Ovarian tissue cryopreservation and transplantation: preliminary findings and implications for cancer patients

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Cancer treatment modalities are increasingly more effective in achieving complete remission and cure. Aggressive chemotherapy and radiotherapy, as well as bone marrow transplantation, results in >90% cure in many cancers of children and young women. As a result of this success however, a new problem has arisen. Many young women survive to live the rest of their lives in menopause, and have no chance of conceiving on their own. Oocyte cryopreservation has resulted in a handful of pregnancies, but the technique may not be applicable to young women and children. Ovarian tissue cryopreservation and transplantation has emerged against this background, first in successful rodent studies, and then in sheep and human ovarian xenograft studies. Because of the encouraging results with animals and xenografts, a human ovarian transplantation trial was launched. Pelvic auto-transplantation of frozen-banked ovarian tissue resulted in ovulation in one patient. Several other patients received fresh grafts subcutaneously, and preliminary results indicated antral follicle development at least in one patient. With the addition of promising data from humans, ovarian tissue cryopreservation from selected patients before cancer treatment, and in those requiring oophorectomy for benign causes, is now advocated.

Key words: cancer/fertility/oophorectomy/ovarian tissue cryopreservation/ovarian transplantation

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Introduction

Cancer treatment modalities are increasingly more effective in achieving complete remission and cure. Aggressive chemotherapy and radiotherapy, as well as bone marrow transplantation, results in >90% cure in many cancers of children and young women (Ries *et al.*, 1999). As a result of this success however, a new problem has arisen, with many young women surviving to live the rest of their lives in menopause, and having no chance of conceiving on their own.

A measure to retain fertility has long been available for men, namely sperm cryopreservation. Since the 1970s, sperm cryopreservation has become a routine technique, and reasonable pregnancy rates have resulted from using frozen spermatozoa (Goldberg *et al.*, 1999). Unfortunately, the search for a similar option has not been as successful for women. Unlike spermatozoa, metaphase II (M-II) oocytes are relatively large (the largest cell in the body) and highly specialized cells with complex intracellular skeletal structure and an extensive cytoplasm (Oktay *et al.*, 1998a). As a result, damage to the cell spindle, oocyte cytoskeleton and zona pellucida frequently occurs after cryopreservation and thawing (Mazur *et al.*, 1972; Trounson and Kirby, 1989; Vincent *et al.*, 1990), which historically translates into low pregnancy rates of <3% (Chen, 1986; Al-Hasani *et al.*, 1987; Van Uem *et al.*, 1987; Porcu *et al.*, 1997; Oktay *et al.*, 1998a). In addition, a recent mouse study showed the possibility of retrieving fertilizable oocytes from antral follicles of frozen–thawed whole ovaries (Sztein *et al.*, 2000). Unfortunately, human ovaries are too large and too fibrous to freeze in their entirety, and consequently ovarian cortex has to be cut into 1 to 2 mm-thick slices (each no larger than 0.5×1 cm dimensions) in order for the cryoprotectants to penetrate the tissue, and for the tissue to fit into the cryovials. It has long been known that mouse oocytes recovered from antral follicles are much less susceptible to cryo-damage (Fuller and Bernard, 1984; Caroll *et al.*, 1993), and therefore results from

mouse studies cannot necessarily be applied to humans (Oktay and Gosden, 1999).

In addition to low pregnancy rates, there are also logistical problems with the cryopreservation of oocytes. A cancer patient is usually started on the first course of chemotherapy within 1–2 weeks of diagnosis. However, more than 3 weeks are usually required for cycle preparation, ovarian stimulation, and oocyte retrieval for egg freezing, and this delay is often unacceptable to oncologists. For the same reasons, IVF and embryo freezing in adult patients with established partners is also not feasible. Another practical problem occurs in the youngest patients, as it is both ethically and technically challenging to perform ovarian stimulation and oocyte retrieval in children.

Therefore, at present the cryopreservation of ovarian tissue pieces, rather than the whole human ovary, or oocytes from antral follicles, provides the most practical and useful means of preserving a patient's infertility.

Experimental studies of ovarian tissue cryopreservation and transplantation

It was because of the aforementioned difficulties with oocyte cryopreservation that the interest in ovarian tissue cryopreservation was rekindled. Several researchers had experimented with ovarian cryopreservation in rodents during the 1950s (Parkes and Smith, 1952; Green et al., 1956; Deanesly, 1957; Parrot, 1960), but because of the unavailability of modern cryoprotectants and lack of automated cryopreservation machines, the results were only minimally successful. Hence, the technique was abandoned until the 1990s.

During the 1960s glycerol was the only cryoprotectant available, and although useful for sperm freezing, it was ineffective for cryopreserving oocytes and ovarian tissue (as shown recently; Newton et al., 1996). Since the 1970s, more effective cryoprotectants have emerged, namely, propanediol, ethylene glycol and dimethylsulphoxide (DMSO). Recent studies found no statistical difference in the effectiveness of these cryoprotectants in human ovarian tissue cryopreservation (Newton et al., 1996).

In the 1990s, when rodent studies were repeated using the modern cryoprotectants, the results were very encouraging. In one study, fresh and cryopreserved fetal ovarian tissue was transplanted to adult syngeneic mice, with fertility being restored in up to 86% of the animals, similar to that in controls (Cox et al., 1996). In another study where autotransplantation was performed, histology and endocrine function of autotransplanted cryopreserved ovaries were similar to those of non-frozen transplanted ovaries and of ovaries in sham-operated groups (Harp et al., 1994). More recent studies have also confirmed the success with orthotopic transplantation of frozen–thawed ovarian tissue in the mouse (Sztein et al., 1998; Candy et al., 2000; Shaw et al., 2000).

One group (Gosden et al., 1994a) chose the sheep model, which was more applicable to humans than the rodent model. Sheep ovaries share similar characteristics (such as follicle density and stromal fibrosity) with human ovaries. In the first of the two studies, frozen-banked strips of ovarian cortical pieces were autotransplanted on the infundibulopelvic ligament (Gosden et al., 1994a). Each animal also had a fresh transplant on the opposite site, which served as a control. The arterial blood supply

was well developed within 1 week of the transplantation, as shown by histological sectioning, and the first signs of ovulation were detected 4 months after transplantation. Two pregnancies occurred—one from a fresh graft, and another from a frozen–thawed graft—and there were no abnormalities in the newborn animals.

In the second study, autotransplants were performed with frozen–thawed tissue in eight sheep, and the animals were monitored for up to 22 weeks (Baird et al., 1999). Two 5×5 mm ovarian cortical pieces were grafted onto each side. All the animals resumed cyclicity and showed hormone production. In that study, baseline FSH concentrations were elevated, but the luteal phase progesterone measurements were normal. However, serum inhibin-A concentrations were found to be relatively low in the luteal phase. Even though the elevated baseline FSH and low inhibin-A concentrations suggest a low ovarian reserve, this did not prevent the pregnancies in either study (Gosden et al., 1994a; Baird et al., 1999). The authors also showed that, while 7% of follicles are lost during freezing and thawing, 65% were lost during the revascularization period. Despite this drastic reduction in follicular reserve, animals continued to ovulate for nearly 2 years. However, towards the end of the study, FSH concentrations rose to near-castration levels again. Because of the limited lifespan of the ovarian grafts, the authors suggested that the transplantation procedure be delayed until the recipient is interested in conception, and we agree with this point of view.

In another sheep study, the function of heterotopic and orthotopic autografts of frozen–thawed ovarian cortex of sheep was compared (Aubard et al., 1999). Fresh and frozen–thawed fragments of ovarian cortex were autografted on the uterine horn of six ewes (orthotopic grafts) and under the skin of the belly in nine ewes (heterotopic grafts). In both fresh and frozen–thawed grafts, preantral and antral follicles were first detectable at 4 and 10 weeks respectively following grafting, but only 5% of the primordial follicles appeared to have survived. Although ovulation resumed in most ewes, none of the ewes grafted orthotopically became pregnant at a synchronized mating. Seven months following grafting, oocytes could be collected from both heterotopic and orthotopic grafts, and matured; some of these oocytes fertilized, but none developed to the blastocyst stage.

These aforementioned animal studies encouraged further investigations using human ovarian tissue as xenografts in severe combined immunodeficiency disease (SCID) mice. These mice carry a mutation which renders them both B cell- and T cell-deficient (Bosma et al., 1983), and they are therefore unable to reject grafts from foreign species. A modification of this technique has been used for human ovarian xenografting (Gosden et al., 1994b). The same group had already shown (using cat and sheep ovarian tissue) that follicle development could be achieved using the SCID-xenograft model (Gosden et al., 1994b), and this model was also used successfully to grow follicles in xenografts from marmosets (Candy et al., 1995).

The SCID model entails grafting 1 mm^3 blocks of ovarian cortical tissue under the kidney capsule, through a dorsal incision. These grafts can also be placed either subcutaneously (Weissman et al., 1999) or intramuscularly (Revel et al., 2000). The fate of the grafts in the immunologically compromised recipients can be followed for 6 months or more if the mice are housed in an appropriate, sterile, environment.

Table I. Modified human ovarian tissue cryopreservation protocol

Freezing

1. Equilibrate 1–3 mm thick, 3×10 mm strips of ovarian cortex for 30 min at 4°C in phenol-free buffered medium (i.e. L-15) containing 1.5 mol/l DMSO (propanediol/ethylene glycol), 20% autologous serum and 0.1 mol/l sucrose. Place the vials on a tissue-roller during incubation to ensure even penetration of cryoprotectant.
2. Load the tissue in cryovials into an automated freezer, starting at 0°C, and cool at 2°C/min to –7°C.
3. Equilibrate for 10 min before manual seeding.
4. Continue to cool at 0.3°C/min to –40°C.
5. Cool at the faster rate of 10°C/min to –140°C.
Transfer to liquid nitrogen dewar flask for storage.

Thawing

7. Thaw at room temperature for 30 s.
8. Place in 37°C water bath for 2 min.
9. Wash tissues stepwise in progressively lower concentrations of cryoprotectant media with 20% serum plus 0.1 mol/l sucrose; gently agitate tissue for 5 min in each step (1.5, 1.0, 0.5, 0 mol/l).
10. Perform the last wash with media containing 20% serum only.
Transfer to the operating room for transplantation in fresh media with serum, on ice.

In a similar study, human ovarian cortical pieces were cryopreserved using various cryoprotectants, and later thawed and grafted to SCID mice (Newton *et al.*, 1996). After 18 days, the grafts were removed and the outcomes determined by calculating the percentage of primordial follicles surviving relative to the fresh controls. With the exception of glycerol, all cryoprotectants (propanediol, ethylene glycol, DMSO) performed well, and 44 to 84% of the follicles survived. The results with glycerol were extremely poor, which explains the low success rates obtained with earlier studies.

In a later study, the survival rates of primordial follicles were quantified by the use of viability stains following cryopreservation (Oktay *et al.*, 1997a). Ovarian cortical pieces (approximately 2×2 mm in size) were cryopreserved using a slow-freeze protocol (Table I). After thawing, the tissues were partially digested using collagenase type IA (Sigma, St Louis, MO, USA), followed by microdissection of the primordial follicles, after which the follicles were incubated with viability stains. It was found that while ~70% of follicles had survived this process and were viable, a larger degree of survival was found when only oocytes were considered. There was no statistically significant difference between fresh and frozen–thawed specimens in primordial follicle or oocyte survival, however.

In order to study the long-term survival of human ovarian xenografts, two studies were performed in SCID mice (Oktay *et al*, 1998b, 2000). In the first study, 1 mm³ ovarian cortical pieces from a 17-year-old patient were grafted under the kidney capsules of hypogonadal-oophorectomized-SCID mice (Oktay *et al.*, 1998b). During the last 6 weeks of the 17-week grafting period, one group of animals received FSH stimulation, while the control group received only saline injections. Serum oestradiol concentrations were measured after 17 weeks, when the animals were killed. In the FSH-treated group, antral follicles up to 5.0 mm were found (Figure 1), and oestradiol concentrations peaked at >2000 pmol/l; in addition, the uteri showed clear signs of oestrogenization (Table II).

In the second study, frozen–thawed human ovarian tissues were used for xenografting into SCID mice (Oktay *et al.*, 2000).

Because these animals were not hypogonadal, no FSH was administered. The grafts were recovered 22 weeks later and examined histologically. Many follicles had initiated growth and, interestingly, follicle growth initiation rates were significantly higher in the xenografts than in the ungrafted controls (12.5 ± 1.9 versus 5.6 ± 2.4, *P* < 0.05), although there was still a significant number of primordial follicles (75 ± 6.8) remaining in each graft. Presumably, because no exogenous FSH was given, follicles did not develop beyond the one- to two-layer stage. In a similar study (Weissman *et al*., 1999), cryopreserved human ovarian tissues were xenografted subcutaneously to non-obese diabetic (NOD)-SCID mice, which led to antral follicle development after FSH stimulation. The same group recently reported antral follicle development and oocyte retrieval after intramuscular xenografting of human ovarian tissue to nude mice (Revel *et al*., 2000). Consequently, the stage was now set for human trials of ovarian transplantation.

Human ovarian transplantation trials

Encouraged by the mounting evidence from animal autograft and human xenograft studies, the first ovarian transplantation trial was initiated in humans with, in the initial phase, the procedure being performed on two patients with benign ovarian disease. Two different approaches were chosen for transplantation: (i) pelvic or orthotopic, where the tissue is grafted near the infundibulopelvic ligament—in such a case a natural pregnancy could be achieved if the Fallopian tubes are intact; and (ii) forearm or heterotopic, where the tissue is grafted into the subcutaneous space above the brachioradialis fascia in the forearm.

The latter technique has been used successfully for autografting fresh (Wells *et al*., 1976) and frozen-banked parathyroid gland tissue (Wagner *et al*., 1991) for many years. In addition, there have been several reports on the feasibility of heterotopic sites for ovarian transplantation. For example, a case was reported (Leporrier *et al*., 1987) where ovarian tissue was grafted into the medial aspect of the left arm before radiotherapy for Hodgkin's disease. Initially, the skin was expanded by implanting
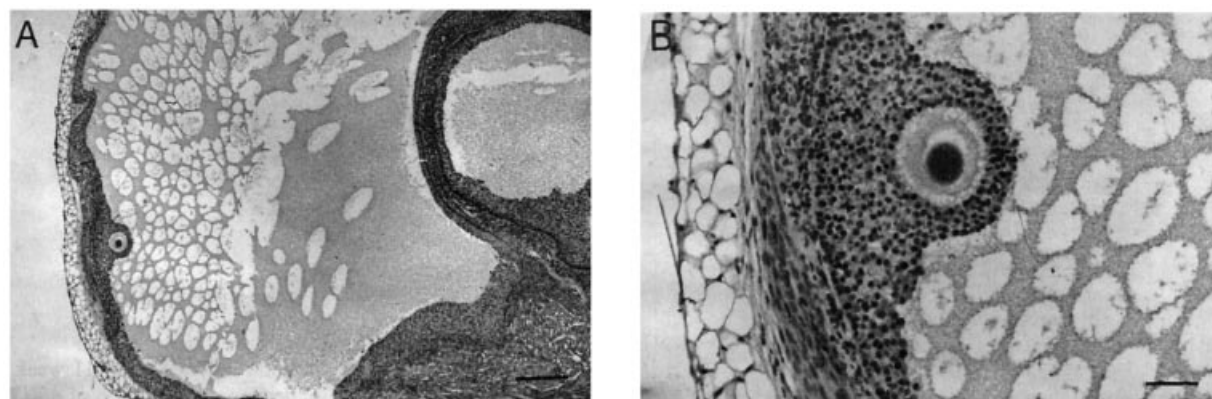
Figure 1. (**A**) Low magnification of a Graafian follicle (3 mm diameter) in human ovarian tissue xenografted 17 weeks previously and stimulated with FSH for the last 6 weeks (original magnification, ×20). (**B**) Higher magnification of the same follicle showing a germinal vesicle oocyte surrounded by cumulus cells (original magnification, ×20). Haematoxylin and eosin staining. (From Oktay *et al.*, 1998b reproduced with permission.)

Table II. Assessment of oestrogen secretion by ovarian xenografts in SCID/*hpg* mice stimulated with FSH. (From Oktay *et al.*, 1998b.)

Graft	No. of antral follicles (mm diameter)	Serum oestradiol (pmol/l)	Uterine weight (mg)	Vaginal introitus
1	1 (5)	2070	212 (ballooned)	Patent
2	2 (3.4)	780	131	Patent
3	0	35	123	Patent
4	1 (2.5, haemorrhagic)	286	126	Patent

a testis prosthesis subcutaneously, and 2 months later two microsurgical teams worked side-by-side to anastomose ovarian vessels to the left humeral vascular bundle. A right oophoropexy was also performed. Despite this, the patient continued her cycles uninterrupted, with intermittent ovulation being demonstrated (using ultrasound) in the forearm. A post-mature oocyte was retrieved from the graft 1 year later (Leporrier *et al.*, 1987). It is most likely due to the complicated nature of the procedure that no further such procedures have been reported. In 1997, the accidental transplantation of ovarian tissue subcutaneously was reported (Marconi *et al.*, 1997) when, during a laparoscopic resection of an endometrioma, a piece of ovarian tissue was accidentally left in the subcutaneous area. Several months later, the patient developed a swelling in the umbilical area, which exploration and histological analysis proved to be functional ovarian tissue with antral follicle development. Later, fresh ovarian cortical biopsies from nine patients were grafted to the uterine subserosa (Nugent *et al.*, 1998). After 14 weeks of follow-up, the tissues were removed, whereupon histological analysis showed follicle development to the two- to three-layer stages. On average, 25% of the follicles survived after grafting compared with the ungrafted controls (Nugent *et al.*, 1998); the authors attributed the lack of antral follicle development to suppression by these patients' functioning ovaries.

The above-mentioned reports (Marconi *et al.*, 1997; Nugent *et al.*, 1998) established that ovarian function can be restored without vascular reanastomosis, albeit at a cost of losing 70–75%

of the follicular reserve. In that respect, ovarian grafting can be likened to skin grafting, where spontaneous revascularization is achieved within days. Studies in rats showed that revascularization of ovarian grafts occurs within 48 h (Dissen *et al.*, 1994). Likewise in sheep, the arterial blood supply was well established within 1 week of grafting (Gosden *et al.*, 1994). Unlike the growing follicles which perish prior to revascularization, relatively quiescent primordial follicles have a better chance of surviving the initial period of ischaemia.

Indications for ovarian cryopreservation

In the ovarian tissue banking and transplantation trial, the following indications were used for cryopreservation. Some cancer types may not be ideal for transplantation at the present time, because the risk of ovarian involvement is high (e.g. in acute leukaemias). As discussed later, however, alternative technologies may become available to utilize these tissues in order to obtain mature oocytes for fertilization.

Malignancies of childhood and youth

Some 2% of all malignancies occur during infancy and childhood (age <14 years), totalling 8600 new cases per year in the USA (Linet *et al.*, 1999; Ries *et al.*, 1999). The most common childhood cancers, which place females under the risk of ovarian failure due to chemotherapy and/or radiotherapy, are leukaemia, neuroblastoma, Hodgkin's lymphoma, osteosarcoma, Ewing's sarcoma, rhabdomyosarcoma, Wilm's tumour and non-Hodgkin lymphoma. Although advances in chemotherapy, radiotherapy and bone marrow transplantation have led to many of these cancers becoming curable (Apperly and Reddy, 1995), this translates into more than 4000 young females being exposed to sterilizing chemotherapy/radiotherapy each year.

Malignancies in women of reproductive age

Breast cancer: Some 5% of all invasive breast cancers occur under the age of 40 years, and this amounts to 8600 new cases each year. Approximately 600 of these patients are aged less than 30 years (Ries *et al.*, 1999; SEER, 1999), and many of these young women opt for breast-conserving treatment. Conservative treatment protocols often require additional chemotherapy and radiotherapy,

which commonly result in ovarian failure. In these patients, ovarian tissue can be obtained during the lumpectomy, avoiding additional risk of exposure to general anaesthesia.

Cervical carcinoma: Cervical squamous cell carcinoma can be seen as early as the second decade of life. Each year, approximately 12 800 invasive cancers are diagnosed (Chen *et al.*, 1999; SEER, 1999), and fewer than 3000 of these are in women of reproductive age. Although ovarian involvement is extremely uncommon (<0.2%) in invasive cervical cancer (even in stage III), it is still a good precaution to perform frozen sections on biopsies at the time of oophorectomy.

Leukaemia: Approximately 6500 new cases of leukaemia are diagnosed annually in females, with the majority in children and women of reproductive age (Chen *et al.*, 1999; Linet *et al.*, 1999; SEER, 1999). However, the major concern with freezing ovarian tissue from leukaemic patients is the systemic nature of the disease, since these patients carry a high probability of ovarian involvement. The good news is that many molecular markers are now available to screen for the presence of malignant cells in the tissue (see below) and, even when ovaries are involved, future technologies such as in-vitro follicle growth may enable the diseased tissue also to be utilized.

Bone marrow transplantation patients

Bone marrow transplantation (BMT), which was initially developed for the treatment of leukaemia, is performed increasingly more liberally in other cancerous and non-cancerous conditions. For example, BMT is a treatment option in aplastic anaemia, autoimmune and immune-deficiency diseases, severe forms of rheumatoid arthritis, as well as sickle cell anaemia (Tyndall and Millikan, 1999; Walters, 1999). BMT is also used in the treatment of types of cancer that do not originate from the bone marrow, such as lymphoma and breast cancer (Anderson-Reitz, 1999; Rauck and Grouas, 1999; Thomas, 1999). Before BMT is carried out, an intensive chemotherapy and radiotherapy regimen is used to destroy the pre-existing bone marrow, but unfortunately this regimen results in ovarian failure in up to 92% of the patients (Meirow, 2000).

Oophorectomy as an adjunct treatment

Endometriosis: In the management of endometriosis, oophorectomy is occasionally needed in cases where there is no response to medical treatment. At the time of oophorectomy, healthy portions of the ovary can be isolated under a dissection microscope. The tissue is then frozen-banked for future orthotopic or heterotopic transplantation. If recurrence of endometriosis is of concern, ovarian tissues may in the future be used for in-vitro growth or xenografting.

Breast cancer: Because many invasive breast carcinomas are oestrogen-dependent, there is currently a trend towards performing ovarian ablation in women of reproductive age with breast cancer in order to improve survival rates (Clark, 1998). In these patients, ovarian tissue can also be cryopreserved without a need for a second procedure. However, the transplantation of ovarian tissue to patients with oestrogen-dependent neoplasms is likely to create a debate similar to the one faced when deciding whether or not to give hormone replacement or recommend pregnancy to those patients.
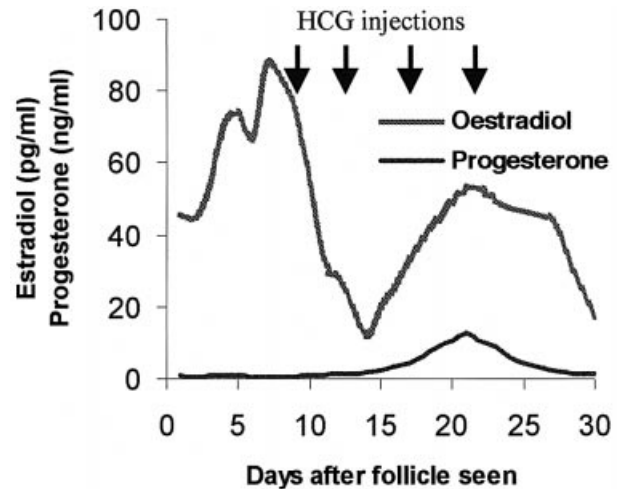

Figure 2. Oestrogen and progesterone production after transplantation of frozen–thawed autologous ovarian tissue.

Prophylactic oophorectomy

A slightly controversial indication for ovarian tissue cryopreservation occurs in patients with familial ovarian cancer. If the patient feels that she has temporarily completed childbearing, and is in her early thirties or younger, then ovarian cryopreservation can be offered. This would in theory maintain her risk for ovarian cancer development at the age of removal. When a pregnancy is desired, ovarian tissue could be utilized for transplantation or in-vitro growth. Once the pregnancy has been accomplished, the remaining tissue could be removed. For this purpose, a heterotopic site would be more suitable as it would allow the tissue to be closely monitored and, if necessary, to be easily removed (i.e. subcutaneous grafting).

Benign ovarian tumours

When ovaries are removed for extensive or recurrent benign ovarian cysts, healthy tissue is separated from the damaged tissue under a dissection microscope and cryopreserved. When there is a history of recurrence, subcutaneous placement may be superior because of the ease of monitoring, as well as the ease of removal.

Preliminary findings from the New York-Cornell Human Ovarian Transplantation Trial

In the first case of our ovarian transplantation trial (a 32-year-old woman who had lost her other ovary due to another cystadenoma at the age of 17 years), 90% of the patient's remaining ovarian tissue was removed because of a recurrent benign cystadenoma; the normal portions were cryopreserved for later use. A small portion of non-frozen ovarian tissue (4×4×1 mm) was grafted into the forearm, using the method employed for parathyroid gland transplantation (Wells *et al.*, 1976) to test the feasibility of this site as a recipient. Tissue was also grafted into the broad ligament, adjacent to the uterine artery. At 4 months after the procedures, ultrasound follow-up was used to visualize the grafts, and 2–3 mm antral follicles were noted in the forearm by high-frequency ultrasound probes. A >10% gradient was detected for oestradiol between the antecubital vein (into which the graft drains) and the wrist veins, indicating hormonal production by the
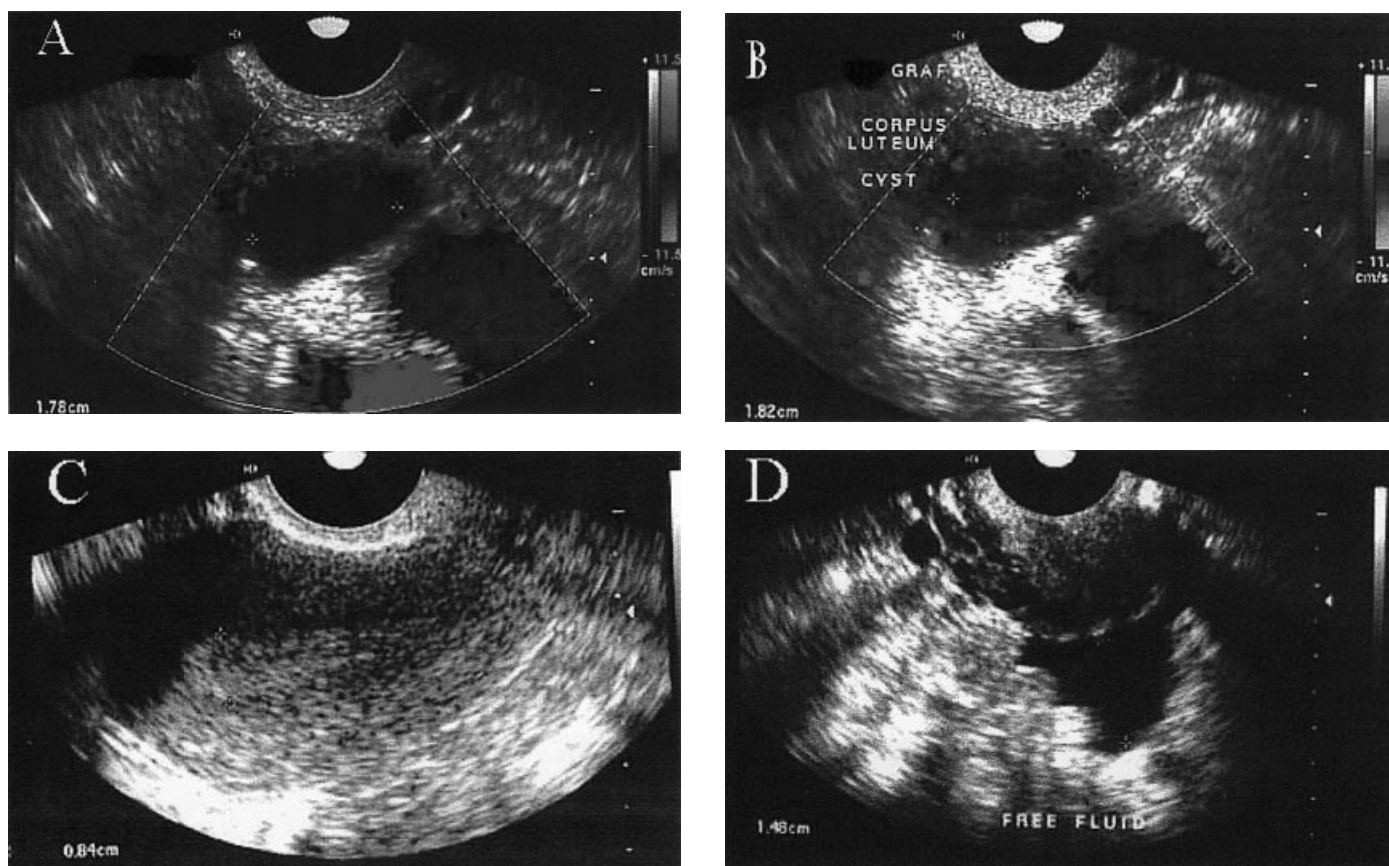
Figure 3. Demonstration of antral follicle development and ovulation by ultrasound in transplanted ovarian tissue. (**A**) Antral follicle development in the transplanted ovarian tissue; (**B**) corpus luteum; (**C**) free fluid in the cul de sac after ovulation; (**D**) endometrial transformation after ovulation.

graft. Because this patient still had a small portion of her ovary remaining, further follicle development appears to have been suppressed in the grafts. Her remaining frozen ovarian tissue will be transplanted when the patient experiences ovarian failure.

The second patient had undergone ovarian tissue cryopreservation at the age of 29 years when it was removed for an unspecified indication. Propanediol and a slow freeze technique (Oktay and Gosden, 1999; Table I) were utilized. The tissues were shipped to the authors' centre after an 8-month period of storage, whereupon 80 pieces of ovarian tissue with sizes ranging from 2×2 to 5×10 mm were thawed using a rapid-thaw protocol (Table I). The tissues were then threaded onto three separate 6/0 Vicryl sutures, and the three strings were then anchored by suturing to a triangular frame made from an absorbable cellulose membrane (Surgicell®; Ethicon Inc., NJ, USA). Using a laparoscopic technique, the grafts were sutured into a pocket created by dissection in the pelvic sidewall, and posterior to the broad ligament. The patient received 80 mg of aspirin daily for 3 weeks, starting 1 week before surgery. She also received 150 IU FSH (Fertinex®; Serono, Norwell, MA, USA) by i.m. injection daily for the first seven postoperative days. FSH was given based on the evidence that gonadotrophin administration can enhance graft survival (Imthurn *et al.*, 2000).

Blood flow to the grafts was demonstrated by Doppler-ultrasound examination as early as 3 weeks after surgery. At 15 weeks after the procedure, the patient was stimulated with human menopausal gonadotrophin (HMG) (Pergonal®; Serono). After 11

days of stimulation, a dominant follicle emerged in the ovarian graft, and the hormone replacement was discontinued. Post-stimulation testosterone concentrations were significantly higher than those pre-stimulation (18.3 ± 3.0 ng/dl versus 35.6 ± 2.0, $P = 0.0002$). Serial ultrasound examinations demonstrated continual follicle growth, though the HMG dose had to be increased gradually from 150 IU/day to 675 IU/day in order to sustain this. The serum oestradiol concentration peaked at 93 pg/ml (Figure 2), and the HMG dose was increased to 675 IU. However, because the oestradiol concentration fell to 75 pg/ml the next day, 10 000 IU HCG was given to trigger ovulation. Since the patient had a history of hypothalamic dysfunction, the luteal phase was supplemented with HCG (10 000 IU), given every 3 days for an additional three doses. Mean (± SE) progesterone concentrations rose from a pre-HCG value of 0.7 ± 0.06 ng/ml to a luteal phase value of 4.5 ± 1.4 (range 1.2–13) ng/ml ($P = 0.01$). After 24 days of stimulation, the largest follicle diameter reached 16.5–17 mm (Figure 3A). Ovulation was confirmed by sonographic demonstration of the collapse of the follicle (Figure 3B), and the appearance of free fluid in the cul-de-sac (Figure 3C). The endometrium also transformed into a uniform luteal phase pattern and reached 9 mm thickness (Figure 3D). The progesterone concentration peaked at 13 ng/ml (see Figure 2), and the patient had spontaneous menses 16 days after the first HCG injection (Oktay and Karlikaya, 2000).

On the third day of her following menstrual cycle, the patient's FSH concentration was 16 mIU/ml, LH 12 mIU/ml and oestradiol

Table III. Risk of ovarian involvement in various cancer types

Low risk (<0.2%)	Moderate risk (0.2–11%)	High risk (>11%)
Wilm's tumour	Breast cancer	Leukaemia
Non-Hodgkin lymphoma	Adenocarcinoma of	Neuroblastoma
Hodgkin lymphoma	uterine cervix	
Nongenital-rhabdomyosarcoma		
Osteogenic sarcoma		
Squamous cell carcinoma of		
uterine cervix		
Ewing's sarcoma		

17 pg/ml. On her fifth cycle day, the patient elected to restart oestradiol replacement at 0.1 mg/day. A follicle measuring 4 mm was noted in the graft on cycle day 6; this follicle reached 6 mm but ceased growth on cycle day 11, and became atretic on cycle day 16.

Clearly, the latter patient had a low ovarian reserve, which could in part be explained by the expected losses during the freeze–thaw and transplantation processes. However, because the tissue was not frozen in our laboratory, the possibility of variations in freezing methodology could not be ruled out. For example, the ovarian pieces were thicker and larger than would normally have been prepared for tissue cryopreservation.

Safety concerns with ovarian transplantation

There is a legitimate concern for the reseeding of cancer cells when performing ovarian transplantation. At least one animal study raised this question, although a very aggressive form of lymphoid cancer was used as an example (Shaw *et al.*, 1996). In that study, ovarian tissue collected from mice with lymphoma was grafted to healthy female mice. In one group, in which the origin of lymphoma could be traced, all females which received a single 1 mm^3 graft of fresh or cryopreserved (DMSO, ethylene glycol, propanediol or glycerol) ovarian tissue developed lymphoma of donor origin and subsequently died for this reason.

In a recent abstract, human frozen–thawed ovarian tissue from patients with Hodgkin's disease ($n = 5$), Non-Hodgkin's lymphoma (NHL; $n = 13$), acute lymphoblastic lymphoma (ALL; $n = 2$) and acute myeloblastic leukaemia (AML; $n = 2$) were xenografted to NOD/*LtSz*-SCID mice (Kim *et al.*, 1999). While none of the tissues from patients with NHL resulted in cancer recurrence, one xenograft from a patient with Hodgkin's disease resulted in recurrence (one out of five). The results from AML and ALL were inconclusive.

What is clear from these studies is that the cancer transmission rate of ovarian transplants will depend greatly on the type of cancer. While many cancer types never metastasize to ovaries, others are systemic in nature and pose a greater risk to the recipient (Table III).

Ovarian involvement in Wilm's tumour, lymphomas (with the exception of Burkitt's), osteosarcomas, Ewing's sarcoma, and extragenital rhabdomyosarcomas is extremely rare (Young and Scully, 1994). Likewise, in squamous cell cervical cancer, ovarian involvement is <0.2%, including the most advanced stages.

In contrast, aggressive leukaemias and neuroblastomas carry a high risk of involving the ovary. Because leukaemia is a systemic
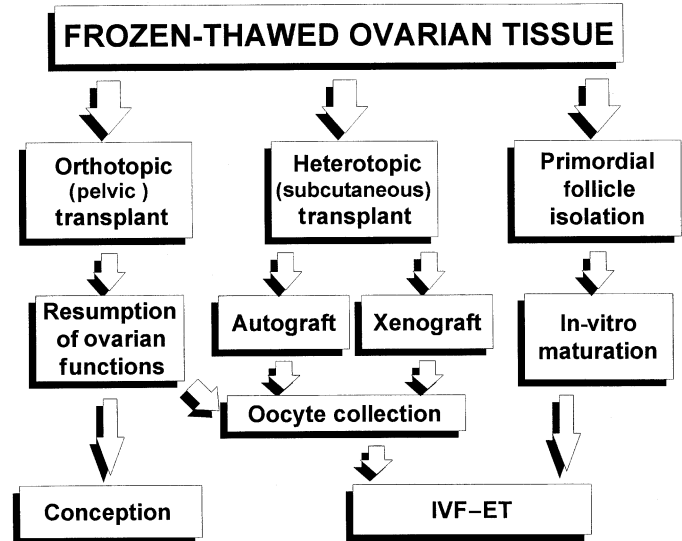

Figure 4. Experimental options for the utilization of banked human ovarian tissue. Cryopreserved tissue can be transplanted in the pelvis, on or around the original pedicle. If the Fallopian tubes are intact, natural conception can be achieved. In other cases, the tissue may be grafted to a heterotopic site, either as an autograft (i.e. subcutaneously) or xenograft (in immunodeficient animals such as SCID mouse). A third alternative is to isolate primordial follicles from the ovarian tissue and mature them *in vitro*. Mature oocytes obtained from heterotopic grafts and by in-vitro maturation will need to be fertilized *in vitro* to achieve pregnancy. ET = embryo transfer.

disease, it is expected that the blastic cells will be present in vascular channels in the ovary at the time of removal. The fact that the ovarian cortex is relatively avascular and that the tissues are taken through multiple washes may reduce the likelihood of transmission, but contamination with even a few cells cannot be tolerated. Therefore, a diagnosis of leukaemia remains to be a relative contraindication for autotransplantation at the present time. Neuroblastomas are less aggressive compared with acute leukaemias, but still pose a significant risk to the transplant recipient. In an autopsy series, ovaries contained metastases in 25–50% of the neuroblastoma cases (Young and Scully, 1994).

Breast cancer carries a low to intermediate risk of ovarian involvement (Table III). In the absence of clinical and radiological evidence of distant metastasis, ovarian involvement has been reported in 2–11% of the cases (Young and Scully, 1994). In most cases, a thorough clinical and radiological evaluation rules out ovarian involvement.

Strategies to minimize risk of cryopreserving ovarian tissue with cancer

At the time of ovarian removal, multiple samples are taken routinely from the ovary in order to perform a thorough histological analysis. In leukaemia and lymphomas, chromosomal and other markers can be studied using immunochemical and molecular biological techniques to screen for the presence of cancer cells in the ovary. For example with polymerase chain reaction (PCR), one neoplastic cell among >10^5 cells can be detected (Lee *et al.*, 1987). In this setting, PCR has been used to evaluate specimens for specific chromosome translocations, for example t(14;18) in follicular lymphoma, t(10;14) in T-cell acute lymphoblastic leukaemia/lymphoma, t(8;21) and t(15;17) in acute
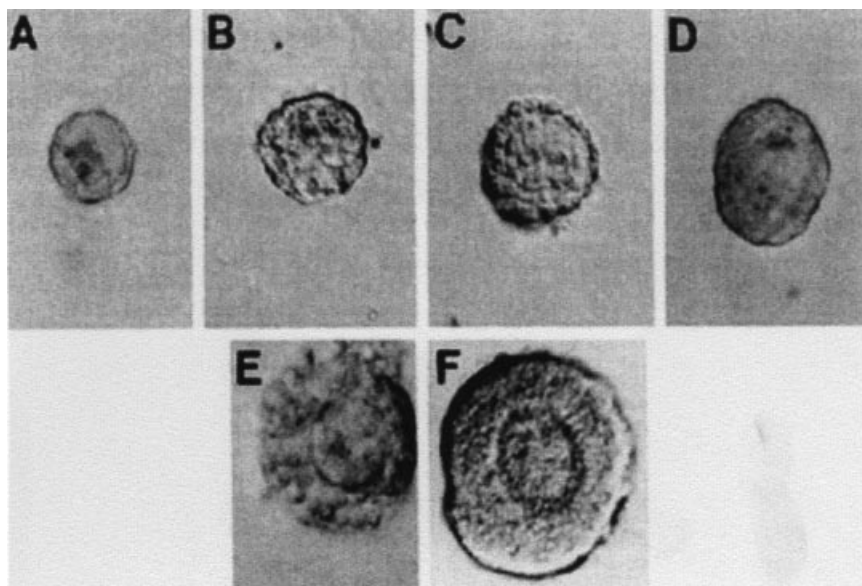
Figure 5. Human ovarian follicles isolated with partial digestion and microdissection technique. (**A**) A primordial follicle; (**B**) an early primary follicle with only few granulosa cells showing signs of enlargement; (**C**) a primary follicle: one layer of cuboidal granulosa cells; (**D**) a two-layer follicle; (**E**) a three-layer preantral follicle; (**F**) a large preantral follicle. (original magnification, ×300). (Reproduced with permission of the © Endocrine Society from Oktay, K., Briggs, D. and Gosden, R.G. Ontogeny of FSH receptor gene expression in isolated human ovarian follicles (1997). *J. Clin. Metab.*

myeloblastic leukaemia, and t(9;22) in chronic myelocytic leukaemia and acute lymphoblastic lymphoma. Immunohistochemistry and Northern or slot-blot analysis can be performed to detect myeloperoxidase expression in acute myeloblastic leukaemia (Abruzzo and Stass, 1996).

Because of the rapid progress in the field of cancer treatment, it is imperative to retain close communication with the patient's medical oncologist. Indeed, the oncologist's approval of the patient's eligibility for ovarian tissue banking is often required, as well as to confirm the safety of transplantation on an individual basis.

If these precautions are taken, the risk of cryopreserving an ovary infested with cancer cells is remote. In the future, other options for when the ovaries are involved might become available, but these methods will not be problem-free (Figure 4). It has been possible to isolate primordial follicles from human ovarian cortex (Oktay *et al.*, 1997a,b) (Figure 5), and to obtain pregnancies from in-vitro-matured primordial follicles in the mouse (Eppig and O'Brien, 1996). However, it is not yet possible to grow the human primordial follicles to maturity *in vitro*. In addition, the only mouse which did result from an in-vitro-matured oocyte showed some metabolic disorders, including severe obesity. Thus, it may be several years before an in-vitro maturation procedure is perfected and becomes a standard procedure. Even if techniques are developed for in-vitro follicle growth, because human primordial follicles take several months to reach the antral stages (Oktay *et al.*, 1998b), lengthy culture periods may be needed, and these may not be practicable in a clinical setting. Alternatively, in selected patients, ovarian follicles can also be grown in immunodeficient animals and the oocytes retrieved to perform an IVF–embryo transfer procedure (Revel *et al*, 2000). The in-vitro fertilization of these oocytes is likely to stir controversy, however. Finally, it must be noted that

ovary is not an immunologically privileged organ (Scott *et al.*, 1981, 1987), and ethical concerns regarding the development of allograft techniques for ovarian transplantation are unfounded (Robertson, 2000).

References

Abruzzo, L. and Stass, S. (1996) Molecular genetics of hematopoietic neoplasms. In Henry, J.B. (ed.), *Clinical Diagnosis and Management by Laboratory Methods*. W.B. Saunders, Philadelphia, pp. 1362–1373.

Al-Hasani, S., Diedrich, K., van der Ven, H. *et al.* (1987) Cryopreservation of human oocytes. *Hum. Reprod.*, **2**, 695–700.

Anderson-Reitz, L., Mechling, B.E. and Hertz, S.L. (1999) High-dose chemotherapy for breast cancer. *JAMA*, **10**, **282**, 1701–1703.

Apperley, J.F. and Reddy, N. (1995) Mechanisms and management of treatment related gonadal failure in recipients of high dose chemotherapy. *Blood Rev.*, **9**, 93–116.

Aubard, Y., Piver, P., Cogni, Y. *et al.* (1999) Orthotopic and heterotopic autografts of frozen–thawed ovarian cortex in sheep. *Hum. Reprod.*, **14**, 2149–2154.

Baird, D.T., Webb, R., Campbell, B.K. *et al.* (1999) Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at –196°C. *Endocrinology*, **140**, 462–471.

Bosma, G.C., Custer, R.P. and Bosma, M.J. (1983) A severe combined deficiency mutation in the mouse. *Nature*, **301**, 527–530.

Candy, C.J., Wood, M.J. and Whittingham, D.G. (1995) Follicular development in cryopreserved marmoset ovarian tissue after transplantation. *Hum. Reprod.*, **10**, 2334–2338.

Candy, C.J., Wood, M.J. and Whittingham, D.G. (2000) Restoration of a normal reproductive life span after grafting of cryopreserved mouse ovaries. *Hum. Reprod.*, **15**, 1300–1304

Carroll, J., Wood, M.J. and Whittingham, D.G. (1993) Normal fertilization of frozen-thawed mouse oocytes: protection of certain molecules. *Biol. Reprod.*, **48**, 606–612.

Chen, C. (1986) Pregnancy after human oocyte cryopreservation. *Lancet*, **i**, 884.

Chen, V.W., Wu, X.C. and Andrews, P.A. (eds) (1999) *Cancer in North America, 1991–1995. Volume One: Incidence.* Sacramento, North America Association of Central Cancer Registries.

Clark, M.J. (1998) Ovarian ablation in breast cancer, 1896 to 1998: milestones

along hierarchy of evidence from case report to Cochrane review. *Br. Med. J.*, **317**, 1246–1248.

Cox, S.L., Shaw, J.M. and Jenkin, G. (1996) Transplantation of cryopreserved fetal ovarian tissue to adult recipients in mice. *J. Reprod. Fertil.*, **107**, 315–322.

Deanesly, R. (1957) Egg survival in immature rat ovaries grafted after freezing and thawing. *Proc. Roy. Soc.*, **147**, 412–421.

Dissen, G.A., Lara, H.E., Fahrenbach, W.H. *et al.* (1994) Immature rat ovaries revascularized rapidly after autotransplantation and show a gonadotropin-dependent increase in angiogenic factor gene expression. *Endocrinology*, **134**, 1146–1154.

Eppig, J.J. and O'Brien, M.J. (1996) Development *in vitro* of mouse oocytes from primordial follicles. *Biol. Reprod.*, **54**, 197–207.

Fuller, B.J. and Bernard, A. (1984) Successful *in vitro* fertilization of mouse oocytes after cryopreservation using glycerol. *Cryo-Letters*, **5**, 307–312.

Goldberg, J.M., Mascha, F., Falcone, T. and Kovacs, G.T. (1999) Comparison of intrauterine and intracervical insemination with frozen donor sperm: a meta-analysis. *Fertil. Steril.*, **72**, 792–795.

Gosden, R.G., Baird, D.T., Wade, J.C. and Webb, R. (1994a) Restoration of fertility to oophorectomized sheep by ovarian autografts stored at –196°C. *Hum. Reprod.*, **9**, 597–603.

Gosden, R.G., Boulton, M., Grant, K. and Webb, R. (1994b) Follicular development from ovarian xenografts in SCID mice. *J. Reprod. Fertil.*, **101**, 619–623.

Green, S.H., Smith, A.U. and Zuckerman, S. (1956) The number of oocytes in ovarian autografts after freezing and thawing. *J. Endocrinol.*, **13**, 330–334.

Harp, R., Leibach, J., Black, J. *et al.* (1994) Cryopreservation of murine ovarian tissue. *Cryobiology*, **31**, 336–343.

Imthurn, B., Cox, S., Jenkin, G. *et al.* (2000) Gonadotrophin administration can benefit ovarian tissue grafted to the body wall: implications for human ovarian grafting. *Mol. Cell. Endocrinol.*, **163**, 141–146.

Kim, S.S., Gosden, R.G., Radford, A.J. *et al.* (1999) A model to test the safety of human ovarian tissue transplantation after cryopreservation: xenografts of ovarian tissues from cancer patients into NOD/LtSz-SCID mice. American Society for Reproductive Medicine Annual Meeting, Toronto, Ontario, Canada, September 25–30, Abstract O-003.

Lee, M.-S., Chank, K.-S., Cabanillas, F. *et al.* (1987) Detection of minimal residual cells carrying the t(14;18) by DNA sequence analysis. *Science*, **237**, 175–178.

Leporrier, M., Theobald, P., Roffe, J.-L. and Muller, G. (1987) A new technique to protect ovarian function before pelvic irradiation: heterotopic ovarian transplantation. *Cancer*, **60**, 2201–2204.

Linet, M.S., Ries, L.A.G., Smith, M.A. *et al.* (1999) Recent trends in childhood cancer incidence and mortality in the United States. *J. Natl Cancer. Inst.*, **91**, 1051–1058.

Marconi, G., Quintana, R., Rueda-Leverone, N.G. and Vighi, S. (1997) Accidental ovarian autograft after a laparoscopic surgery: case report. *Fertil. Steril.*, **68**, 364–366.

Mazur, P., Leibo, S.P. and Chu, E.H.Y. (1972) A two-factor hypothesis of freezing injury. *Exp. Cell Res.*, **71**, 345–355.

Meirow, D. (2000) Reproduction post-chemotherapy in young cancer patients. *Mol. Cell Endocrinol.*, **169**, 123–131.

Newton, H., Aubard, Y., Rutherford, A. *et al.* (1996) Low temperature storage and grafting of human ovarian tissue. *Hum. Reprod.*, **11**, 1487–1491.

Nugent, D., Newton, H., Gosden, R.G. and Rutherford, A.J. (1998) Investigation of follicle survival after human heterotopic autografting (abstract). *Hum. Reprod.*, **13** (Abstract Book 1), 22–23.

Oktay, K. and Gosden, R.G. (1999) Cryopreservation of human oocytes and ovarian tissue. In Brinsden, P. (ed.), *A Textbook of In Vitro Fertilization and Assisted Reproduction*. Parthenon Inc., Lancaster, UK, pp. 303–310.

Oktay, K. and Karlikaya, G. (2000) Ovarian function after transplantation of frozen, banked autologous ovarian tissue. *N. Engl. J. Med.*, **342**, 1919.

Oktay, K., Nugent, D., Newton, H. *et al.* (1997a) Isolation and characterization of primordial follicles from fresh and cryopreserved human ovarian tissue. *Fertil. Steril.*, **67**, 481–486.

Oktay, K., Briggs, D. and Gosden, R.G. (1997b) Ontogeny of FSH receptor gene expression in isolated human ovarian follicles. *J. Clin. Endocrinol. Metab.*, **82**, 3748–3751.

Oktay, K., Newton, H., Aubard, Y. *et al.* (1998a) Cryopreservation of immature human oocytes and ovarian tissue: an emerging technology? *Fertil. Steril.*, **69**, 1–6.

Oktay, K., Newton, H., Mullan, J. and Gosden, R.G. (1998b) Development of human primordial follicles to antral stages in SCID/hpg mice stimulated with follicle stimulating hormone. *Hum. Reprod.*, **13**, 1133–1138.

Oktay, K., Newton, H. and Gosden, R.G. (2000) Transplantation of cryopreserved human ovarian tissue results in follicle growth in SCID mice. *Fertil. Steril.*, **73**, 599–603.

Parkes, A.S. and Smith, A.U. (1952) Regeneration of rat ovarian tissue grafted after exposure to low temperatures. *Proc. Roy. Soc.*, **140**, 455–467.

Parrot, D.M. (1960) The fertility of mice with orthotopic grafts derived from frozen tissue. *J. Reprod. Fertil.*, **1**, 230–241.

Porcu, E., Fabbri, R., Seracchioli, R. *et al.* (1997) Birth of healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. *Fertil. Steril.*, **68**, 724–726.

Rauck, A.M. and Grouas, A.C. (1999) Bone marrow transplantation in adolescents. *Adolesc. Med.*, **10**, 445–449, xi–xii.

Revel, A., Raanani, H., Leyland, N. and Casper, R.F. (2000) Human oocyte retrieval from nude mice transplanted with human ovarian cortex (abstract). European Society of Human Reproduction and Embryology Annual Meeting, June 26, Bologna, Italy.

Ries, L.A.G., Percy, C.L. and Bunin, G.R. (1999) Introduction. In Ries, L.A.G., Percy, C.L., Smith, M.A., Gurney, J.G. *et al.* (eds), *Cancer Incidence and Survival among Children and Adolescents: United States SEER Program 1975–1995*. National Cancer Institute, SEER Program, NIH Pub. No. 99-4649, Bethesda, MD, pp. 1–15.

Robertson, J.A. (2000) Ethical issues in ovarian transplantation and donation. *Fertil. Steril.*, **73**, 443–446.

Scott, J.R., Keye, W.R., Poulson, A.M. and Reynolds, W.A. (1981) Microsurgical ovarian transplantation in the primate. *Fertil. Steril.*, **36**, 512–515.

Scott, J.R., Hendrickson, M., Lash, S. and Shelby, J. (1987) Pregnancy after tubo-ovarian transplantation. *Obstet. Gynecol.*, **70**, 229–234.

SEER*Stat Software, version 2.0 (1999) (SEER Cancer Incidence Public-Use Database, 1973–1996, August 1998 Submission) National Cancer Institute.

Shaw, J.M., Bowles, J., Koopman, P. *et al.* (1996) Fresh and cryopreserved ovarian tissue samples from donors with lymphoma transmit the cancer to graft recipients. *Hum. Reprod.*, **11**, 1668–1673.

Shaw, J.M., Cox, S.L., Trounson, A.O. and Jenkin, G. (2000) Evaluation of the long-term function of cryopreserved ovarian grafts in the mouse, implications for human applications. *Mol. Cell. Endocrinol.*, **161**, 103–110.

Sztein, J.M., Sweet, H., Farley, J. and Mobraaten, L. (1998) Cryopreservation and orthotopic transplantation of mouse ovaries: new approach in gamete banking. *Biol. Reprod.*, **58**, 1071–1074.

Sztein, J.M., O'Brien, M.J., Farley, J.S. *et al.* (2000) Rescue of oocytes from antral follicles of cryopreserved mouse ovaries: competence to undergo maturation, embryogenesis, and development to term. *Hum. Reprod.*, **15**, 567–571.

Thomas, E.D. (1999) Bone marrow transplantation: a review. *Semin. Hematol.*, **36** (Suppl. 7), 95–103.

Trounson, A. and Kirby, C. (1989) Problems in the cryopreservation of unfertilized eggs by slow cooling in dimethylsulphoxide. *Fertil. Steril.*, **52**, 778–786.

Tyndall, A. and Millikan, S. (1999) Bone marrow transplantation. *Baillières Best. Pract. Res. Clin. Rheumatol.*, December 13, 719–735.

Van Uem, J.F.H.M., Siebzehnrubl, E.R., Schuh, B. *et al.* (1987) Birth after cryopreservation of unfertilized oocytes. *Lancet*, **i**, 752.

Vincent, C., Pickering, S.J. and Johnson, M.H. (1990) The hardening effect of dimethylsulphoxide on the mouse zona pellucida requires the presence of an oocyte and is associated with a reduction in the number of cortical granules present. *J. Reprod. Fertil.*, **9**, 253–259.

Wagner, P.K., Seesko, H.G. and Rothmund, M. (1991) Replantation of cryopreserved human parathyroid tissue. *World J. Surg.*, **15**, 751–755.

Walters, M.C. (1999) Bone marrow transplantation for sickle cell disease: where do we go from here? *J. Pediatr. Hematol. Oncol.*, **21**, 467–474.

Weissman, A., Gotlieb, L., Colgan, T. *et al.* (1999) Preliminary experience with subcutaneous human ovarian cortex transplantation in the NOD-SCID mouse. *Biol. Reprod.*, **60**, 1462–1467.

Wells, S.A., Jr, Ellis, G.J., Gunnels, J.C. *et al.* (1976) Parathyroid autotransplantation in parathyroid hyperplasia. *N. Engl. J. Med.*, **295**, 57–62.

Young, R.H. and Scully, R. (1994) Metastatic tumors of the ovary. In Kurman, R.J. (ed.), *Blaustein's Pathology of the Female Genital Tract*, 4th edition. Springer-Verlag, New York, pp. 939–974.