

## Ovarian cryopreservation and grafting: its potential for human reproductive biology and animal conservation

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### Abstract

Ovarian cryobanking has considerable potential for fertility preservation and restoration and has been used to establish term pregnancies in mice, rats, sheep and humans, yet there is scope for progress towards *in vitro* and *in vivo* strategies to A) screen and improve outcomes of cryopreservation procedures and to minimize ischemic damage following grafting, B) monitor folliculogenesis and hormonal feedback, C) screen for, and remove, malignant cells, D) generate antral follicles containing normal mature fertilizable oocytes even when orthotopic autografting is not possible, and E) combine ovarian cryopreservation with more advanced reproductive technologies such as nuclear transfer (for animals only). In species such as mice a very diverse range of both cryopreservation and grafting strategies (including xenografting) has successfully generated live young. Human ovarian grafting is still a rare procedure and it is therefore encouraging that several babies have now been born. Progress has been slowest for species where compatible recipients are seldom available, such as with rare and endangered species. For these, further significant breakthroughs will be needed before cryobanked material can be reliably and efficiently used to generate new offspring.

**Keywords:** animal, cryopreservation, grafting, human, ovary, reproduction, wildlife.

### Introduction

The ovary is the sole source of female gametes and is central to reproduction and fertility. In instances when it becomes desirable to protect a female's germ line- if for example premature loss of oocytes or ovarian function is likely, or inevitable, several strategies can be employed including different assisted reproductive technologies (ART's) (for recent reviews see Shamonki and Oktay 2005; Donnez *et al.*, 2006; Fabri, 2006; Hasegawa *et al.*, 2006; Oktay *et al.*, 2006; Serebrovska *et al.*, 2006; Lee, 2007; Lornage and Salle, 2007). Table 1 summarizes these different ART approaches, and

outlines some advantages and disadvantages of each. At present, no single ART option is ideal but ovarian tissue cryopreservation does complement and enhance other approaches very well.

A significant advantage associated with ovarian tissue cryopreservation over most other ART approaches is the availability of material. Collections are largely independent of the female's age, cycle and hormonal status and can therefore be organized at almost any time and with minimal notification. Collections are usually straightforward, unless there are complications such as severe adhesions, scarring, severe endometriosis or a transmittable disease, and will in almost every case result in material that contain a large number of oocytes and has the potential to restore fertility to a suitable recipient. How much tissue needs to be collected to achieve success (fertility restoration) will however depend on biological variables such as the donor's and recipient's age, and scheduled treatments such as gonadotoxic cancer therapy. Because the ovarian cortex is so rich in primordial follicles even a limited amount of tissue may be sufficient to subsequently restore a female's fertility, as demonstrated by restoration of endocrine function and/or live young being born to recipients of cryopreserved whole ovaries (Cox *et al.*, 1996, 2000; Candy *et al.*, 2000; Shaw *et al.*, 2000a, b, c; Wang *et al.*, 2002a, b; Courbiere *et al.*, 2008), pieces of ovary (e.g., half or 1/4 of one ovary or even less; Salle *et al.*, 2002; Snow *et al.*, 2002; Yin *et al.*, 2003; Almodin *et al.*, 2004; Bordes *et al.*, 2005) and isolated follicles (Carroll and Gosden, 1993; Liu *et al.*, 2001). Removing both ovaries will cause the donor to become menopausal and is only justified in cases where the patient will almost inevitably lose all ovarian follicles.

In humans menopause and loss of endocrine function predisposes the donor to additional health related complications including cardiovascular and bone health, vasomotor symptoms and urogenital changes (Reich, 2001). Presently, cryopreserved transplanted grafts restore at least temporary hormonal activity to previously sterile females, but it is not possible to predict either how long any one graft will remain functional.

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Table 1. Female germline ART approaches: advantages and disadvantages summarized.

Approach	Advantages	Disadvantages
Embryo cryopreservation	<p>Established cryopreservation protocols (CP) for at least 22 species including cattle, sheep, and humans (Mazur <i>et al.</i>, 2008)</p> <p>Pregnancies achieved in many species</p> <p>Recipient does not need to be related to donor</p> <p>Reasonably predictable outcome following transfer</p>	<p>All species: sperm needed</p> <p>Cycle dependent</p> <p>Limited embryo numbers obtainable</p> <p>CP protocols not available for all species (Mazur <i>et al.</i>, 2008)</p> <p>May require hormonal stimulation/synchronization/superovulation</p> <p>May require surgical collection</p> <p>Some individuals yield few embryos</p> <p>Human: only suitable for females of reproductive age</p> <p>Ethical issues</p> <p>Time storage limit in many countries (legislation)</p>
Oocyte cryopreservation	<p>Successful CP in some species</p> <p>Male/sperm not needed at time of collection.</p> <p>Suitable for: single women/animals married women</p> <p>Applicable to species in which effective hormonal stimulation protocols do exist</p> <p>Pregnancies achieved</p> <p>Recipient can be unrelated to donor</p> <p>Human: fewer ethical issues compared to embryo cryopreservation</p>	<p>Most species: fertilization required at a later stage</p> <p>Cycle dependent</p> <p>Risk of cryodamage to the oocyte</p> <p>Limited numbers</p> <p>May require hormonal stimulation/synchronization/superovulation*</p> <p>May require surgical collection</p> <p>Some individuals yield few oocytes</p> <p>Human: only females of reproductive age</p> <p>May be time storage limit</p>
Ovarian cryopreservation	<p><u>Ovary collection</u></p> <p>Easy to obtain by surgery</p> <p>Collection is age &amp; cycle independent</p> <p>Possible to collect from recently deceased animals.</p> <p>Male/sperm not needed at collection</p> <p>Collection does not require hormonal treatment of donor</p> <p>Large numbers of eggs easily collected</p> <p>Scope for natural selection of eggs as they mature</p> <p>Direct <i>in vitro</i> maturation of harvested oocytes from antral follicles (prior to freezing) possible</p> <p>Direct <i>in vitro</i> culture of follicles (prior to freezing) possible</p> <p><u>Tissue cryopreservation</u></p> <p>Cryopreservation simple</p> <p>Graft may restore full fertility, i.e., capacity to cycle, conceive &amp; gestate without ART or other intervention</p>	<p><u>Tissue cryopreservation</u></p> <p>Potential histocompatibility issues: recipient must be histocompatible with donor or immunosuppressed</p> <p>Both collection and re-grafting is invasive</p> <p>Ischemia during collection, processing and after grafting all reduce graft function</p> <p>Still experimental to a certain stage and possibly not optimized</p> <p>Reproductive function takes time to return</p> <p>Risk of re-introduction of disease</p> <p>Graft longevity unpredictable (usually short)</p> <p>Graft (ortho or heterotopic) may require subsequent ART</p> <p>Recruitment pattern after grafting may differ from normal (fast rate of loss)</p> <p><i>In vitro</i> culture of oocytes from primordial follicles collected after cryopreservation not yet possible</p> <p><u>Additional disadvantages in whole ovary re-anastomosis:</u></p> <p>Fractures may compromise ovarian integrity</p> <p>Anastomosis requires microsurgery</p> <p>Anatomy of blood supply complicates/prevents cryoprotectant perfusion &amp; anastomosis in some species*</p> <p>Cryopreservation of large ovaries is experimental</p>
Nuclear transfer	<p>Somatic cells are exceptionally easy to collect (any age, stage, status)</p> <p>Somatic cells are exceptionally easy to cryopreserve</p> <p>Advantage in using oocytes as they are genetically unique (somatic cells are identical to the donor)</p>	<p>Proof is needed that cryopreserved nuclei can be used as donors</p> <p>Requires compatible recipient oocyte*</p> <p>Requires appropriate imprinting of donor material*</p> <p>Proof is needed that cryopreserved primordial follicles can be cultured to maturity (a stage at which imprinting is complete)</p> <p>Nuclear transfer requires <i>in vitro</i> manipulations and culture*</p> <p>Ethics and legal issues still to be resolved (and NOT permitted for humans)</p>

\*limits the usage of this tool in many rare and endangered species.



There is no single method by which ovarian tissue is cryopreserved and there are also many different ways in which transplanted tissue sustain cyclicity and fertility (e.g., Karow and Critser, 1997). The most common approach is to use pieces of ovarian cortex. One advantage of this approach is that the individual pieces may, even when used separately, support hormonal activity and even term pregnancies. This allows pieces to be grafted on separate occasions and thereby prolong the period of function tissue, or to separate graft sites, or multiple recipients (commonly done in animals), or to be used for analysis or *in vitro* culture. In animals graft success (for non-reanastomosed ovaries) falls dramatically with age due to both falling follicle abundance and somatic aging. Very follicle rich material e.g., ovaries from fetuses and very young donors can completely, or almost completely, restore a young recipients fertility and reproductive lifespan including number of progeny (Vom Saal *et al.*, 1994; Candy *et al.*, 2000; Shaw *et al.*, 2000; Liu *et al.*, 2008; Wang *et al.*, 2008). However, even very follicle rich (non-frozen) ovaries perform poorly when grafted into ageing, or aged, recipients (reviewed in Vom Saal *et al.*, 1994), and this may correlate with e.g., changes in VEGF expression in the ovary (Yeh *et al.*, 2008) and slower wound healing with age. An as yet unanswered question is whether this decline would also hold true for recipients of intact, reanastomosed, ovaries. Cryobanking of a whole intact ovary and its blood vessels and surgically re-attaching (re-anastomosing) it to the recipient's blood supply, is an alternative to cryopreserving pieces which has the potential to reduce damage due to ischaemia as it is independent of neo-vascularization of the graft. This approach has been applied to species as small as the rat (Wang *et al.*, 2002a, b) and as large as the sheep (Salle *et al.*, 2002; Arav *et al.*, 2005; Imhof *et al.*, 2006; Baudot *et al.*, 2007) and is becoming of interest to clinicians.

Upon transplantation surviving follicles need to complete their maturation, which entails extensive developmental changes (including morphological, cytoplasmic, nuclear and functional). The time taken to develop into oocytes that are sufficiently mature to sustain fertilization is 5 weeks for mouse fetal ovaries, which do not yet contain follicles (Cox *et al.*, 1996, 2000), 3 weeks for newborn mouse ovaries, which contain mainly primordial follicles; (Cox *et al.*, 1996, 2000; Eppig and O'Brien, 1996), but is reduced if the tissues contain growing follicles at the time of collection. In species such as the human, complete folliculogenesis is estimated to take several months (Gougeon, 1985; Oktem and Oktay, 2008), and approximately 12 and 17 weeks in cattle and sheep respectively (Britt, 1991; Bordes *et al.*, 2005). It becomes important to optimize procedures for all follicle stages. Although follicles in ovaries from young animals and fetal mice generally develop and mature as

expected even if grafted to highly atypical situations such as males of the same species (Waterhouse *et al.*, 2004), or to other species (Snow *et al.*, 2002; Cleary *et al.*, 2003), there are some interesting exceptions. Ovaries of young wallabies (marsupials) collected before follicular development is complete, form testis like chords rather than follicles after xenografting to mice (Mattiske *et al.*, 2002), they also become similarly masculinized if allografted into young male wallabies (Whitworth *et al.*, 1996).

Another anomaly seen in grafted ovaries is that they often contain many large luteinized structures, which may reflect that the tissue is exposed to high menopausal levels of gonadotropins before normal cyclicity is established. Xenografted ovarian tissue followed by regular ultrasound assessment (Fassbender *et al.*, 2007) may allow *in vivo* graft assessment and could provide a powerful tool for assessing the efficiency of hormone (superovulation) regimes and narrowing the optimal time frame for oocyte retrieval. *Ovarian cryopreservation and grafting is clearly both a fascinating research topic and a technique that does have both human and animal applications. Its usefulness for both clinical and animal applications stems from its flexibility and versatility, but it is our opinion that further developments are needed for this technique to reach its full potential.*

### **Fundamental aspects of ovarian tissue cryopreservation**

#### *Advantages of ovarian cryopreservation*

One advantage of ovarian cryopreservation is that it circumvents cryopreservation of fully grown mammalian oocytes. Full sized, mature, oocytes are among the rarest cells in the body and are short lived, with the result that their availability is limited and strongly cycle, season and age specific. Even though their availability can, in some species, be boosted by the use of hormones, the main problem is that mature oocytes of most species are very difficult to cryopreserve (reviewed in Critser *et al.*, 1997; Gook and Edgar, 2007; Leibo, 2008). Furthermore, in the human, the maximum storage time may be limited by legislation. An ovary of a female of reproductive age is, by contrast, not only easy to collect but usually contains numerous oocytes and tolerates cryopreservation and storage at -196°C very well. A range of cryopreservation procedures, including very cheap and simple ones, have been successful for ovarian tissue, and although storage times may be regulated by national laws, storage limits and ethical issues may be less stringent than those relating to mature oocytes (Dudzinsky, 2004). Table 2 summarizes and compares these and other complexities, advantages and disadvantages of cryopreservation of ovarian tissue versus whole ovary.



Table 2. Comparative analysis of complexities, advantages and disadvantages of cryopreservation of ovarian tissue versus whole ovary.

	Whole ovary and its vessels CP Aim for vascular anastomosis	Whole ovary without vessels for CP	Ovarian piece(s) for CP	Isolated follicles for CP	Oocytes (derived from ovary before CP starts)
Ease of collection at surgery	Very variable, depends on vasculature (impossible for some)	Very easy all species	Very easy	Primordial may be impossible Growing are easy	Very easy (release by puncturing or opening follicles)
Cryoprotectant entry	Perfusion to ensure rapid & uniform entry	Limited CP entry unless the ovary is very small or the time long	Dependent on thickness, size, temperature and CP solution etc	Normal oocyte (or embryo) CP methods can work	Normal oocyte (or embryo) CP method
Cryopreservation method & outcome	Slow cooling: variable outcome Vitrification: very variable outcome	Variable outcome with all tested CP methods	Most methods work to some extent (Slow, Rapid & Vitrification)	Limited information but oocyte or embryo protocols can work	Immature oocytes have poor prognosis. Mature(ed) MII oocytes can be processed using normal MII procedures
Storage container	Must be large enough for whole ovary and vessels may require bag or equivalent	Must be large enough for whole ovary may require bag or equivalent	Wide range of options (size dependent, but usually vials)	Types normally used for embryos or oocytes	Types normally used for embryos or oocytes
Replacement	Requires microsurgery May be impossible Most likely to orthotopic site.	Easy (surgery to ortho- or heterotopic site)	Easy (surgery to place at ortho- or heterotopic site)	Easy (injection or surgery to place at ortho- or heterotopic site)	Transfer as oocyte to lumen of fallopian tube (Surgical GIFT) or embryo to lumen of uterus (non-surgical ET)
Follicle outcome	Big losses if blood supply fails.	Many follicles lost	Many follicles lost	Limited information	NA
Fertility outcome	Pregnancy rat Live young sheep	Pregnancy rat, mouse	Live young rat, mouse, sheep, human	Live young mouse	Live young

*Physiological aspects of ovarian tissue cryopreservation*

The reason for the flexibility of ovarian cryobanking stems from basic biology. Although the ovaries of mammals contain the female's lifetime supply of oocytes, most of these are present as quiescent primordial follicles (each containing one oocyte) in developmental arrest. In most mammals the primordial follicles are predominantly located within the thin (usually 1 mm or less) fibrous, outer layer of the ovary (the cortex). This superficial location simplifies collection and aids permeation by antifreeze compounds, water and gasses. In addition, it may benefit survival *in vitro* and *in vivo* after grafting. Because most of the female's oocytes are concentrated

in the cortex, one of the most common collection strategies is to simply strip thin sheets of ovarian cortex off the ovary (either *in situ* or after removal). If thin sheets cannot be obtained, the ovary may be cut into small pieces (e.g., ~1 mm thick or 1 mm<sup>3</sup>) because it is known that the protective "antifreeze" chemicals (cryoprotectants) penetrate ovarian tissue relatively slowly (Paynter *et al.*, 1997; Gerritse *et al.*, 2008). Since cryopreservation of ovarian tissue commenced, many different slow and rapid cooling (including vitrification) protocols have been used successfully, using an immensely wide range of media, cryoprotectants (types and concentrations), equilibration conditions (times and temperatures) cryoprotectant addition and removal strategies, cooling and warming rates, seeding conditions, break point and storage temperatures and



containers (Smith, 1961; Shaw *et al.*, 2000a, b, c; Newton and Illingworth, 2001; Salehnia *et al.*, 2002; Aubard *et al.*, 2003; Isachenko *et al.*, 2003; Migishima *et al.*, 2003; Snow *et al.*, 2004; Devireddy, 2005; Gandolfi *et al.*, 2006; Hasegawa *et al.*, 2006; Li *et al.*, 2006). Most of these studies have focused on species with small ovaries or have used small pieces and equilibrated these with the cryoprotectant simply by immersion. An alternative approach, which can be used for intact ovaries which are collected together with their blood supply, is to pump (perfuse) solutions through them to achieve uniform cryoprotectant distribution within ~20 minutes (reviewed in Paynter *et al.*, 1997).

Most studies to date have used cryopreservation by slow (controlled) cooling, the remainder have evaluated rapid cooling (which includes vitrification) procedures (Shaw *et al.*, 2000a, b, c; Salehnia *et al.*, 2002; Isachenko *et al.*, 2003). The rationale for using vitrification for mature oocytes or embryos is very clear as it is simple, fast and inexpensive and can achieve better results than slow cooling procedures (Oktay *et al.*, 2006; Vajta and Nagy, 2006). Most recently published results support this view and show that for well researched species, for which vitrification methods for oocytes and embryos have been developed, they are often more efficient and reliable than slow freezing (Oktay *et al.*, 2006; Vajta and Nagy, 2006; Gook and Edgar, 2007). This is not yet the case for ovarian tissue since slow cooling can be performed using simple, cheap equipment and has fewer attendant equilibration and toxicity problems than vitrification. Ovarian tissues have therefore been cryopreserved simply and inexpensively with both slow and rapid cooling procedures, with methods that can be adopted for use under field conditions and/or on a low budget (Smith, 1961; Cleary *et al.*, 2001; Salehnia *et al.*, 2002; Isachenko *et al.*, 2003). A potential benefit with slow cooling is that the low cryoprotectant concentrations used with this procedure are less toxic than those used for rapid cooling/vitrification protocols. The slow cooling techniques allow quite long equilibration times and high equilibration temperatures without compromising survival (Snow *et al.*, 2001). An issue with rapid protocols, and in particular vitrification, is the use high cryoprotectant concentrations and relatively short equilibration times. High cryoprotectant concentrations are toxic but short equilibration times do not permit full permeation. As a result the outer portion of ovarian tissues, in particular any edges or corners (which can include the cortex), get greater exposure than inner portions, resulting in non-uniform protection of the tissues. This case has been shown by greatly differing immediate survival of follicles (as assessed by uptake/exclusion of live dead stains) in different parts of tissue pieces (Choo *et al.*, 1999). Whether vitrification would be better suited to equilibration by perfusion, is not clear although numerous attempts to vitrify another whole model organ (the kidney) has to date met with

very limited success, possibly because of damage to the vascular endothelium (Fahy *et al.*, 2002). At present slow cooling protocols have had the most wide and diverse use, and have proven at least partially effective for ovaries of species including insects and amphibians (Mochida *et al.*, 2003; Wooi *et al.*, 2008).

### Overview of banking and the use of ovarian material

*Cryopreservation of ovarian tissue pieces: background, its relative value in female germline preservation and outcomes*

Ovarian grafting commenced in the first half of last century and contributed greatly to advances in our understanding of endocrinology and graft rejection. The discovery of the protective role of cryoprotectants for sperm in 1949 (Polge *et al.*, 1949), then stimulated a decade of research on ovarian tissue cryopreservation during which pregnancies were established (reviewed in Smith, 1961) and live mouse pups born (Parrott, 1960). From 1960 to 1990 ovarian grafting continued to be used as a research tool (Vom Saal *et al.*, 1994), but research on ovarian cryopreservation was largely ignored in favor of studies on oocyte and embryo *in vitro* culture and cryopreservation. In the 1990's interest in ovarian cryopreservation re-emerged as its practical value as a strategy for banking genetic material for laboratory, companion and domestic species as well as rare and endangered species and humans was realized (Cecim *et al.*, 1995; Karow and Critser, 1997; Shaw *et al.*, 2000b; Paris *et al.*, 2004; Donnez *et al.*, 2006; Jewgenow and Paris, 2006).

As indicated earlier thin pieces of cortex have in most studies proved to be particularly easy to cryopreserve and graft. The main disadvantages of only processing pieces of ovarian cortex are the difficulties in assessing cryosurvival and the fact that present primordial follicles in these tissue pieces A) have a tendency to become recruited in large numbers which shortens the working lifespan of the graft, B) take a long time to fully mature (weeks in mice, months in human and sheep depending on the time folliculogenesis normally takes *in vivo*), and C) are prone to ischaemia and die if oxygen does not become available (e.g., through vascular in growth from the surrounding tissue, or by diffusion across a thin membrane or fluid layer). These problems have stimulated interest in improving cryopreservation procedures for whole ovaries with its associated vasculature to permit vascular re-anastomosis. Although whole mouse and rat ovaries have been cryopreserved since the 1950's they are small and have usually been replaced without vascular re-anastomosis. Parrott (1960) demonstrated over 30 years ago that frozen ovarian tissue pieces autografted to mice could restore fertility. Since then, reports on autografting without re-anastomosis range from reduced fertility (Gosden *et al.*, 1994; Stzein *et al.*, 1998) to no



detectable difference between recipients of control (non-frozen) grafts and cryopreserved grafts (Candy *et al.*, 2000; Shaw *et al.*, 2000a; Chen *et al.*, 2006; Liu *et al.*, 2008; Wang *et al.*, 2008). Success is most likely for ovarian tissue with high follicle density, low donor age, low recipient age, and a large amount of tissue available for transfer, but the exact outcomes cannot presently be predicted. It is particularly difficult to predict in advance which cryopreservation protocol will be best as the research over the last 50 years shows that a range of cryoprotectant types and concentrations, cooling rates and cryopreservation methods can all work at least to some extent.

In all studies where only the cryopreserved ovarian cortex is returned to a previously ovariectomized recipient, ovarian function returns gradually at a pace commensurate with the rate of follicle development meaning that it may take months before the first ovulation (e.g., sheep and human). In studies where both the cortex and some amount of other underlying ovarian tissue is cryopreserved and grafted both primordial and growing follicles are sometimes able to survive and a more rapid return of fertility is possible. Evidence for this includes several studies in which mature oocytes have been collected in a much shorter timepoint than it takes for a primordial follicle to grow to maturity. A good example is shown by Candy *et al.*, (1995), who observed fully grown antral follicles in Marmosets 7 days after grafting. It is also possible to successfully cryopreserve just antral and pre-antral follicles (Lima *et al.*, 2006), and get live young (Caroll and Gosden, 1993; Kagawa *et al.*, 2007). There is also some evidence from humans, since transplanted cryopreserved ovarian tissue obtained from women who immediately prior to cryopreservation received gonadotoxic treatment requires a considerable longer time period to regain ovarian function as compared to women who did not receive treatment, indicating that follicles which have already started follicular development survive the cryopreservation procedure (Andersen *et al.*, 2008)

Interestingly it has been demonstrated that oocytes obtained directly from antral follicles immediately after thawing can be used to produce live young (Sztejn *et al.*, 1998). *However, our opinion is that in cases where preservation of the germ line is of utmost importance, and established protocols are available, then it is recommended that oocytes that are released as the ovary is processed for cryopreservation (and which are usually at the germinal vesicle stage with a compact cumulus), are cryopreserved at the same time, or after a period of maturation, or as an embryo after fertilization.*

Several studies focused on the effect of the age of the donor and recipient on the outcome on terms of fertility and showed a clear decline in time (Vom Saal *et al.*, 1994; Hani *et al.*, 2006). Hani and colleagues specifically showed that grafted mouse ovarian tissue

from younger donors had a higher pup birth rate compared to tissue from older donors. In addition, it was also shown that younger recipients had a higher pup birth rate compared to older recipients. *We believe that this indicates that further animals studies are required before such evidence can be translated into human guidelines for tissue freezing and grafting (in terms of up to which age cryopreservation and grafting should be recommended).*

#### *Practical applications of ovarian tissue cryopreservation and grafting: mouse germline banking*

Ovarian grafting alone or in combination with cryopreservation is useful when used in combination with grafting for a range of mouse mutants (Agca, 2000; Glenister and Thornton, 2000; Shaw and Nakagata, 2002; Nagy *et al.*, 2003) and transgenic mice lines (Harari *et al.*, 1997). For species such as mice, in which inbred and immunocompromised strains are available, cryobanking ovaries can be cheaper and more efficient than banking oocytes or embryos, in particular for individuals that are infertile, sterile or difficult to superovulate (Rall *et al.*, 2000). Ovarian grafting is an effective option for mice because the ovaries can be collected from very young donors including newborns, which minimizes feed and husbandry issues, and each donor ovaries can subsequently be grafted to one or more recipients. Mature oocytes and embryos, by contrast, cannot be collected until the females are old enough to be superovulated ( $\geq 3$  weeks old) or naturally mated ( $\geq 6$  weeks old). Obtaining live young from cryobanked ovarian tissue is efficient in that it involves only one surgical event in which a young prepubertal recipient has her own ovaries removed and replaced by grafts (orthotopic grafting). Pregnancies can then be established once she matures simply by placing her with a fertile male. Each recipient may, by the conclusion of her breeding life, produce many litters without any further interventions. Protocols for cryobanked embryos are more complex and considerably more costly in that the recipient has to be mature and pseudopregnant at the time of embryo transfer. To ensure that she is pseudopregnant requires considerable manpower as it either involves making vaginal smears (for a number of mature females) every day to monitor their cycle stages and then using manual stimulation on the females in estrus to induce a pseudopregnant state. An alternative approach is to place mature females with vasectomised males. These males detect which females are in estrus and by mating with them induce pseudopregnancy. As female mice on average cycle every 4 days, each time a transfer is to be performed 4 (or more) females have to be placed with males to get 1 recipient. The pseudopregnant females are then anaesthetized for surgical transfer of embryos to either their oviducts or uteri (Nagy *et al.*, 2003). Following the weaning of the resulting pups the surrogate is usually euthanized.



Procedures for oocytes are comparable to those for embryos except that the oocytes have to be fertilized before use and their use must therefore be combined with IVF or ICSI (with sperm from either a freshly killed male or frozen stocks). The cryopreservation outcomes for oocytes and embryos of well researched strains are exceedingly good and reliable, but may be less so for less well researched strains, mutants and genetically modified mice. Oocyte and embryo banking therefore requires considerably more resources than ovarian banking in terms of time, money, labor, animals, skills and planning.

There is however one important advantage that oocyte and embryo banking currently have over ovarian banking and that is that any female of the same species can serve as a recipient for embryos and carry these to term, because the reproductive tract supports and will not reject even unrelated oocytes, embryos and fetuses. Ovarian tissues are by contrast usually rejected unless the recipient is histocompatible or immunologically compromised (naturally or with drugs) or the graft is protected by placing it in an immunologically privileged site or within a protective container that excludes immune cells (Shaffer and Hulka, 1969; Gosden *et al.*, 1994; Wolvekamp *et al.*, 2001). We are therefore hopeful that our increasing understanding of immune rejection will ultimately allow us to eliminate problems associated with graft rejection, and thereby be able to perform ovarian tissue allografts to non-histocompatible recipients for all species and possibly even perform xenografting easier across species barriers to recipients other than immunocompromised rodent recipients.

#### *Practical applications of ovarian tissue cryopreservation and grafting: human germline banking*

Girls and women afflicted with a malignant disease have over the last few decades experienced increased survival rates, often as a result of more aggressive chemotherapy regimes being used. This has led to an increase in the number of girls and women, who as a side effect become sterile. In combination with the advance in technical possibilities to preserve fertility, human germline banking has become an important issue in the management of the quality of life to the growing population of cancer survivors treated during their fertile years. A number of techniques are now being developed to preserve their fertility. Currently, the mostly used techniques include cryopreservation of mature oocytes, embryos and ovarian cortex. Although cryopreservation of mature oocytes and especially embryos has now become established procedures in infertility treatment, they suffer from some disadvantages in the context of sick women. They require the woman to undergo ovarian stimulation with administration of exogenous hormones, which is often a lengthy process, requiring a time period of several weeks in a situation where the woman may

require immediate treatment in order to combat a malignant disease. Even if ovarian stimulation can be accomplished the harvest of mature oocytes is usually limited to around 10-15 at the best. With the current efficacy for cryopreservation of both oocytes and the derived embryos these numbers are too low to guarantee a high chance of conception. Finally, oocytes and embryos have to be returned to a uterine environment in which the endometrial lining is primed for implantation. If the woman is not cycling naturally this can only be achieved through exogenous hormonal support. Ovarian grafts by contrast re-establish a cyclic endocrine hormone milieu including appropriate conditions for conception, gestation and parturition.

Cryopreservation of ovarian tissue fulfil a number of shortcomings, it is available at short notice, and a large number of oocytes can be collected, but the technique is still experimental and the efficacy has not yet been determined, although encouraging results are now being collected (Andersen *et al.*, 2008; Donnez *et al.*, 2008). In addition, removal of ovarian tissue or indeed one entire ovary does not seem to affect (or only marginally by one or two years) fertility and the age at which the woman will enter menopause. If one ovary is removed and cryopreserved the remaining ovary will in case the gonadotoxic treatment only marginally affect the follicular pool sustain preovulatory follicle development in each cycle and may contain enough follicles to sustain the around 400-500 ovulations that a woman experience in her entire reproductive life. If the gonadotoxic treatment induces premature ovarian failure (POF) the cryopreserved tissue may be thawed and transplanted. On the other hand, if the gonadotoxic treatment does not induce menopause, the cryopreserved ovary can remain in liquid nitrogen for possible later use if POF occur earlier than expected. So, once a functional program has been established, each woman mainly needs to consider the (low) risks of the operation for removal of ovarian tissue.

It is estimated that several thousand women have already had ovarian tissue cryopreserved worldwide. This is almost exclusively women who faced gonadotoxic treatment, often a malignant disease like Morbus Hodgkin's disease or mammary cancer, but also patients with non-malignant diseases like various autoimmune diseases and genetic diseases like Turner syndrome have at an early age had tissue cryopreserved (Andersen and Hreinsson, 2005). The demand for this technique is likely to increase considerably in parallel with efficacy being documented and improved. In Denmark, which is a country with a fairly well established service, the activity is currently on an annual basis around 15 cryopreservations per million inhabitants and is estimated to increase over the next coming years.

Recently, it has been shown that ovarian tissue sustains an ischemic period prior to cryopreservation without total loss of follicular viability (Schmidt *et al.*,



2003). If the tissue after removal from the patient is cooled on ice, it can be transported at least up to 5 hours before being cryopreserved. This has recently been documented by the birth of two healthy babies following transplantation of such cryopreserved tissue (Andersen *et al.*, 2008). This allow hospitals who treat women, that may not want to or cannot be moved to another hospital where cryopreservation expertise is available, to remove the ovarian tissue locally and send it on ice to a central unit, that perform the cryopreservation procedure.

Although ovarian tissue from many girls and women has been cryopreserved, experience with transplantation is only accumulated at a slow pace. There is usually a considerable time period (i.e. at least two years) until the woman is cured and considered fit for receiving transplantation. Furthermore, only around half of the women who had tissue cryopreserved actually entered treatment induced menopause (Rosendahl *et al.*, 2008) and a relative large fraction of young women and girls may not have found a partner yet and will save tissue until the wish to get pregnant. Equally important is the fact, that safety of the procedure is not yet fully established for a number of different diseases. Is there a risk of reintroducing the malignant cells that may be harbored by the grafts in connection with transplanting? Currently, a lot of research focuses on the safety aspects of transplanting ovarian tissue.

Around 25 women have globally received transplantation of cryopreserved ovarian tissue after having entered menopause as a result of gonadotoxic treatment. None of the women seem to have experienced a relapse as a result of transplantation. The tissue has regained ovarian function in all women. Following a period of three to six months after transplantation levels of gonadotropins gradually returned to pre-menopausal levels. Thereafter, the grafts support regular menstrual cycles in some periods, whereas irregular cyclicality may occur in other periods, probably reflecting that the pool of functional follicles is rather limited and sometimes there is no recruitable follicles, securing a negative feedback of pituitary FSH release Andersen *et al.*, 2008; Donnez *et al.*, 2008). This is likely to be explained by a considerable follicle loss during the cryopreservation procedure and until re-vascularization has occurred in the transplanted tissue. The functional life-span of the grafts varied from just a few menstrual cycles to four years (and still being functional in a few cases; (Andersen *et al.*, 2008; Donnez *et al.*, 2008). The longevity of the transplants is affected by a number of factors, including the age of the woman at cryopreservation, and the amount of tissue transplanted. However, based on the present knowledge, cryopreserved ovarian tissue restore the endocrinological function of the ovary effectively.

Although most of the transplanted women had a pregnancy wish, some actually did not and some only

supported ovarian function for a few menstrual cycles with much reduced chances of conception. Currently, five women have each given birth to a healthy child following transplantation of cryopreserved ovarian tissue (Donnez *et al.*, 2004; Meirow *et al.*, 2005; Demestere *et al.*, 2006; Andersen *et al.*, 2008). Three conceived following assisted reproduction, whereas two naturally achieved pregnancy. In addition, two women have miscarried, one who's pregnancy resulted from an oocyte retrieved from a heterotopic transplant (Rosendahl *et al.*, 2006), the other had conceived from an orthotopic graft (Andersen *et al.*, 2008). The number of recruitable follicles which develop in the transplants are often reduced to only one or two per cycle and even the use of ovarian stimulation with exogenous hormones may not augment the number of preovulatory follicles. Therefore no or only a small dose of exogenous hormones is used for these patients. However, if the oocytes are aspirated from the preovulatory follicles and treated as in conventional IVF treatment, the fertilization rate and implantation potential appears to be similar to that normally seen (Andersen *et al.*, 2008).

The remaining inactive ovary is used mostly for transplantation and it appears to support follicular development well in the transplanted tissue. Oocytes developed in grafts transplanted to the remaining ovary has supported all the current pregnancies. However, tissue transplanted heterotopically also appears to support follicular development.

Taken together, transplanted cryopreserved ovarian tissue support endocrine function with good efficacy and functional life-span is most often sufficient to allow the woman a chance for becoming pregnant. The chance of giving birth to child appears currently to be similar to the use of cryopreserved oocytes or embryos, but needs to be followed closely in the coming years. Further, the safety issue also needs further optimization although no one has yet experienced a relapse due to replacement of ovarian tissue.

#### *Practical applications of ovarian cryobanking: conservation of threatened wildlife species*

Human activities are causing a decline in the abundance, diversity and distribution of many animal species. Cryobanking has been envisioned (Wildt, 2000), and started to be implemented (Paris *et al.*, 2004), as a strategy which will allow genetic material of rare and endangered species to be held for future use. Although this material has not yet been proved to generate live young for endangered species, there is every reason to believe that it will work because ovarian tissue auto or allografts have restored cyclicality and fertility for a variety of common species including moths (Mochida *et al.*, 2003), mice (Parrott, 1960), sheep (Gosden *et al.* 1994; Salle *et al.*, 2002; Gutierrez *et al.*, 2003), rats (Dorsch *et al.*, 2007) and humans





(Donnez *et al.*, 2004). Progress for rare species is however likely to be slow and raise many welfare and ethical issues, because several aspects of the procedure (e.g., capture and invasive surgery to collect or replace ovaries) has the potential to harm the animal and is therefore unlikely to be justifiable except (possibly) for individuals that are overrepresented in the gene pool. Ovaries can however be collected, usually as a last resort, from dying or dead individuals as the follicles may remain viable and retain some developmental potential for some time after collection/death (Cleary *et al.*, 2001 Snow *et al.*, 2001). In the human most grafts are intended for autografting, but this is unlikely to be of much use for rare or endangered species. In laboratory rodents allografting is widely used, but in wildlife conservation work this approach will be complicated by issues of graft rejection and disease

transmission (Gosden, 2007; Petroianu *et al.*, 2007; Wei *et al.*, 2007). If problems of rejection can be overcome then cryopreserved ovarian tissues could potentially be an immense asset for rare and endangered species as it would let material be used largely independent of time and place (see Table 3). It would also allow material from one donor to be placed in multiple recipients to allow more rapid expansion of valuable genetic material in small or pocketed populations. However other issues will also need to be addressed before such technology is applied to threatened wildlife species. Feasibility issues will complicate the use of cryobanking for all large animal species (e.g., Rhinoceros, Elephant, and Pygmy Hippopotamus). It is also likely that as in Europe, policies governing how captive Zoo based animals can be propagated will become increasingly strict and will favor “hands-off” approaches.

Table 3. Outcome of cryopreserved parts of the ovary in *in vitro* or *in vivo* studies.

Cryopreserved part of the ovary	Outcome following <i>in vitro</i> culture	Outcome following auto or allografting	Outcome following xenografting
Isolated primordial follicles	Recruitment	?	Not done?
Isolated growing follicles	Recruitment	Birth	?
Ovarian cortex alone	Recruitment	Birth (sheep, human)	Stasis (in ovo) Recruitment Maturation
Whole ovary	Recruitment	Implant Maturation (sheep) Birth (mouse) Anastomosed: maturation (sheep) pregnancy (rat)	As implant Recruitment Maturation Births (after ART)
Oocytes from cryopreserved follicles	Term	NA	NA

Transplantation of ovarian tissue of one species to another (xenografting also known as heterografting) has been shown to support follicle development for a number of species including marmoset, elephant, hamster, dog, wallaby, wombat, cat, macaque, cow, pig, rabbit, goat (Paris *et al.*, 2004). Although the potential value of ovarian tissue xenografting for rare and endangered species has been recognized, its true value for rescuing female gametes of these species is not yet established. Snow *et al.*, (2002) succeeded in generating live young from xenografted ovarian tissue but this required IVF and embryo transfer, and it could therefore be anticipated to be a problem for any species in which hormonal stimulation, *in vitro* culture, IVF and embryo transfer protocols are not well established. In addition, this work was performed in a concordant xenogeneic model (mouse to rat) and it is not clear whether such successes can be booked across greater xenogeneic barriers. On a more positive note, the technology of ovarian xenografting allows one ovary, to be divided

into many small usable pieces. In the case of wombat ovaries approximately 60 pieces can be grafted and harvested (Paris, unpublished). This is of greatest value in species that form only a single, dominant, antral follicle per cycle. Ovarian xenografting has already proved to be an interesting model system which has allowed full sized oocytes and antral follicles to develop for a diverse range of species including wallabies, cats and humans (Gook *et al.*, 2001; Mattiske *et al.*, 2002; Fassbender *et al.*, 2007). Xenografting does have limitations and one of them is that the most versatile recipients found to date are mice and rats with non-functional immune systems (e.g., SCID and Nude strains). These recipients are normally housed in sterile, specific pathogen free, conditions (Nagy *et al.*, 2003) and it can be difficult to get clearance from the animal house manager or management committee to surgically graft material from animals of unknown disease status into the bodies of these animals. Although live young have been successfully derived from mouse ovarian tissue xenografted into Nude



rats (Snow *et al.*, 2002), this model is less appropriate for species which normally form very large antral follicles, species with prolonged folliculogenesis, and species whose endocrinological or physiological requirements are different to or possibly even incompatible with those of the graft recipient. Even in closely related species it is quite likely that xenografting will compromise the developmental of growing follicles and oocytes to some extent.

As stated above, there is a current lack of knowledge about the normality and fertilizability of oocytes harvested from xenografts. It is advised that further ovarian xenografting research should be done that starts by evaluating fresh tissue in a well studied model (e.g., mouse of cow) and compare the competency of any resulting oocytes with that of normal controls. Aspects which could be evaluated include mitochondrial status and glucose-6-phosphate-dehydrogenase activity of the oocyte, expression patterns of IGFBPs, and the steroid levels within the follicular fluid. This would give some insight whether the grafting procedure per se leads to oocytes with a decreased competency. An interesting study by Gook *et al.* (2003) showed that human ovarian tissue xenografted to mice could form antral follicles, but only if the recipient was regularly administered human FSH over a prolonged period of time. It is likely that many rare and endangered species would also benefit from exogenous support, but in their case it will not be known how much or what type of hormone will be needed, and thus for each species a specific stimulation protocol will need to be established.

Although *in vitro* culture and nuclear transfer can be applied to material from rare and endangered species these two approaches are even less well developed than ovarian grafting. Thus cryobanked ovarian material from valuable individuals of threatened species may have to remain in storage for the time being, while strategies for generating normal, fertilizable oocytes from this material are developed and improved. *In conclusion, in our opinion the most welcome advance however would be a capacity to prevent immune rejection without (aggressive) immunosuppressive drugs, as this would permit allografting which would ensure that follicles and oocytes could be grown in a physiologically compatible environment, unless it is shown that grafting across a xenogeneic barrier does not compromise the oocytes in any possible way.*

#### **Identified areas for further development to improve the outcomes of ovarian tissue cryopreservation and grafting**

##### *Reducing damage caused by ischaemia*

*We believe that, as summarized in the section above, current cryopreservation protocols can successfully and reliably preserve the female germ line.*

*Following warming this material can then be used to help restore fertility. We believe that outcomes could be improved by enhancing follicle survival, in particular that of the more advanced stages of follicular development.* There are several recognized factors that would potentially enhance overall survival including: A) optimizing equilibration and cryopreservation to account for cryoprotectant permeation, toxicity and osmotic effects (Krohn, 1977; Snow *et al.*, 2004), B) minimizing the ischemic damage that is associated with cryopreservation and grafting, and C) reducing losses through apoptosis in the follicles, vascular endothelium, and ovarian epithelium. Although the fertility of normal mice is often better than ovarian graft recipients the difference between recipients of fresh and cryopreserved ovarian grafts may be small or undetectable (Candy *et al.*, 2000; Shaw *et al.*, 2000a, c; Liu *et al.*, 2008; Wang *et al.*, 2008). The interpretation is that most damage is inflicted by the grafting rather than the cryopreservation step. Following grafting the revascularization commences within 24 hours in the mouse (Schneider-Kolsky, 1997) and rat (Dissen *et al.*, 1994) and can now be monitored *in vivo* by MIR (Israely *et al.*, 2004) and ultrasound (Fassbender *et al.*, 2007). Ischaemic injury and possibly also re-perfusion injury during this period of revascularization usually kills a substantial proportion of all follicles. Research has aimed at establishing how much damage is caused by ischaemia (Cleary *et al.*, 2001; Snow *et al.*, 2001; Schmidt *et al.*, 2003) and at evaluating whether ischemic damage can be minimized by growth factors (e.g., VEGF; Schnorr *et al.*, 2002); gonadotropins (Imthurn *et al.*, 2000), antioxidants (Nugent *et al.*, 1998), melatonin (Sapmaz *et al.*, 2003); the use of anti-apoptotic agents (Cummings, 2004), and location (Israely *et al.*, 2003; Yang *et al.*, 2006). It is important to realize that angiogenesis within the ovary is constantly required for follicle and corpus luteum growth and development (Reynolds *et al.*, 2000; Fraser, 2006) and that it is particularly adapted for this to happen (Israely *et al.*, 2003, 2004). The growth rate of the capillaries in the corpus luteum are outstanding as indicated by the fact that fully functional capillary networks traverse newly formed corpora lutea within days of ovulation, even though these structures are commonly 2 cm or more in diameter (Reynolds *et al.*, 2000; Fraser, 2006). Prominent putative angiogenic factors in the ovary include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor- $\alpha$  (TGFL $\alpha$ ), and the three mammalian members of the TGF/3 family (Dissen *et al.*, 1994). Pretreatment with compounds such as VEGF, which are known to stimulate neovascularization, has however not been associated with any substantial benefit in terms of restoration of graft function (Schnorr *et al.*, 2002). Administration of antioxidants such as vitamin A and vitamin C following grafting to reduce ischemia has proved effective in some but not all



models (Kim *et al.*, 1994; Nugent *et al.*, 1998; Cleary, 2004). Israely *et al.*, (2004) showed that transplantation into angiogenic granulation tissue (prewounded muscle tissue) improved graft vascularization and follicular survival with functional blood vessels detected within 2 days, at least 24 hours prior to intact controls. Our own (Shaw, unpublished) studies found that in mice some circulation was evident within 24 h for autografts placed on the kidney (a very rich capillary supply) and in the bursa, but was minimal at this time for subcutaneous grafts. This may, at least in part, explain why Yang *et al.*, (2006) who intensively studied the influence of orthotopic versus heterotopic grafting in mice found that graft site influenced both the number and quality of oocytes produced by ovarian grafts.

Many factors, including follicle number and type and graft site, influence the duration and efficacy of fertility restoration, and this makes it impossible to accurately predict the outcome of an ovarian tissue graft will be in terms hormonal and fertility outcomes for any given patient. As a consequence it is very difficult to establish guidelines for graft management. Further development of *in vivo* monitoring tools following grafting such as endocrine monitoring (Gosden *et al.*, 1994), but also ultrasound assessment of follicular growth (Fassbender *et al.*, 2007) are crucial to generate insights into the outcome of ovarian tissue following grafting and thereby determine how to best protect the ovaries throughout cryopreservation and grafting.

#### *Controlling the risk of disease transmission*

The risks associated with applying ovarian grafting to donors with cancers or disease were revealed in a study in which female mice died of cancer (lymphoma) shortly after receiving ovarian tissue from donors with cancer (Shaw *et al.*, 1996). This indicated that both healthy and malignant cells within the ovary could survive cryopreservation and be transferred within the graft. This is relevant to clinicians who chose treatment strategies for women with cancer. If these women, can have their ovarian tissue cryobanked before treatment they could potentially have this ovarian tissue return if the cancer therapy causes sterility (e.g. as a result of premature menopause). In these cases it is important to ensure that all material collected, before all cancer cells have been eliminated, are free of viable cancer cells. This is however only likely to be a major issue for women with cancers that are metastatic, or circulating via the lymph or blood, or located within the ovary itself. This has now been confirmed by research assessing the likelihood of transmission in the human (Meirow *et al.*, 2008) and has been accompanied by research to develop strategies to prevent transmission, such as isolation, purification, and *in vitro* culture of follicles (O'Brien *et al.*, 2003; Dolmans *et al.*, 2006). In animals the risk is less likely to be associated with cancers than infectious diseases, in particular when

ovarian tissues are recovered from non-captive animals and animals for which the cause of death is not known.

#### *Improving the capacity to retrieve normal, fertilizable, oocytes*

Although ovarian orthotopic grafting can permit conception without human intervention, there are instances where it is desirable to collect oocytes for investigation or to allow the use of other assisted reproductive technologies such as IVM, IVF or ICSI. One approach which simplifies is the use of heterotopic sites (Yang *et al.*, 2006) in combination with auto, allo or xeno grafting. Even though this approach can result in viable oocytes even after xenografting (Snow *et al.*, 2002) there are indications that outcomes at these sites may differ from those in the anatomically correct, orthotopic site (Yang *et al.*, 2006). Thus hormonal stimulation to trigger follicular development and oocyte maturation may be less effective or even ineffective for heterotopic grafts, and in these cases *in vitro* maturation followed by *in vitro* fertilization has to be used. Attempts to use longer periods of *in vitro* maturation are rarely used for cryopreserved material. In part this is because culture conditions are greatly inferior to *in vivo*, but the slow rate at which follicles develop and mature (either *in vitro* or *in vivo*) is also important. It takes a long time and very specific conditions for oogenesis and folliculogenesis, and many essential changes take place during this time. Essential findings include A) the revelation that oocytes undergo nuclear imprinting as they grow. This means that even though it is technically possible to move a nucleus from an immature oocyte into an enucleated mature oocyte it will not give rise to a normal fetus (Kono *et al.*, 1991). B) the culture of (non-cryopreserved) ovaries from newborn mice can give rise to fertilizable oocytes (at low efficiency) and live young, but care is needed to verify that the offspring are fully normal (O'Brien *et al.*, 1996). C) Non-frozen primordial oocytes can grow to full size and acquire appropriate imprinting patterns *in vitro* but could only form viable embryos through the use of micromanipulation to perform serial nuclear transfers into the cytoplasm of mature oocytes (Kono *et al.*, 1991). To date the mouse is the only species in which primordial follicles have been successfully grown from the primordial oocyte and the primordial follicle stage to maturity and then yielded developmentally competent embryos (Kono *et al.*, 1991; Eppig and O'Brien, 1996). Significant advances in the fields of *in vitro* culture and nuclear reprogramming are therefore possibly required before *in vivo* grafting can be omitted from strategies to produce mature oocytes from cryopreserved ovarian tissues.

#### *Controlling the recruitment of primordial follicles*

The ovaries of newborn human girls contain more than a million primordial follicles, the majority are



in developmental arrest, with some already recruited into the growth phase (Reynaud *et al.*, 2004). By the time menopause is reached at ~50 years of age (Elder *et al.*, 2008) there are few, if any, primordial follicles left. In normal females the decline results from a combination of recruitment into the growth phase and atresia and usually happens at a relatively predictable rate (Jones *et al.*, 2007; Westergaard *et al.*, 2007). Accelerated rates of loss (or low initial numbers as in Turner syndrome; Reynaud *et al.*, 2004) will result in premature ovarian failure (POF), but POF can also be caused by iatrogenic treatments including cancer therapy. It can be speculated that any treatment which would stop primordial follicles from becoming recruited, might be a powerful tool to preserve the fertility of cancer patients. The reasoning is that most but not all cancer drugs act by targeting, and killing, actively dividing cells. They therefore also target and kill the actively proliferating granulosa, cumulus and theca cells of growing follicles. Unfortunately there is currently no known way of stopping or even suppressing primordial follicle recruitment *in vivo*, and it may even be that recruitment increases when growing follicles are ablated thereby speeding up the onset of premature menopause. Giving GnRH analogs to downregulate the pituitary and ovary throughout the period of chemotherapy is claimed to have reduced the incidence of premature menopause in cancer patients (Blumenfeld, 2007). Although there is no evidence that this modifies recruitment, it could be speculated that downregulation may slow the death of recruited follicles by slowing their cells cleavage rate. Primordial follicle recruitment can be triggered by both *in vitro* culture and grafting and is particularly noticeable in small ovarian pieces with a limited number of follicles predominantly of the same type (e.g., ovarian cortex with only primordial follicles). At its most extreme this “induced” recruitment results in a large, essentially synchronous, cohort of growing follicles and a reduced reserve of non-recruited follicles and will in these cases significantly reduce the functional lifespan of the graft. Grafts with high follicle densities, including those from fetal or young donors may undergo “premature” recruitment but a sufficiently large number of follicles remain to support prolonged, or even completely normal, fertility (Candy *et al.*, 2000; Shaw *et al.*, 2000a, c). It is less clear what happens in whole, anastomosed ovarian grafts, but it seems likely that they will retain their normal (stable) rate of recruitment, providing that their growing follicles survive. It would in our opinion be valuable to establish the mechanisms that inhibit primordial follicle recruitment, in particular if this factor(s) could be isolated and used safely to inhibit recruitment *in vitro* and *in vivo*. The ultimate goal would be to slow or stop follicular recruitment for patients at risk of premature menopause (natural or iatrogenic), and for ovarian grafts. At present it is not known what does control primordial follicle recruitment

but rapid progress is being made through a combination of functional and genetic analysis of normal and genetically modified animals as well as *in vitro* cultured and grafted material (Holt *et al.*, 2006; Hutt *et al.*, 2006a, b; Knight and Glister 2006; Nilson *et al.*, 2006, 2007; Choi *et al.*, 2007; Dharma *et al.*, 2008).

#### *Improving the capacity to cryopreserve and graft whole ovaries*

To date most studies on ovarian cryopreservation have focussed on ovarian tissue pieces. Although this approach allows the germline to be preserved, and is convenient and versatile (in that individual pieces can be accessed at different time for different purposes) it may not be the most efficient way by which to restore fertility due to the ischemic damage that occurs in connection with transplantation. One way to reduce ischemic damage is to develop procedures which permit whole cryopreserved ovaries to be re-anastomosed to the blood supply of the recipient. The technical feasibility of whole (non-frozen) ovary grafting was demonstrated by researchers in the field of endocrinology (Goding 1966; Goding *et al.*, 1967a, b) who showed that ovarian autotransplants resumed activity with minimal interruption even at heterotopic sites. The first success with this approach for cryopreserved reproductive tissue was reported by Wang *et al.*, (2002a, b) who retransplanted portions of rat reproductive tracts (including the upper third of the uterus, the oviduct and the ovary). Following grafting, reproductive function was fully restored and pregnancy was observed, but only in a proportion of recipients. Since then whole ovary cryopreservation and grafting has also been evaluated for sheep (Arav *et al.*, 2005; Baudot *et al.*, 2007; Onions *et al.*, 2008).

Taken together, research in large animals has included the cryopreservation of ovarian cortex ranging in size from ~1 mm<sup>3</sup> to sheets of several cm<sup>2</sup>. After warming, this material has been either cultured *in vitro* or grafted into recipient(s). Cryopreserving small pieces will have some advantages over whole ovary cryopreservation including flexibility in both when and how that material is used (providing that the tissue pieces are stored in many separate containers). This approach can increase the potential for success as proven recently for a woman who did not achieve a term pregnancy until her second round of grafting (Andersen *et al.*, 2008). Small pieces can potentially be returned by a simple non-surgical method (e.g., to the arm; Wolner-Hanssen *et al.*, 2005) but follicle survival and development may be perturbed at heterotopic sites (Yang *et al.*, 1996). Whole ovary autografting is however a challenge. Goding (1966) and Goding *et al.* (1967a, b) showed that although their (non-frozen) ovarian autotransplants resumed activity with minimal interruption even at heterotopic sites, such locations could modify the subsequent dynamics of the hypothalamic–



pituitary-gonadal (HPG) axis. A subsequent study (on non-cryopreserved material) helps to emphasize the significance of the blood supply of the ovary within its normal orthotopic site, by showing that female ewes HPG axis could be massively perturbed solely by re-routing the blood vessel leaving the ovary (Garces *et al.*, 2000). In rats ovarian grafts that drained either into the liver or into the vena cava (greater omentum and retroperitoneum) resulted in differing estradiol levels, cyclicity, and ovarian cysts and corpora lutea (Petroianu *et al.*, 2005).

We now know that it is possible to establish pregnancies and obtain live young following orthotopic grafting of entire ovaries in conjunction with re-anastomosis of the ovarian blood supply (Wang *et al.*, 2002a, b; Baudot *et al.*, 2007). Although there is increasing interest in whole ovary cryopreservation and vascular re-anastomosis, a large proportion of the grafts fail (Courbiere *et al.*, 2007). We speculate that this may be because the cryopreservation procedures used on ovaries/ovarian tissues are derivatives of cryopreservation procedures developed for oocytes and embryos, and not somatic cells. They may well be suboptimal for somatic cells and in particular cell such as vascular endothelial cells which are critical for vascular integrity, maintenance of normal blood flow and clotting. A problem is that even grafts in which the vasculature has failed may restore fertility because of compensatory *de-novo* vascular ingrowth from the surrounding tissues (which means that the nominally intact graft is in fact equivalent to an implant).

### Conclusions

Ovarian cryopreservation is a research tool but is also used, alone or in combination with other assisted reproductive technologies, to preserve the female germline for a range of species, including humans. Attributes that have made ovarian tissue valuable for germ line banking include the ease with which it can be collected and cryopreserved, the diversity of donors and recipients that can be used, it usually preserves large number of germ cells, and the fact that it can be used to generate oocytes, embryos, and normal live young. Although a diverse range of cryopreservation and grafting procedures, are effective for ovarian tissue, optimizing methods to have greater post-thaw and grafting survival is still a priority. This review indicates that there remains considerable scope to improve the success, safety and versatility of both ovarian cryopreservation and grafting.

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