Chapter 8
Ovarian Tissue Cryopreservation and Transplantation: Banking Reproductive Potential for the Future

David Lee, MD

Transplantation of cryopreserved ovarian tissue is a technology that holds promise for preserving reproductive potential for the future. It may be apropos for cancer survivors who will undergo treatment with sterility-inducing chemotherapy or radiation. Although there is some evidence suggesting cellular and molecular injury with the freezing and thawing process, there are examples in both animals and humans that transplantation of cryopreserved ovarian tissue can lead to successful restoration of fertility. Currently, cryopreservation of ovarian tissue is the only option available to preserve fertility in prepubertal girls or women who cannot delay their cancer treatment. For this patient population, ovarian tissue banking and subsequent transplantation is the only fertility-preserving method that has resulted in live-born pregnancies. The technology of ovarian tissue banking is currently at the forefront of the emerging field of oncofertilty.

Indications for Ovarian Tissue Banking

Scope of the Clinical Problem and Incidence of Ovarian Failure

There are more than 9 million cancer survivors living in the United States today. Furthermore, it is estimated that by 2010, 1 in every 250 people will be a survivor of cancer [1]. The prognosis for patients with childhood cancers is excellent, with greater than 70% surviving, and therefore, attention can be focused on patients' quality of life rather than just survival. Unfortunately for many young women, the chemotherapy or radiation therapy used to treat them is toxic to their ovaries and renders them infertile and dependent upon hormone replacement therapy. The incidence of ovarian failure may approach over 90% in patients undergoing high-dose chemotherapy [2]. Given that 1 in 52 females between birth and age 39 are diagnosed with cancer [3], many people are potentially affected.

One potential solution is to remove, freeze, and bank ovarian tissue before a patient undergoes gonadotoxic treatment, thereby removing the ovaries from harm, and then transplant the tissue back after completing treatment (autografting). Alternatively, ovarian tissue could be transplanted to an immunocompromised

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mouse host in order to minimize the risk of cancer transmission within the grafted ovarian tissue (xenografting), or oocytes isolated from the tissue could be matured in culture (in vitro maturation). Clinical decisions must always weigh the potential risks and benefits. Since there has been limited success with the aforementioned strategies, and since ovarian tissue banking requires removal of ovarian tissue, it is necessary to have a clear idea of the risk of ovarian failure from chemotherapy and/or radiation therapy. If the risk of ovarian failure is inevitable, it is reasonable to undertake these fertility-preserving strategies.

Gonadotoxicity of Chemotherapy

Chemotherapy can cause sterility in 38–56% of Hodgkin's lymphoma patients and the majority of bone marrow transplant patients [2]. The clinical course can be unpredictable. Oligomenorrhea can be followed by normal menses or premature ovarian failure (POF). Treatment with alkylating agents is particularly harmful (Table 8.1) [4,5]. The incidence of ovarian failure is dependent on the agent, dose, and age of the patient. Younger patients are more resistant to the gonadotoxic effects of the chemotherapy (Table 8.2) [6–9]. Offspring born to women who have received prior chemotherapy do not appear to be at increased risk for birth defects.

Table 8.1 The gonadotoxicity of chemotherapeutic agents

In 168 patients who received combination chemotherapy, the overall ovarian failure rate was 34%, representing an odds ratio of 1.0. The odds ratio of ovarian failure was calculated in exposed and non-exposed patients.

Group	Mechanism	Agents	Odds ratio for ovarian failure
Alkylating agents	Crosslinks DNA strands Inhibits RNA formation	Cyclophosphamide (Cytoxan) Cholorambucil Mustine Melphalan Busulfan Carmustine Lomustine	4.0
Platinum derivatives	Crosslinks DNA strands	Cisplatin Carboplatin	1.77
Vinca alkaloids	Disrupts microtubules and spindle	Vincristine Vinblastine	1.0
Antimetabolites	Inhibits pyrimidine or purine synthesis or incorporation into DNA	Cytarabine Methotrexate	0.3
Antibiotics	Multiple (transcription inhibition, DNA intercalation)	Adriamycin Bleomycin	0.25
Others	Unknown	Procarbazine	Unknown

Table 8.2 The gonadotoxicity of cyclophosphamide is dose and age dependent

Dose of cyclophosphamide before amenorrhea	Age of patient (years)
5,200 mg (5 g)	40
9,300 mg (10 g)	30
20,400 mg (20 g)	20
>50,000 mg (50 g)	Prepubertal

Table 8.3 Dose estimated to cause ovarian failure in 97.5% of patients as a function of age

Age (years)	Ovarian dose (cGy)
Birth	20.3 Gy (2,030 cGy)
10	18.4 Gy (1,840 cGy)
20	16.5 Gy (1,650 cGy)
30	14.3 Gy (1,430 cGy)

Gonadotoxicity of Radiation Therapy

Radiation therapy can adversely affect the ovaries, uterus, and hypothalamic-pituitary-ovarian axis such that future fertility is severely compromised.

Ovary

Radiation is harmful to the oocytes within the ovary. The LD50 of irradiation to the oocyte is 4 Gy [10], and some estimate that 5–10 Gy of radiation to the ovary causes ovarian failure in 97% of women. Younger patients are more resilient to radiation. Wallace et al. estimated that 18–20 Gy of ovarian radiation are necessary to induce ovarian failure in 97.5% of patients (Table 8.3) [11]. More conservative estimates by Chiarelli et al. showed that childhood cancer survivors receiving 20 Gy of abdominal irradiation had a relative risk of ovarian failure of only 1.02 [8]. Doses of 20–35 Gy caused infertility in 22% of patients, and doses greater than 35 Gy caused infertility in 32% of patients.

Uterus

High doses of abdominal irradiation (20–30 Gy) [12] and lower doses used in total body irradiation (14.4 Gy) [13] can adversely affect the growth and blood flow of the uterus. If subsequent pregnancy occurs, there is a statistically significant risk of preterm labor, low birth weight babies, and miscarriage.

Hypothalamic-Pituitary-Ovarian Axis

Cranial radiation to treat brain tumors can adversely affect the hypothalamic-pituitary-ovarian axis. Doses greater than 24–50 Gy are associated with delayed puberty [14,15], while lower does of cranial irradiation are associated with precocious puberty [14,16].

Limitations of Fertility: Preserving Techniques

Potential approaches to preserving fertility in women surviving cancer include ovarian suppression with gonadotropin releasing hormone (GnRH), pexying the ovaries outside the field of radiation, embryo freezing, oocyte freezing, and ovarian tissue banking with subsequent in vitro oocyte and follicle maturation. Each of these options has unique problems as summarized in Table 8.4.

Ovarian pexying involves surgically moving the ovaries medially behind the uterus, which is subsequently shielded, or laterally, outside the field of radiation. The ovaries can be sutured to prevent subsequent migration. The surgery can compromise the blood supply to the ovary, however, and transposition of the ovary does not always remove it from the field of radiation. Kwon reported that ovarian failure can still occur in 30–80% of cervical cancer patients undergoing pelvic irradiation after ovarian transposition. In addition, pexying does not protect the ovary from chemotherapeutic agents [17].

Gonadotropin releasing hormone agonist treatment may reduce the risk of POF; however, equivocal results indicate additional controlled trials are needed [18,19]. The observation was made that prepubertal girls had lower rates of ovarian failure after chemotherapy and radiation therapy than post-pubertal patients. With this in mind, some postulate that continuous GnRH exposure leads to downregulation of the pituitary and induction of a prepubertal state. This ovarian quiescence during cancer treatment might decrease susceptibility to gonadotoxic treatments. While some data in monkeys [20,21] and in small, non-randomized clinical studies [18,22] show benefit with GnRH therapy, one prospective randomized trial [23] showed no benefit. Primordial follicles in humans do not have follicle-stimulating hormone (FSH) receptors, so suppression of FSH with a GnRH agonist theoretically would not be protective. Younger patients may be less susceptible to chemotherapy because they have a greater number of oocytes, not because their ovaries are quiescent during chemotherapy.

In vitro fertilization (IVF) with embryo cryopreservation can be performed with pregnancy rates of 20–30% per frozen embryo transfer, but this approach requires ovarian stimulation and a male partner. Consequently, it is not applicable to children and can also create “orphan embryos” should the patient not survive. In addition, 2–6 weeks are required for ovarian stimulation and egg retrieval, which often delays initiation of cancer therapy.

Table 8.4 Summary of treatment options to preserve fertility

Method	Description	Advantages	Disadvantages	Efficacy	Cost
GnRH agonist	• Monthly injection to induce prepubertal state	• May decrease damage by chemotherapy	• Of no benefit in prepubertal girls or with radiation	• May decrease oocyte loss by 40%	$600–$900/ month
Ovarian pexy	• Laparoscopically moving the ovaries outside the field of irradiation	• Minor surgical procedure	• Requires surgery • Not effective with chemotherapy • Ovary may migrate • Blood supply for ovary may decrease	• May decrease the dose of radiation to the ovary to 10% • Ovarian failure rate of 30–80%	$5,000
Ovarian tissue banking	• Laparoscopically remove ovarian tissue or ovaries and freeze • Maturation of eggs in the future by trans-plantation or in vitro methods	• Many oocytes preserved • Does not require stimulation • Does not delay cancer treatment • Appropriate for prepubertal girls	• Requires surgery • Eggs within the ovarian tissue need maturation before use • Limited efficacy	• Four human pregnancies to date	$5,000
Oocyte freezing	• Stimulation of ovaries with gonadotropins • Egg retrieval • Freeze mature unfertilized eggs	• Clinical efficiency improving • Appropriate for single young women • Not appropriate for prepubertal girls	• Requires 2–6 weeks for stimulation and retrieval • Only 20 oocytes banked per cycle	• 200 pregnancies worldwide • 1–3% pregnancy/ oocyte frozen	$8,000–$18,000
Embryo freezing	• Stimulation of ovaries with gonadotropins • Retrieve eggs • Fertilize with partner's or donor sperm	• Proven technique	• Requires 2–6 weeks for stimulation • Not appropriate for prepubertal girls • Requires sperm • Limited number of embryos can be banked	• 20–30% pregnancy/ embryo transfer	$8,000–$18,000

Cryopreservation of mature oocytes eliminates the need for a male partner and prevents creation of orphan embryos. To date, there have been 148 pregnancies in the world via oocyte freezing [24]. However, it too requires time and resources for ovarian stimulation. Second, ovarian stimulation is inappropriate in prepubertal girls because it initiates pubertal changes. Third, monitoring the growth of follicles and extraction of oocytes requires transvaginal ultrasound, which can be problematic in virginal or young patients. Fourth, a limited number of oocytes (15–20) are typically obtained at retrieval. Fifth, mature oocytes are challenging to freeze. The spindle is temperature sensitive, the zona pellucida hardens, and their relatively large size predisposes them to intracellular ice formation. Finally, the success rates are less than 2% per frozen oocyte [24–27].

Cryopreservation of immature oocytes with subsequent short-term in vitro maturation is another alternative and has been demonstrated in the mouse [28]; however, it has not yet yielded human embryos [29]. To date, there is no clear solution to this overwhelming clinical problem.

The Promise of Ovarian Tissue Banking

One potential solution is to freeze and bank ovarian tissue before patients undergo gonadotoxic treatment. Ovarian tissue banking involves surgically removing and cryopreserving ovarian tissue prior to the patient undergoing gonadotoxic cancer therapy, thereby removing the oocytes from harms way. The technology involves freezing immature primordial follicles in situ within the ovarian cortex or whole ovaries. Once the ovarian tissue is frozen, there are several options available for its future utilization, including autografting, xenografting, and in vitro maturation.

Ovarian tissue banking has several theoretical advantages over other fertility-preserving strategies. First, a 1-mm^3 piece of ovarian cortex may contain hundreds of oocytes [4]. Thus, cryopreservation of ovarian tissue is a potentially more efficient method of storing reproductive potential. Second, unlike collection of oocytes and production of embryos, which require time-consuming hormonal stimulation, oophorectomy does not delay cancer treatment. Oophorectomy or ovarian biopsy can usually be performed laparoscopically in less than an hour on an emergency basis. Third, primordial follicles consist of immature oocytes surrounded by a single layer of flattened pre-granulosa cells. These oocytes are much smaller, metabolically less active, and are not arrested at a stage where the spindle is present. All of these characteristics may make them better suited for cryopreservation than mature metaphase II oocytes. Finally, the immature oocytes within the ovarian tissue would be matured much later in life, thereby obviating the need for exogenous gonadotropin stimulation. Thus, ovarian tissue banking is appropriate for prepubertal girls.

Ovarian tissue banking has its disadvantages as well. First, surgery is required to obtain the ovarian tissue. Second, ovarian cortex is theoretically difficult to freeze because of its heterogeneity. Each cell type that comprises ovarian tissue

(oocytes, granulosa cells, interstitial cells) has unique biological characteristics that require different freezing protocols. Finally, oocytes within ovarian tissue are immature, and require maturation before fertilization can occur. Follicles within ovarian tissue are arrested in early meiosis and cannot be fertilized. The process of follicular maturation is complex and requires multiple steps. The primordial to primary follicle transition involves numerous factors, primarily of the transforming growth factor (TGF) and platelet-derived growth factor families [30–35]. The formation of a fluid filled cavity, the antrum, within the layers of granulosa cells signifies the next stage of follicle growth and development, and is dependent on increased follicular vascularization and permeability of the blood vessels. As the follicle continues to grow, it resumes meiosis. In the primate, it is estimated that 150 days are required for growth from the primordial to the large preantral stage, followed by up to 70 days to reach the preovulatory stage [36,37]. Hence, with ovarian tissue banking, transplantation or extensive culture are needed before the harvested oocytes can be fertilized. Emerging technology utilizing three-dimensional follicle culture systems have led to successful in vitro maturation of mouse follicles [38–41]; IVF performed with these cultured oocytes has led to the birth of live, viable offspring [42]. These developments hold promise for the future use of banked, cryopreserved ovarian tissue for in vitro maturation and IVF. The current status of how these disadvantages have been overcome will be discussed below.

Cryopreservation of Ovarian Tissue has been Successful

Ovarian tissue banking is a two-step process. First, ovarian tissue must be cryopreserved with viable oocytes recovered upon thawing. Second, the primordial follicles within the frozen/thawed tissue must be matured. The freezing and thawing process can damage cells by both the formation of intracellular ice as well as the toxicity of the cryoprotectants. Cryoprotectants are molecules that help to prevent intracellular ice formation (see Mullen and Critser, this volume). The majority of pregnancies from banked oocytes have come after slow-rate freezing. Slow-rate (or equilibrium) freezing involves low, non-toxic concentrations of cryoprotectants and dehydration during cooling. Slow cooling involves the precipitation of water as ice, resulting in the separation of water from the solution. In contrast, vitrification involves very rapid freezing where solutions go directly from the aqueous phase to the glass state (amorphous solid) without going through the crystalline solid state in which damage can occur. Much higher concentrations of cryoprotectant are needed for this technique.

Oktay et al. [43] showed that ovarian tissue could be cryopreserved employing 1.5 M ethylene glycol and 0.1 M sucrose [44] and a slow-rate freezing process. A high percentage of primordial follicles survived the freezing/thawing process [43]. Our lab developed a novel system for vitrifying ovarian tissue (Fig. 8.1) [45]. We demonstrated that follicle viability was equivalent with vitrification (70.4 % ± 4.8%, $n = 1{,}705$) and slow-rate freezing (67.3% ± 4.7%, $n = 1{,}895$). Thus, this first step in ovarian tissue banking has been successful.

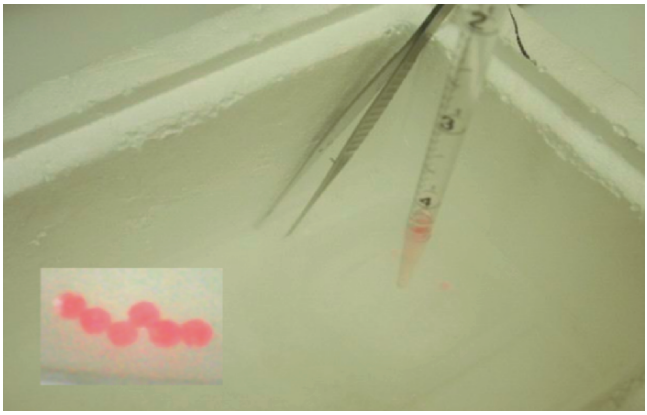

Fig. 8.1 A novel containerless system for vitrifying ovarian tissue developed by the authors. Pieces of ovarian cortex were placed into cryoprotectant and drops of the solution containing tissue were added directly to liquid nitrogen. Frozen droplets were then transferred into cryovials filled with liquid nitrogen for storage

Autotransplantation of Ovarian Tissue has been Successful

The second step in ovarian tissue banking involves maturation of immature follicles. Primordial follicles are immature eggs, arrested in the dictyotene stage of prophase I, and are surrounded by a single layer of flattened, pre-granulosa cells. Oocytes within primordial follicles cannot be fertilized before undergoing maturation. The maturation process is thought to take about 200 days, and the initial stages of growth are not dependent on FSH [36].

Autografting involves transplanting the ovarian tissue back into the donor from whom it was obtained. With autografting, the thawed, transplanted, immature oocytes would mature in vivo, thereby obviating the need for exogenous gonadotropin stimulation. Autografting of ovarian tissue would theoretically preserve a woman's endocrinologic function, unlike IVF and oocyte cryopreservation, which only address fertility.

History of Ovarian Transplantation

Ovarian transplantation is not new; it has a long history dating back to the early 1900s. People believed that waning sex steroids resulted in somatic cell aging, and that transplantation held the key to rejuvenation and eternal youth. However, it was not until the turn of the twentieth century that widespread interest was generated in reproductive organ transplantation. Despite many attempts of allogeneic ovarian transplantation in the 1900s, no clear clinical benefit was realized, primarily due to immune reactions. A breakthrough occurred in 1948 when the first cryoprotectant,

glycerol, was discovered. The development of freezing methods using cryoprotectants led to work on the transplantation of cryopreserved gonadal tissue in the 1950s [46,47], eventually leading to viable offspring in mice [48]. In the 1990s, investigators begun to realize the potential clinical applications of cryopreservation, and research began again using new cryoprotectants.

Cortical Strips

Most recent experience with ovarian transplantation has utilized strips of ovarian cortex. Most of the primordial follicles in ovarian tissue lie in the "outer skin", just beneath the tunica albuginea (see Fig. 8.2). After the ovary is removed, it can be bi-valved, and the inner medullary tissue dissected away, leaving a thin "rind" of ovarian tissue that contains most of the eggs. Thinness (1 mm) of the cortical tissue is important to allow adequate exposure to and diffusion of cryoprotectants into the ovarian tissue prior to cryopreservation. In addition, since ovarian cortical strips are transplanted without vascular anastamoses, thinness of the tissue is important since the graft must initially survive via simple diffusion until neovascularization can occur.

Heterotopic vs. Orthotopic Grafts

The ideal location for transplantation of ovarian tissue has not yet been defined. Orthotopic transplantation is grafting tissue back to its native site. For ovarian tissue, this would include transplantation of cortical tissue back to the ovarian hilum or a nearby location such as the pelvic sidewall [49]. Orthotopic transplantation provides the potential for spontaneous pregnancy without IVF (i.e., the oocyte can ovulate from the transplanted ovarian tissue, be picked up by the tube, fertilized, and implant in the uterus) [50,51].

Heterotopic transplantation involves grafting tissue back to a non-native, ectopic site. Ovarian tissue has been transplanted into the arm and abdomen [52,53]. Heterotopic transplantation allows for easier monitoring of follicular development

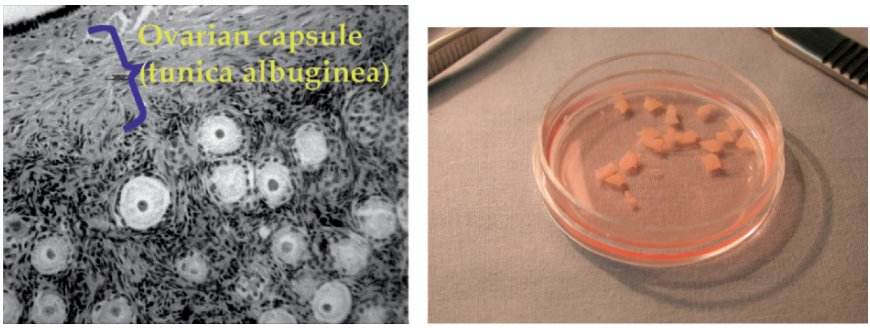

Fig. 8.2 (**A**) The density of primordial follicles is greatest just under the tunica albuginea. (**B**) The outer ovarian cortex has been cut in preparation for transplantation

and retrieval of oocytes. It also allows for easier monitoring of cancer growth within the transplanted ovarian tissue.

Animal Data

To date, successful cryopreservation and transplantation of ovarian tissue has been achieved in various animals. Cryopreservation of mouse ovarian tissue was found to produce good results with restoration of fertility after transplantation [48,54,55]. Fertility has also been restored using autografts stored at −196°C in ovariectomized sheep, whose ovaries more closely resemble those of humans [44]. Schnorr et al. transplanted autologous ovarian tissue into the upper arm of cynomolgus monkeys [56]. Menstrual cyclicity resumed in 5/6 (83%) fresh transplants and in 2/4 (50%) of thawed transplants.

Our lab [53] performed laparoscopic bilateral oophorectomies on seven rhesus macaques, and subsequently autologously transplanted fresh ovarian cortical tissue to the arm, abdomen, and kidney. Ovarian cortex was cut into 1×3×4 mm pieces ($n=219$) in 4°C Leibovitz medium and transplanted immediately to the animal of origin in subcutaneous pockets or flaps juxtaposed to muscle or kidney (Fig. 8.3A, B). Four monkeys had transplants to both the arm and abdomen ($n=23$–54), two to the kidney and abdomen ($n=18$–42), and one to the arm only ($n=26$). When 4 mm follicles developed, oocytes were collected via follicle excision 26–30 h after injecting 1,000 IU of human chorionic gonadotropin (hCG). Mature oocytes were fertilized via intracytoplasmic sperm injection (ICSI). All monkeys demonstrated estradiol (E2) levels greater than 50 pg/ml within 70–150 days post-transplantation. Estradiol and progesterone (P4) levels were higher in the local venous drainage of an arm transplant than in systemic venous blood, indicating the presence of functional grafted ovarian tissue (Fig. 8.3C). One monkey with renal and abdominal grafts showed repeated increases in P4 levels greater than 3 ng/ml approximately every 60 days, which is longer than the normal 28-day cycle (Fig. 8.4). FSH rose in this animal to 10.5 mIU/ml 84 days post-transplantation, but then declined to 2.79 mIU/ml by day 169, indicating adequate estrogen production. Several animals developed multiple follicles without exogenous gonadotropin stimulation; abdominal subcutaneous grafts showed the best follicular development (50%, Table 8.5). Follicles were excised ($n=23$) from 4 hCG-treated monkeys; 16 oocytes were obtained. Eight were mature; six were fertilized via ICSI and cleaved in vitro. A five-cell, an eight-cell, and two morula-stage embryos were transferred laparoscopically to the oviducts of three recipient monkeys. A normal singleton gestation resulted from the transfer of the morulas, and ended in the birth of a healthy, 500-gram female in 2003 (Fig. 8.3D). She is named BRENDA for Bilateral oophorectomy, Resumption of ENDocrine function and Abdominal follicle pregnancy.

From the BRENDA data, several important conclusions can be drawn. First, transplantation into subcutaneous sites resulted in endocrine function and follicular development. Second, the abdomen appeared to be the best transplant site. Third, the resumption of endocrine function after about 130–150 days post-transplant is consistent with the time frame required for progression of primordial to antral

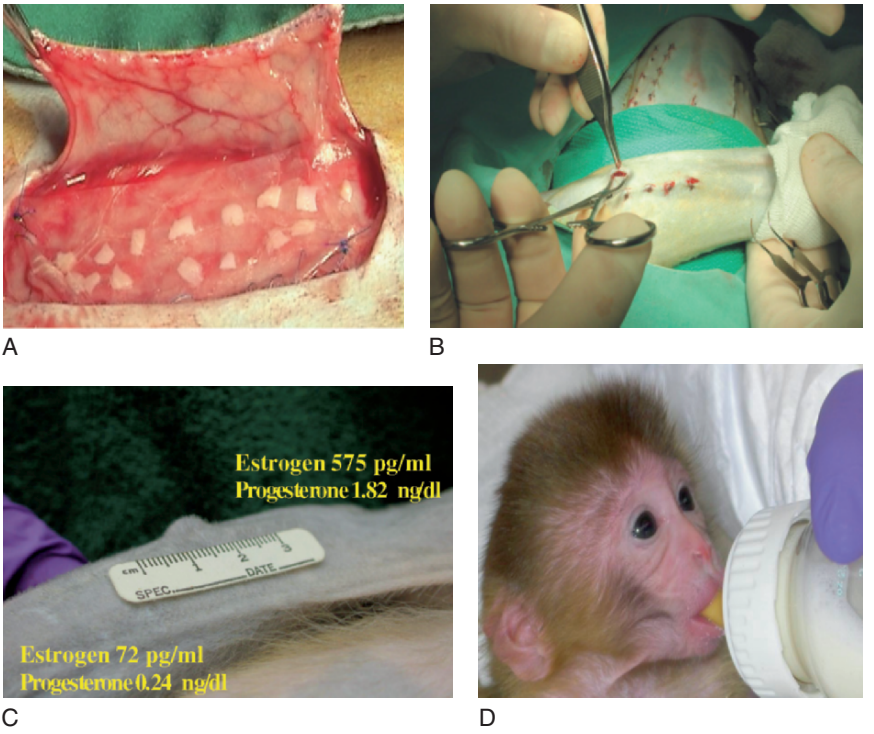

Fig. 8.3 (**A**) Ovarian tissue transplanted to the abdomen in flaps (**B**) and to subcutaneous pockets in the arm and abdomen (**C**) An ovarian follicle developing in the arm shows high local estrogen and progesterone secretion. Estradiol increased from 72 to 575 pg/ml in venous blood from the transplanted tissue. (**D**) Oocytes retrieved from heterotopic grafts were fertilized resulting in a healthy, term live-born monkey. From Lee DM et al. Nature 2004;428:137–138

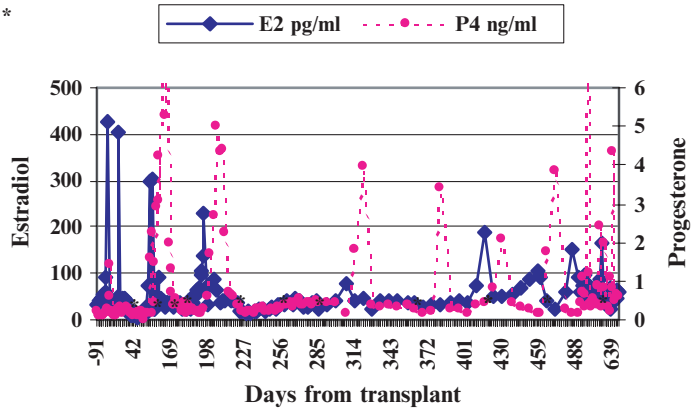

Fig. 8.4 Estradiol and progesterone are secreted cyclically after transplantation of ovarian tissue to the kidney and abdomen

Table 8.5 Of 219 ovarian tissue transplants, 49 developed follicles, and 23 were retrieved. Of 16 oocytes, 8 were mature, 6 were fertilized, and 3 embryos were transferred, resulting in 1 pregnancy

Site	#Graft	#Foll US	#Foll Ret	#Egg	#MII	#Fert	#Cleav	#ET	#Preg
L Arm	46	7	3	2	1	1	1	1	0
L Abd	54	27	15	11	6	5	5	2	1
L Kid	15	7	0	0	0	0	0	0	0
R Arm	44	5	4	3	1	0	0	0	0
R Abd	46	4	1	0	0	0	0	0	0
R Kid	14	1	0	0	0	0	0	0	0
Total	**219**	**49**	**23**	**16**	**8**	**6**	**6**	**3**	**1**
		22%	**47%**	**70%**		**75%**	**100%**		

Abd=abdomen; Cleav=cleaved; ET=embryo transfer; Fert=fertilized; Graf=grafted; Kid=kidney; L=left; MII=resumed meiosis; R=right; Ret=retrieved; US=ultrasound

follicle development [36,57,58]. Therefore, most likely, antral follicles are lost in the tissue preparation and transplantation process, and subsequent antral follicles represent in vivo maturation of the remaining primordial follicles.

Human Data

Oktay and colleagues first reported that ovulation occurred in autografted human ovarian tissue after gonadotropin stimulation [49,59]. A 29-year-old woman had undergone bilateral oophorectomy for benign indications. The ovarian tissue was cryopreserved in 1.5 M propanediol, thawed, and transplanted laparoscopically to the pelvic sidewall. The patient had follicular development documented by ultrasound with high doses of gonadotropins. In another case [52], ovarian tissue was transplanted to the forearm, and E2 measurements showed a gradient between the hand and cubital fossa, demonstrating functionality of the graft.

Radford et al. reported successful orthotopic transplantation of ovarian cortical tissue from a patient treated with chemotherapy for Hodgkin's lymphoma [60]. Seven months after transplanting ovarian cortical strips to the ovaries, she had resolution of hot flashes, E2 in the serum, a 10-mm endometrial lining, and a 2-cm diameter follicular structure seen by ultrasound.

Oktay et al. reported the first embryo derived from cryopreserved ovarian tissue that was heterotopically transplanted to the abdomen of a 30-year-old breast cancer patient [61]. Since then, four human pregnancies have been reported using both fresh and cryopreserved orthotopic ovarian tissue [50,51,62,63]. Donnez et al. [50] reported a 25-year-old patient with Hodgkin's lymphoma who underwent laparoscopic left ovarian cortical biopsies prior to MOPP/ABV chemotherapy and 38 Gy of radiation. She became amenorrheic with an FSH level of 91 mIU/ml. She then underwent laparoscopic peritoneal excision to promote vessel formation prior to ovarian tissue transplantation, followed by laparoscopic ovarian tissue transplantation to the pelvic sidewall 7 days later. A second laparoscopic transplant was also performed. She developed a follicle and became spontaneously pregnant. Although this is the first reported human pregnancy after ovarian tissue transplantation, it is

possible that the pregnancy may have originated from an oocyte released from the ovary left in situ, and not from the transplanted ovarian tissue.

Silber et al. [51] subsequently reported a pregnancy from orthotopic transplantation of fresh ovarian tissue between monozygotic twins discordant for POF. One of two 24-year-old twins developed POF at age 13. The other twin went on to conceive three children spontaneously. After unsuccessful donor egg IVF, the sterile twin received a transplant of ovarian cortical tissue (fresh) from her sister via a mini-laparotomy. Within 3 months, the recipient's cycles resumed, and she conceived on the second cycle.

Meirow et al. [62] reported a definitive pregnancy from orthotopic transplantation of frozen/thawed ovarian tissue after chemotherapy-induced ovarian failure. The patient was a 28-year-old non-Hodgkin's lymphoma patient who had ovarian tissue harvested after first-line chemotherapy but before high-dose chemotherapy. Her FSH levels were consistently elevated (40–104 mIU/ml). Ovarian cortical tissue was transplanted via strips onto one ovary and via injection of a tissue slurry into the other ovary (Fig. 8.5). FSH levels decreased; Müllerian inhibiting substance and inhibin B increased. She conceived after natural cycle IVF.

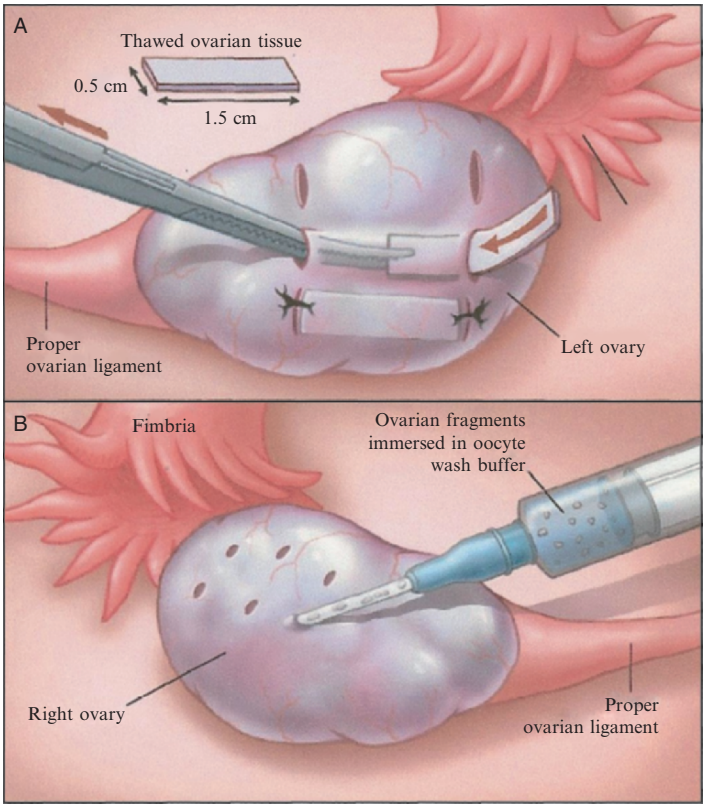

Fig. 8.5 From Meirow D et al. New Engl J Med 2005;355:318–21. Two methods for ovarian tissue transplantation: **(A)** Cortical strips, **(B)** Injection of ovarian tissue "slurry"

Demeestere et al. [63] performed simultaneous orthotopic and heterotopic transplantation of ovarian tissue. Follicles developed at the ovary, peritoneum, and abdomen. A spontaneous pregnancy ensued, but unfortunately ended in a miscarriage secondary to aneuploidy.

Whole Ovary Transplantation by Vascular Anastomosis

Transplanted ovarian cortical pieces rely upon simple diffusion for survival until new blood vessels form. Initially, the grafts are subject to ischemia. As an alternative approach, some have examined whether the intact ovary can be cryopreserved and subsequently transplanted via vascular anastomosis [64–67]. Wang et al. cryopreserved and then transplanted the upper uterus, fallopian tubes, and ovaries in mice, with a subsequent pregnancy [65]. Leporrier et al. performed heterotopic transplantation of an ovary to the arm using vascular anastomosis with extraction of a post-mature egg [64]. Bedaiwy et al. performed whole ovary transplantation in sheep, but in 8 of 11 animals, the vascular anastomosis had occluded completely [66]. Recently, Imhof et al. reported a live-born sheep from transplantation of a whole, frozen/thawed ovary [67]. One ovary was removed and the vessels cannulated so that the entire ovary could be perfused with cryoprotectant. After freezing and thawing, the contralateral ovary was surgically removed, and the thawed ovary transplanted back to the vascular pedicle. One of nine sheep became pregnant.

Problems with Ovarian Transplantation: Re-Introduction of Cancer

Although autografting seems promising, it is not without potential risks. Theoretically, ovarian tissue could carry micro-metastases that could “re-infect” a patient who had been previously cured of her cancer. Ovarian transplantation might be particularly concerning with blood-born malignancies, such as leukemia, where the cancer cells are already in the blood, and therefore presumably within the cryopreserved ovarian tissue. Shaw et al. showed that fresh and cryopreserved ovarian tissue samples taken from donors with lymphoma transmitted the cancer into previously healthy graft recipients [68]. This may bode poorly for the future of autografting, particularly for hematogenous malignancies like leukemia, or for patients with cancers known to metastasize to the ovary.

On the other hand, another study utilizing human tissue suggests that autologous ovarian transplantation is safe [69]. In this study, ovarian tissue from lymphoma patients was xenografted into immunodeficient mice. None of the mice developed lymphoma. However, when lymph nodes from the lymphoma patients were xenografted, mice transplanted with lymph nodes from the Hodgkin’s disease patients did develop lymphoma (positive control).

Table 8.6 Risk of cancer metastases within the ovary [70]

Low risk of ovarian involvement	Squamous cell, cervix
	Ewing's sarcoma
	Breast cancer
	Stage I–III
	Infiltrative ductal
	Wilms' tumor
	Non-Hodgkin's lymphoma
	Hodgkin's lymphoma
	Osteogenic sarcoma
	Non-genital rhabdomyosarcoma
Moderate risk of ovarian involvement	Breast cancer
	Stage IV
	Infiltrative lobular
	Colon cancer (including tumors of rectum and appendix)
	Adeno/adenosquamous, cervix
	Upper gastrointestinal system
Cancers with high risk of ovarian involvement	Leukemia
	Burkitt's lymphoma
	Neuroblastoma
	Genital rhabdomyosarcoma

Hence, it is necessary to develop screening methods to detect minimal residual disease in ovarian tissue to eliminate the risk of cancer cell transmission with transplantation, or to consider xenografting or in vitro maturation, which would minimize re-introduction of cancer cells. A recent review attempts to stratify the risk of ovarian metastases (Table 8.6) [70].

Xenografting as a Potential Solution

Xenografting is another option for maturation of oocytes within cryopreserved ovarian tissue. Xenografting involves transplantation of ovarian tissue from one species (human) to another [severe combined immunodeficient (SCID) mice]. Because of the concern about re-introduction of cancer into patients via transplanted ovarian tissue, investigators have explored transplanting frozen-thawed ovarian tissue into an animal host that would serve as a biological incubator. With this technique, the possibility of cancer transmission and relapse can be minimized since maturation of the primordial follicles occurs in the animal host. When the follicle has matured in the mouse, a single egg can be isolated and fertilized, thereby theoretically eliminating exposure of the patient to cancer cells. However, xenografting may raise some ethical considerations; the concept of maturing human oocytes within another species is distasteful to some. Furthermore, xenografting raises the possibility of transmitting infectious agents or potentially altering the human genome.

Animal Data

Xenografting of cryopreserved ovarian tissue from non-human primates is feasible. Ovarian tissue from marmoset monkeys, which had been frozen and grafted into immunodeficient mice, developed viable, estrogen-producing follicles. Our lab [71] showed that rhesus ovarian tissue could be xenografted to SCID mice and that pre-antral and antral development could occur upon prolonged gonadotropin stimulation. The kidney capsule was a better site than subcutaneous sites for the grafts. There have already been live births reported from xenografting cyropreserved mouse ovarian tissue [72,73].

Human Data

Transplantation of frozen-thawed ovarian tissue into an animal host with subsequent gonadotropin stimulation and oocyte retrieval may offer considerable advantages to cancer survivors. Several groups have shown that human ovarian tissue can survive and grow to large antral stages in immunodeficient mice when transplanted subcutaneously, over the peritoneum, or under the kidney capsule [74–77]. Revascularization is critical for graft survival, and these sites are well vascularized, especially the subcapsular region of the kidney. Finally, Cha's group demonstrated that human fetal ovarian tissue could be vitrified in ethylene glycol and xenografted into NOD-SCID mice with resumption of follicular growth [78].

While these results are promising, a recent study raised the question of whether oocytes derived from xenografted ovarian tissue are ultrastructurally and

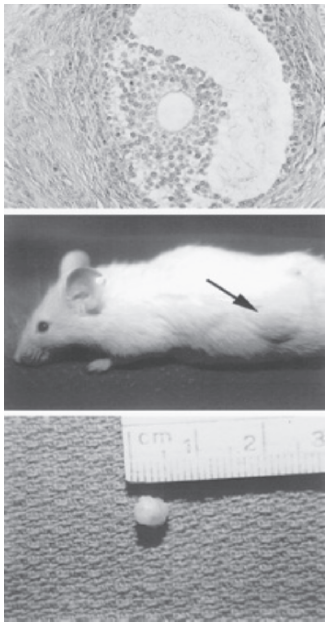

Fig. 8.6 Xenografted human ovarian cortex transplanted into a male NOD-SCID mouse following gonadotropin stimulation produces antral follicles [74]

reproductively competent [79]. When these oocytes were analyzed immunocytochemically, the microtubule organization and chromatin configuration were abnormal. It is possible that xenografting of human ovarian tissue will be more valuable as a research tool than as a clinical treatment. Xenografts could be used to examine which conditions might optimize autologous transplant conditions. Numerous factors, including anti-apoptotic agents [71], antioxidants like vitamin E or ascorbic acid, and angiogenic factors like vascular endothelial growth factor (VEGF), TGF, and FSH have been postulated to be beneficial. Further research is necessary to maximize the efficiency of ovarian tissue transplantation.

Conclusion

Ovarian tissue cryopreservation and transplantation is currently the most effective fertility-preserving treatment for prepubertal girls undergoing gonadotoxic cancer treatment and for women whose chemotherapy or radiation therapy must start immediately. Although there have been human pregnancies reported utilizing these methods, the underlying principles of cryobiology and transplantation biology must be further refined within the new field of oncofertility before widespread clinical application is possible.

References

1. Blatt J. Pregnancy outcome in long-term survivors of childhood cancer. Med Pediatr Oncol 1999;33:29–33.
2. Meirow D, Nugent D. The effects of radiotherapy and chemotherapy on female reproduction. Hum Reprod Update 2001;7:535–543.
3. Greenlee RT, Murray T, Bolden S, et al. Cancer statistics, 2000. CA Cancer J Clin 2000;50:7–33.
4. Meirow D. Ovarian injury and modern options to preserve fertility in female cancer patients treated with high dose radio-chemotherapy for hemato-oncological neoplasias and other cancer. Leuk Lymphoma 1999:33:65–76.
5. Meirow D. Epidemiology and infertility in cancer patients. In: Gosden R, Tulandi T, editors. Preservation of fertility. London: Taylor and Francis, 2004:21–38.
6. Koyama H, Wada T, Nishizawa Y, et al. Cyclophosphamide-induced ovarian failure and its therapeutic significance in patients with breast cancer. Cancer 1977;39:1403–1409.
7. Goldhirsch A, Gelber RD, Castiglione M. The magnitude of endocrine effects of adjuvant chemotherapy for premenopausal breast cancer patients. The International Breast Cancer Study Group. Ann Oncol 1990;1:183–188.
8. Chiarelli AM, Marrett LD, Darlington G. Early menopause and infertility in females after treatment for childhood cancer diagnosed in 1964–1988 in Ontario, Canada. Am J Epidemiol 1999;150:245–254.
9. Falcone T, Attaran M, Bedaiwy M, et al. Ovarian function preservation in the cancer patient. Fertil Steril 2004;81:243–257.
10. Wallace WH, Shalet SM, Hendry JH, et al. Ovarian failure following ovarian irradiation in childhood: the radiosensitivity of the human oocyte. Br J Radiol 1989;62:995–998.

11. Wallace WH, Thomson AB, Saran F, Kelsey TW. Predicting age of ovarian failure after radiation to a field that includes the ovaries. Int J Radiat Oncol Biol Phys 2005;62:738–744.
12. Critchley HOD, Wallace WHB, Shalet SM, et al. Abdominal irradiation in childhood: potential for pregnancy. Br J Obstet Gynaecol 1992:99:392–394.
13. Bath LE, Critchley HO, Chambers SE, et al. Ovarian and uterine characteristics after total body irradiation in childhood and adolescence: response to sex steroid replacement. Br J Obstet Gynaecol 1999;106:1265–1272.
14. Rappaport R, Brauner R, Czernichow P, et al. Effect of hypothalamic and pituitary irradiation on pubertal development in children with cranial tumors. J Clin Endocrinol Metab 1982; 154:1164–1168.
15. Davies HA, Didcock E, Didi M, et al. Growth, puberty and obesity after treatment for leukemia. Acta Paediatr Suppl 1995;411:45–51.
16. Ogilvy-Stuart AL, Clayton PE, Shalet SM. Cranial irradiation and early puberty. J Clin Endocrinol Metab 1994;78:1282–1286.
17. Kwon JS, Case AM. Preserving reproductive function in women with cancer. Sexuality, Reproduction & Menopause 2004;2(2):222–229. (A Publication of the American Society for Reproductive Medicine) http://www.srmjournal.org/home
18. Blumenfeld Z. Preservation of fertility and ovarian function and minimalization of chemotherapy associated gonadotoxicity and premature ovarian failure: the role of inhibin-A and –B as markers. Mol Cell Endocrinol 2002;187:93–105.
19. Revel A, Laufer N. Protecting female fertility from cancer therapy. Mol Cell Endocrinol 2002;187:83–91.
20. Ataya K, Pydyn E, Ramahi-Ataya A, et al. Is radiation-induced ovarian failure in rhesus monkeys preventable by luteinizing hormone-releasing hormone agonists: preliminary observations. J Clin Endocrinol Metab 1995;80:790–795.
21. Ataya K, Rao LV, Lawrence E, et al. Luteinizing hormone-releasing hormone agonist inhibits cyclophosphamide-induced ovarian follicular depletion in rhesus monkeys. Biol Reprod 1995;52:365–372.
22. Blumenfeld Z, Avivi I, Linn S, et al. Prevention of irreversible chemotherapy-induced ovarian damage in young women with lymphoma by a gonadotrophin-releasing hormone agonist in parallel to chemotherapy. Hum Reprod 1996;11:1620–1626.
23. Waxman JH, Ahmed R, Smith D, et al. Failure to preserve fertility in patients with Hodgkin's disease. Cancer Chemother Pharmacol 1987;19:159–162.
24. Oktay K, Cil AP, Bang H. Efficiency of oocyte cryopreservation: a meta-analysis. Fertil Steril 2006;86:70–80.
25. Porcu E, Fabbri R, Damiano G, et al. Clinical experience and applications of oocyte cryopreservation. Mol Cell Endocrinol 2000;169:33–37.
26. Fabbri R, Porcu E, Marsella T, et al. Human oocyte cryopreservation: new perspectives regarding oocyte survival. Hum Reprod 2001;16:411–416.
27. Borini A, Bonu MA, Coticchio G, et al. Pregnancies and births after oocyte cryopreservation. Fert Steril 2004;82:601–605.
28. Eppig J, O'Brien M. Development in vitro of mouse oocytes from primordial follicles. Biol Reprod 1996;54:197–207.
29. Cha KY, Chung HM, Lim JM, et al. Freezing immature oocytes. Mol Cell Endocrinol 2000;169:43–47.
30. Parrott JA, Skinner MK. Direct actions of kit-ligand on theca cell growth and differentiation during follicle development. Endocrinology 1997;138:3819–3827.
31. Elvin JA, Yan C, Wang P, et al. Molecular characterization of the follicle defects in growth differentiation factor 9-deficient ovary. Mol Endocrinol 1999;13:1018–1034.
32. Nilsson E, Parrott JA, Skinner MK. Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. Mol Cell Endocrinol 2001;175:123–123.
33. Otsuka F, Moore RK, Shimasaki S. Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. J Biol Chem 2001;276:32889–32895.

34. Nilsson EE, Kezele P, Skinner MK. Leukemia inhibitory factor promotes the primordial to primary follicle transition in rat ovaries. Mol Cell Endocrinol 2002;188:65–73.
35. Yoon S, Kim K, Chung H, et al. Gene expression profiling of early follicular development in primordial, primary and secondary follicles. Fert Steril 2006;85:193–203.
36. Gougeon A. Regulation of ovarian follicular development in primates: facts and hypotheses. Endo Rev 1996;17:121–155.
37. Zeleznik AJ. The physiology of follicle selection. Reprod Biol Endocrinol 2004;2:31.
38. Kreeger PK, Fernandes NN, Woodruff TK, et al. Regulation of mouse follicle development by follicle-stimulating hormone in a three-dimensional in vitro culture system is dependent on follicle stage and dose. Biol Reprod 2005;73:942–950.
39. Kreeger PK, Deck JW, Woodruff TK, et al. The in vitro regulation of ovarian follicle development using alginate-extracellular matrix gels. Biomaterials 2006;27:714–723.
40. Pangas SA, Saudye H, Shea LD, et al. Novel approach for the three-dimensional culture of granulosa cell-oocyte complexes. Tissue Eng. 2003;9:1013–1021.
41. Xu M, West E, Shea LD, et al. Identification of a stage-specific permissive in vitro culture environment for follicle growth and oocyte development. Biol Reprod 2006;75:916–923.
42. Xu M, Kreeger PK, Shea LD, et al. Tissue-engineered follicles produce live, fertile offspring. Tissue Eng 2006;12:2739–2746.
43. Oktay K, Nugent D, Newton H, et al. Isolation and characterization of primordial follicles from fresh and cryopreserved human ovarian tissue. Fert Steril 1997;67:481–486.
44. Gosden RG, Baird DT, Wade JC, et al. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at –196 degrees C. Hum Reprod 1994;9:597–603.
45. Yeoman RR, Wolf DP, Lee DM. Co-culture of monkey ovarian tissue increases survival after vitrification and slow-rate freezing. Fert Steril 2005;83:1248–1254.
46. Deanesly R. Immature rat ovaries grafted after freezing and thawing. J Endocrinol 1954;11:197–200.
47. Parkes A, Smith, AU. Preservation of ovarian tissue at –79 Degrees C for transplantation. Acta Endocrinol 1954;17:313–320.
48. Parrot D. The fertility of mice with orthotopic ovarian grafts derived from frozen tissue. J Reprod Fertil 1960;1:230–241.
49. Oktay K, Aydin B, Karlikaya G. A technique for laparoscopic transplantation of frozen-banked ovarian tissue. Fertil Steril 2001;75:1212–1216.
50. Donnez J, Dolmans MM, Demylle D, et al. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet 2004;364:1405–1410.
51. Silber SJ, Lenahan KM, Levine DJ, et al. Ovarian transplantation between monozygotic twins discordant for premature ovarian failure. New Engl J Med 2005;353:58–63.
52. Oktay K, Economos K, Kan M, et al. Endocrine function and oocyte retrieval after autologous transplantation of ovarian cortical strips to the forearm. J Am Med Assoc 2001;286:1490–1493.
53. Lee DM, Yeoman R, Battaglia DE, et al. Birth of a monkey after heterotopic transplantation of fresh ovarian tissue and assisted reproduction. Nature 2004;428:137–138.
54. Harp R, Leibach J, Black J, et al. Cryopreservation of murine ovarian tissue. Cryobiology 1994;31:336–343.
55. Sztein J, Sweet H, Farley J, et al. Cryopreservation and orthotopic transplantation of mouse ovaries: New approach in gamete banking. Biol Reprod 1998;58:1071–1074.
56. Schnorr JA OS, Toner JP, Hsiu JG, Willams RF, Hodgen GD. Fresh and cryopreserved extrapelvic ovarian transplantation in non-human primates: folliculogenesis, ovulation, corpus luteum function, endometrial development, and menstrual patterns. Abstract presented at: the American Society of Reproductive Medicine 2000 Annual Meeting; October 21–26, 2000; San Diego, California.
57. Gougeon A, Ecochard R, Thalabard JC. Age-related changes of the population of human ovarian follicles: increase in the disappearance rate of non-growing and early-growing follicles in aging women. Biol Reprod 1994;50:653–663.
58. Gougeon A. Ovarian follicular growth in humans: ovarian ageing and population of growing follicles. Maturitas 1998;30:137–142.

59. Oktay K, Karlikaya G. Ovarian function after transplantation of frozen, banked autologous ovarian tissue. N Engl J Med 2000;342:1919.
60. Radford JA, Lieberman BA, Brison DR, et al. Orthotopic reimplantation of cryopreserved ovarian cortical strips after high-dose chemotherapy for Hodgkin's lymphoma. Lancet 2001;357:1172–1175.
61. Oktay K, Buyuk E, Veeck L, et al. Embryo development after heterotopic transplantation of cryopreserved ovarian tissue. Lancet 2004;363:837–840.
62. Meirow D, Levron J, Eldar-Geva T, et al. Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy. New Eng J Med 2005;355:318–321.
63. Demeestere I, Simon P, Buxant F, et al. Ovarian function and spontaneous pregnancy after combined heterotopic and orthotopic cryopreserved ovarian tissue transplantation in a patient previously treated with bone marrow transplantation: case report. Hum Reprod 2006;21:2010–2014.
64. Leporrier M, von Theobald P, Roffe JL, et al. A new technique to protect ovarian function before pelvic irradiation. Heterotopic ovarian autotransplantation. Cancer 1987;60:2201–2204.
65. Wang X, Chen H, Yin HK, et al. Fertility after intact ovary transplantation. Nature 2002; 415:385.
66. Bedaiwy MA, Jeremias E, Gurunluogly R, et al. Restoration of ovarian function after autotransplantation of intact frozen-thawed sheep ovaries with microvascular anastomosis. Fertil Steril 2003;79:594–602.
67. Imhof M, Bergmeister H, Lipovac M, et al. Orthotopic microvascular re-anastomosis of whole cryopreserved ovine ovaries resulting in pregnancy and life birth. Fertil Steril 2006; 85(Suppl 1):1208–1215.
68. Shaw JM, Bowles J, Koopman P, et al. Fresh and cryopreserved ovarian tissue from donors with lymphoma, transmit the cancer to graft recipients. Hum Reprod 1996;11:1668–1673.
69. Kim SS, Hwang IT, Lee HC. Heterotopic autotransplantation of cryobanked human ovarian tissue as a strategy to restore ovarian function. Fertil Steril 2004;82:930–932.
70. Sonmezer M, Shamonki MI, Oktay K. Ovarian tissue cryopreservation: benefits and risks. Cell Tissue Res 2005;322:125–132.
71. Lee DM, Yeoman RR, Yu T, et al. Sphingosine-1-phosphate inhibits apoptosis in rhesus monkey ovarian tissue *in vitro* and may improve reproductive function in xenografts. 2005 Joint American Society for Reproductive Medicine/Canadian Fertility & Andrology Society Meeting, Oct. 15–19, Montreal, Quebec, CANADA. Fertil Steril 2005;84(Suppl 1):S2.
72. Gunasena KT, Lakey JR, Villines PM, et al. Allogeneic and xenogeneic transplantation of cryopreserved ovarian tissue to athymic mice. Biol Reprod 1997;57:226–231.
73. Snow M. Cox SL, Jenkin G, et al. Generation of live young from xenografted mouse ovaries. Science 2002;297:2227.
74. Weissman A, Gotlieb L, Colgan T, et al. Preliminary experience with subcutaneous human ovarian cortex transplantation in the NOD-SCID mouse. Biol Reprod 1999;60:1462–1467.
75. Kim SS, Soules M, Gosden RG, et al. The evidence of follicle maturation and subsequent ovulation in human ovarian tissue xenografted into severe combined immunodeficient (SCID) mice. Fertil Steril 2000;74:S34.
76. Nisolle M, Casanas-Roux F, Qu J, et al. Histologic and ultrastructural evaluation of fresh and frozen-thawed human ovarian xenografts in nude mice. Fert Steril 2000;74:122–129.
77. Oktay K, Newton H, Gosden RG. Transplantation of cryopreserved human ovarian tissue results in follicle growth initiation in SCID mice. Fertil Steril 2000;73:599–603.
78. Lee K, Lee S, SJ Y, et al. Resumption of the human primordial follicle growth in xenografts after vitrification of the ovarian tissues. Fertil Steril 2000;74(3 Suppl 1):S214.
79. Kim SS, Kang HG, Kim NH, et al. Assessment of the integrity of human oocytes retrieved from cryopreserved ovarian tissue after xenotransplantation. Hum Reprod 2005;20:2502–2508.