Chemotherapy and radiotherapy are essential in the treatment of cancers in children and young adults; while, these therapies often result in testicular failure or reduced ovarian function. So far, the priority is exclusively on the treatment of cancer, and these side effects have not been recognized. However, advances in the studies of ovarian cryopreservation are expected to correct the imbalance between the treatment effects and adverse reactions. Particularly for young women with cancer, ovarian cryopreservation before initiation of cancer treatment may give hope for the preservation of fertility. One of the problems in the study of cryopreservation techniques is that use of cryopreserved ovary yields only a very low rate of fertilization. We hypothesized that this is caused by instable blood flow during organ transplantation, so we conducted an experiment in a rat ovarian transplantation model, focusing on vascular anastomosis and cryopreservation. This study is based on the technique of vascular anastomosis, which we call “Super-Microsurgery”. First, we examined the feasibility of the technique for ovarian transplantation. We removed vascularized ovaries from recipient rats and anastomosed the vessels of the ovaries to the subcutaneous vessels of donor recipient nude mice. A histological analysis of the group undergoing anastomosis and the group not undergoing anastomosis revealed that ovarian follicles and ovarian granulosa cells were better preserved in the group undergoing anastomosis, indicating the importance of vascular anastomosis in ovarian transplantation.
Ovarian transplantation in humans

In October 2004, Donnez et al. published a report describing a woman with stage IV Hodgkin's lymphoma giving birth to a healthy girl after transplantation of cryopreserved ovarian tissue fragments. This is the world’s first report of a live birth after transplantation of cryopreserved ovarian tissue. As another example of ovarian transplantation, there is a report that a 24-year-old woman with ovarian failure underwent transplantation of the ovarian tissue of her monozygotic twin sister and gave birth to a healthy girl after a normal period of gestation. Despite all the efforts around the world for research into the transplantation of cryopreserved ovaries, only five live births have been reported as of May 2009. The fragments of ovarian tissue was used in all reported cases.

In the previous method for preservation of the ovaries in gynecology, the fragments of ovarian tissue is transplanted, and anastomosis is not performed. The results of the method are unsatisfactory; estradiol may be secreted for 2 to 8 months but often disappears in 1 year, although the ovary may function for 3 to 4 years in some cases. Permeation of the cryoprotective agent into fragmented tissue of the ovary can reduce crystal formation and freezing injury but, at the same time, blood flow cannot be secured, and thus leading to necrosis of many ovarian follicles and result in poor quality eggs. Although it is very useful, the method is still in the experimental stage and has not achieved effective clinical application.

To avoid ischemic injury due to unstable blood flow, attempts have recently been made to cryopreserve and transplant a vascularized whole ovary. Live birth has already

Figure 1.
In plastic surgery, anastomosis of vessels of about 2.0 mm diameter has been considered possible with a microscope for reconstructive surgery for cancer patients. We enabled anastomosis of about 0.2–0.3 mm diameter, about one-tenth of the conventional technique, by using 12-0 nylon string (50-µm needle) and by continuous development of surgical instruments.

Figure 2. Development of a model for xenotransplantation of the vascularized ovary
Subcutaneous heterotopic xenotransplantation from rats to nude mice
a: The femoral artery and vein of the nude mouse are opened. b: The rat ovary is anastomosed to the femoral artery and vein of the nude mouse. c: Subcutaneous heterotopic xenotransplantation of the ovary.

Figure 3. HE staining
a, b: The transplanted ovary of the anastomosis group. Nearly 100% of the eggs were alive. c, d: The transplanted ovary of the anastomosis group. Almost all ovarian follicles were dead due to ischemic injury. Some ovarian follicles were alive on the surface of the transplanted ovary. The arrow indicates the border between the transplanted organ and the recipient vessel.
been reported in animal experiments\(^6\), but the method is not yet ready for clinical application. This is because vascular anastomosis of the ovary, which is especially small compared with other organs, requires high-level techniques. Technical progress in microvascular anastomosis using microscopy over the past 40 years in plastic surgery has enabled anastomosis of vessels of about 0.3 mm diameter using 12-0 nylon string (50-µm needle) (Figure 1). We named the anastomosis technique “Super-Microsurgery”\(^{11}\). This technique enabled the creation of an experimental model for tissue transplantation that had been considered impossible.

As the first step of research into preservation of fertility, we histologically analyzed the ovarian tissue, which had been transplanted using the super-microsurgery technique, and showed the importance of preserving blood flow in ovarian transplantation. Our present study breaks new ground and focuses on blood flow in transplantation, which will surely lead to new findings.

**Methods**

**Ovarian transplantation in rats**

The donor rats (F344/Jc1, 10 weeks old, 130 g) were administered general anesthesia with intraperitoneally administered pentobarbital and placed in the supine position. Then, an abdominal midline incision was performed. The ovarian artery and vein were identified, and the ovary was removed with the vessels preserved. The right ovarian artery and vein were removed to the level of the bifurcation of the left renal artery and vein. Recipient nude mice (BALB/c-nu/nu, 10 weeks old, 30 g) were administered general anesthesia with intraperitoneally administered pentobarbital and placed in the supine position in the same way. The inguinal skin was then incised, and the femoral artery and vein were removed and opened to prepare the recipient vessel (Figure 2a). Using 12-0 nylon string, we anastomosed the mouse femoral artery to the rat ovarian artery (0.3 mm), as well as the mouse femoral vein to the rat ovarian vein (0.9 mm), and sutured the skin to fix the ovary under the skin of the mouse (anastomosis group, Figure 2b and c). We also subcutaneously transplanted the ovary to mice without anastomosis of the artery and vein and used them as the control (no-anastomosis group). Four weeks after transplantation, the recipient nude mice were again administered general anesthesia with intraperitoneally administered pentobarbital. We removed the transplanted ovaries from the anastomosis and no-anastomosis groups and examined them histologically.

**Histological evaluation**

Each sample (transplanted ovaries of the anastomosis group, transplanted ovaries of the no-anastomosis group) was fixed in 10% formalin solution, embedded in paraffin, and cut into serial sections of 4–5 µm thick. The sections were stained with HE and examined by light microscopy.

We anastomosed the ovarian artery and vein of the rats to the femoral artery and vein of the recipient mice, confirmed the blood flow, and sutured the skin (Figure 2). We then removed the ovaries from the group undergoing anastomosis (anastomosis group) and the group not undergoing anastomosis (no-anastomosis group) four weeks after transplantation and histologically examined the follicular viability (Figure 3).

In the anastomosis group, nearly 100% of the follicles were alive after transplantation. In the no-anastomosis group, on the other hand, central necrosis was observed in the tissue of the transplanted ovaries and the follicular viability was 1–2%.

In the anastomosis group, most of the granulosa cells surrounding the follicles were also alive.

**Results**

We are studying the temporary egg-sitter transplantation method\(^{13}\) and the ovarian whole organ freezing method\(^{14}\) for the preservation of fertility in children with cancer. The former study aims at the preservation of the whole ovary by temporarily transplanting the ovary into someone else (e.g., the child’s mother) before cancer treatment or during remission and returning the ovary to the patient after cancer treatment. Compared with previous studies, this method is advantageous in that freezing of the organ is not required. The latter study, ovarian whole organ freezing, examines the organ freezing method under variable magnetic fields. This method is intended to provide stable blood flow by anastomosis during autotransplantation of the ovarian organs that have been cryopreserved without fragmented and thawed. Both of these studies were conducted in an experimental model of ovarian organ xenotransplantation between rats and mice, which has been considered unfeasible.

Ovarian autotransplantation is a current topic in gynecology. Aspects of the surgical techniques have not been studied very much. Previous reports of foreign cases of pregnancy focused on the follicles remaining on the surface of the ovary not undergoing anastomosis and showed that pregnancy may still be possible. However, the problem still remains that the deteriorated quality of eggs due to ischemic injury results in a low pregnancy rate. Some researchers studied the transplantation of vascularized ovaries but reported that transplantation of the frozen-thawed whole ovary resulted in the frequent occurrence of thrombosis and follicular viability of less than 10%\(^{6,14}\).

In the present study, nearly 100% of the follicles were alive after transplantation in the transplanted ovaries in the anastomosis group. In the no-anastomosis group, on the other hand, few follicles remained alive, and most follicles became...
necrotic due to ischemic injury. Although some follicles remained alive in the no-anastomosis group, these were present on the tissue surface adjacent to the recipient vessel and considered nourished by the exudates from the recipient vessel. The ovary is responsible for secretion of female hormones (estrogen and progesterone) and for fertility. So, once the ovary function is disrupted, women experience sexual difficulties and growing girls fail to develop secondary sexual characteristics. Moreover, juvenile climacteric disturbances may develop. The ovarian transplantation method used in the present study is expected to preserve endocrine function because ovarian granulosa cells were preserved. Resumption of the menstrual cycle after cancer treatment is regarded as a sign of fertility in gynecology, but this understanding is not completely correct. In the no-anastomosis group, the menstrual cycle may resume because some eggs and granulosa cells were alive. However, the quality of the eggs was severely deteriorated due to ischemic injury. While the remaining poor quality eggs and granulosa cells maintain the sexual cycle, fertility is expected to be low. This may be the cause of anovulatory cycles and limited cases of pregnancy in the clinical setting despite resumption of the menstrual cycle after cancer treatment.

In addition, anastomosis to the subcutaneous artery and vein during transplantation experiments instead of transplanting into the abdomen enables easy understanding of the status of the ovary over time, which may lead to further study success. However, although a rat model for transplantation experiments is suitable for basic studies of ovarian transplantation, clinical application in humans is not possible with the data from this model alone. We have attempted cryopreservation and transplantation of the ovary in pigs and cynomolagus monkeys (Figure 4) and think that clinical application of the method in humans is feasible in terms of transplantation techniques.

Conclusion
We emphasize that our focus is on the blood flow of the organ. This study is unique in that use of the super-microsurgery technique enables stabilization of blood flow in the ovarian tissue immediately after transplantation. By combining the technique with our cryopreservation technique, we aim to establish a new fertility preservation method. This is a type of reconstructive surgery using the super-microsurgery technique for preservation of fertility in children with cancer.

<Issue 1: Cryopreservation of the ovary>
The method for cryopreservation of the vascularized ovary has not yet been established even at the animal experiment level. Cryopreservation or alternative methods to preserve the ovary should be established.

<Issue 2: Technological difficulties in the super-microsurgery technique>
Acquisition of the super-microsurgery technique requires considerable training. Technical difficulties should be overcome in both basic experiments and clinical application.

<Issue 3: Metastasis of cancer in the ovary>
It is virtually impossible to demonstrate that there is no cancer remaining in the ovary to be transplanted. For clinical application, patients should be selected for application of the technique.

References