mouse embryos at E15.5, both the upper and lower jaws (mandible and maxilla) were sutured or fixed through the embryonic membrane with 8-0 nylon. The embryos underwent *EU* development[72-75].

Other surgical techniques: Another surgical technique is the resection of parts of the fetal organs. Naruse and Kameyama[76] combined the *EU* system with argon laser irradiation to the extra digits of genetic polydactyly mice. To explore the relationship between agenesis of the olfactory bulb and that of the corpus callosum, Naruse and Keino[77] performed fetal *EU* laser surgery to induce arhinencephaly in mice and clarified that agenesis of the olfactory bulbs induced agenesis of the corpus callosum[78]. In this *EU* system, they induced fetal tissue destruction without damage to the yolk sac membrane and amnion or leakage of amniotic and extra-embryonic fluid to yield embryos with high viability. Sequential observation of NTD by the *EU* method was successfully utilized to analyze the mechanism of generation of anencephaly[46]. In our laboratory, Matsumoto *et al*[46] created anencephaly mouse embryos. Pregnant mice were administered 1 mg/kg body weight 5-azacytidine (Sigma Chemical, St. Louis, Mo.) dissolved in physiologic saline by intraperitoneal injection at E7.5. After that, they observed the sequences of exencephaly, and their subsequent morphological changes, and mechanism of transformation from exencephaly to anencephaly by the *EU* development system at different embryonic days[46]. The most invasive studies to date include amputations of the limb or digit to study regenerative responses. It is possible to transplant tissues between mouse embryos to study cell–cell interactions during development. Examples include studies of the interaction between anterior and posterior tissues during mouse limb development[12] and grafts of digits in association with amputation studies[79]. Amputation studies have also been carried out on mice with targeted mutations to identify genes that are functionally required for a regenerative response[80] and to explore the diastema region of the jaw as a permissive site for the development of a transplanted tooth germ[81]. Other surgical manipulations that have been carried out on mouse embryos using a surgical approach to experimentally induce spina bifida aperta[82].

Restraining movement, amputation, wound healing and tissue grafting surgeries cause significant trauma to the embryo and can compromise embryo survival. In our and other researchers experiences, these types of embryo surgeries can have a high level of success from E13.5 and later, whereas similar manipulations at earlier stages are more challenging yet feasible[12]. This study demonstrates how multiple targeted manipulations can be successfully combined using an *EU* approach.

For both *IU* and *EU* surgery in rodents, Yamada *et al*[13] reviewed in detail about abdominal closure, recovery and post-operative care.

CONCLUSION

Advances in fetal interventions can be predicted over the next decade, driven by novel biological and endoscopic techniques. Developmental biologists have repeatedly used animal models (*e.g.*, mammals such as rodents, sheep, pigs, and monkeys; amphibians; birds) for experimental analyses of histogenesis or organogesis, or to develop powerful tools for studying the function of specific genes during development. We have explained on the methodological procedures of the *IU* (mouse/rat, sheep, pig and monkey) and *EU* (rodents) development system. These systems are useful methods for *in vivo* functional analyses from early/late organogenetic to histogenetic phases. The number of studies using *IU* or *EU* approaches has increased over the past 30 years. Now it is clear that we can successfully probe the *IU* environment of the mammalian embryo both classically (amputation, tissue transplantation, NTD creation and repair) and genetically (electroporation, gene therapy). The *EU* technique is far simpler and more time- and cost-effective than establishing genetically modified mouse/rat lines and provides a convenient experimental design for developmental research. To explore development, especially as it pertains to human health issues, there is clearly a need to develop and expand new strategies that enhance our ability to directly access the post-implantation mammalian embryo.

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P- Reviewer: Shan LP, Wang CC **S- Editor**: Ji FF
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