Untapped Reserves: Controlling Primordial Follicle Growth Activation

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Abstract

Even with the benefit of assisted reproductive technologies (ART), many women are unable to conceive and deliver healthy offspring. One common cause of infertility is the inability to produce eggs capable of contributing to live birth. This can occur despite standard-of-care treatment to maximize the recovery of eggs from growing ovarian follicles. Dormant primordial follicles in the human ovary are a 'reserve' that can be exploited clinically to overcome this problem. We discuss how controlling primordial follicle growth activation (PFGA) can produce increased numbers of high-quality eggs available for fertility treatment(s). We consider the state-of-the-art in interventions used to control PFGA, and consider genetic and epigenetic strategies on the horizon that might improve compromised oocyte quality to increase live births.

Keywords

cryopreservation; follicle; infertility; *in vitro* fertilization (IVF); oncofertility; ovary

Assisted Reproduction and the Supply of High Quality Eggs

Extraordinary advances have been made in the study and treatment of infertility in the last 40 years [1–4]. The first *in vitro* fertilization (IVF) birth in 1978 revolutionized the field of fertility treatment and since that time, overall IVF success rates have been improving steadily year after year [5,6]. Initially, IVF treatments resulted in a low successful pregnancy rate of 9%, and a delivery rate of 7% per egg retrieval procedure (see Glossary) [7]. Recent data from the United States of America report an approximate 38% live birth rate per retrieval; remarkably, this exceeds the fecundability of natural conception cycles in the

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general population on a per cycle basis [8] Access to Assisted Reproductive Technology (ART) services has also steadily improved, with treatments now available within most academic centers and an even greater number of private centers worldwide.

The abovementioned clinical progress has been accompanied by steady progress in our understanding of ovarian physiology and the endocrinology of female fertility. For example, optimization of the amount of injectable gonadotropin medications used during the so-called "controlled ovarian stimulation" prior to a given egg retrieval has been studied exhaustively [9–11]. The number of eggs retrieved is a key parameter within IVF cycles, both as a practical necessity for treatment and a measure of "ovarian response." Historical studies and more recently, regulatory requirements for clinical reporting to large databases, e.g. the Society for Assisted Reproductive Technology Clinical Outcome Reporting System (www.sart.org) [12] in the US have resulted in a more precise accounting of IVF statistics. These data suggest that stimulation is approaching its limits in terms of maximizing the chances of conception and pregnancy within an IVF cycle [9–11]. An additional practical limit for gonadotrophic stimulation for some women is the risk of the adverse reaction termed ovarian hyper-stimulation. Patients that respond to stimulation in this fashion can exhibit major fluid/electrolyte imbalances, blood clots or other severe outcomes that might place the patient and her chance of pregnancy at risk [13].

It is commonly accepted that the greater the number of eggs retrieved, the greater the chances of generating one or more embryos that meet the criteria for transfer back into a patient's uterus. However, the critical factor is that at least one egg needs to be of sufficient quality to give rise to a successful pregnancy. Thus, increasing the supply of high quality eggs for use in assisted reproduction attempts is a desirable goal for all patients, and particularly, for those faced with contraindications that limit this supply. After discussing the profiles of particularly difficult-to-treat IVF patients, we discuss how very recent advances might eventually be applied to enable overcoming infertility based on increasing the number of high quality eggs.

Two large patient sub-populations known to have reduced chances of pregnancy and live birth are those referred to as "advanced maternal age" and "diminished ovarian reserve (DOR)" [14,15], each of whom usually produce very few eggs during treatment. These patient sub-populations are discussed as follows. It is well-known that fertility decreases in women with age. The likelihood of conception per unit time is relatively stable between the onset of puberty and a woman's early thirties. "Advanced maternal age" is typically assumed to start around age 35, and soon after, oocyte quantity and quality can be seen to decline precipitously [16]. At about age 37, several parameters of egg developmental competence have been shown to change significantly [17,18], including markers of cytoplasmic and chromosomal competence that begin to favor aberrant development and chromosomal segregation after fertilization [19,20]. This can manifest in eggs that are developmentally incompetent even if successfully fertilized (for individual or an entire retrieved cohort of eggs). Because many women might seek to delay pregnancy and childbirth to fit lifestyle choices and needs, often until their late thirties [21], the population faced with overcoming poor egg quality will likely increase.

Women with DOR are also characterized by low indirect markers of the "reserve" of ovarian primordial follicles [14]. These markers include serum levels of Antimüllerian hormone (AMH; commonly accepted threshold level < 1ng/ml) or, elevated serum levels of Follicle Stimulating Hormone (FSH; thresholds differ but > 10 IU/ml is commonly accepted) [22], measured on day 2 or 3 of the menstrual cycle. DOR diagnosis is not by definition, age-specific and can be seen in young women attempting to conceive; commonly interpreted as evidence of accelerated ovarian aging characterized by an accelerated loss of primordial follicles. In addition, DOR patients often respond poorly to gonadotropin ovarian stimulation during IVF cycles [14,15]. Critically, exposure to certain chemicals, including chemotherapeutic agents, or ovarian surgery, can result in significant depletion of the ovarian reserve, leading, again, to accelerated ovarian aging [13,24],. General terms for capturing optimal ovarian health at a given time include "fertility preservation" and "oncofertility" [23]. All of the above features of DOR patients correspond to a reduced chance of conception and live birth [25,26], both spontaneously and with assisted conception. Women of advanced age, or, those diagnosed with DOR that have failed assisted reproduction treatments, are often counseled to consider egg donation programs, precluding their ability to give birth to their own genetic offspring.

A central question for the field is therefore determining a means to increase the number of available high-quality eggs for all women undergoing IVF, but especially those with advanced chronological age, DOR, or for women with low yield of oocytes during a given cycle of treatment. An increasingly plausible strategy may be to access dormant primordial follicles within the ovarian cortex (Figure 1). Specifically, during normal ovarian function within the menstrual cycle, a small number of primordial follicles begin to grow daily, supplying the ovary with its population of growing follicles [27,28]. The vast majority of follicles will die within the ovary during normal reproductive cycling in a process referred to as atresia [29,30], usually leaving only a single dominant follicle [31] that survives to ovulate a mature, fertilization-competent egg. The above-mentioned hormonal stimulation strategies are largely relevant to this growing proportion of follicles. The remaining dormant population of ovarian follicles [32]-- including the primordial pool of follicles -- is currently being probed for their potential clinical utility. Of relevance, primordial follicles of many species, including humans and mice, remain growth-arrested, even if isolated and placed into "permissive" tissue culture conditions [33]. Thus, overcoming the signaling cues that enforce growth-arrest of primordial follicles may be a critical step to clinically exploit this pool of ovarian follicles.

When considering possible fertility treatments, the isolation of primordial follicles from the ovarian cortex (in addition to non-growing, transitional and primary follicles [32]) might offer several advantages in terms of numbers and accessibility. For instance, the number of primordial follicles exceeds the number of growing follicles until very late in reproductive life [32]. The human ovary is organized into distinct cortex and medullary layers (Figure 1) [34]. The cortex is a thin layer of fibrous, dense tissue analogous to the peel of an orange, and this is where most primordial follicles are found [34]. The medullary region, akin to the fruit within the orange peel, mostly consists of stromal tissue surrounding blood vessels, lymphatic ducts, and connective tissue, contiguous with the ovarian ligament/hilus. Because the primordial follicles are superficial within the organ, it is relatively trivial for an

experienced practitioner to collect cortical tissue *via* a minimally invasive, laparoscopic approach, and a simple scalpel "shaving" of the surface. Also, human ovarian cortex is highly compatible with cryopreservation and particularly, vitrification, so biopsied tissue can be stored for long periods of time prior to thawing and use [35–37]. Depending on the overall primordial follicle content in an ovary, anywhere from a few to tens of primordial follicles can be present in a single cortical piece approximately 10 mm x 10 mm x 1 mm deep [33,38]. Such collection and preservation of cortical tissue is now being routinely performed for cancer patients, envisioning that mature eggs might be one day produced for fertilization purposes [23,24]. We expand upon existing workflows and additional experimental strategies below.

Clinical Strategies and Workflows

In vivo: Cortex removal, preservation, and replacement

One clinical strategy that has been used successfully is the collection, cryopreservation, thawing, and surgical re-delivery of ovarian cortex tissue to the surface of a patient's ovary [39]. Patients newly diagnosed with cancer may not be able to take advantage of an IVF cycle in order to cryopreserve oocytes or embryos for fertility preservation prior to undertaking gonadotoxic therapies, due to either the time-sensitive need for treatment, or the clinical need to avoid gonadotrophic stimulation. However, in these situations, removal and cryopreservation of ovarian cortex pieces might be undertaken. Re-delivery of pieces of ovarian cortex to the surface of the ovary after patient recovery (process described as orthotopic transplantation; [40]) has resulted in the resumption of menstrual cycling for predictable lengths of time, and has led to a growing number of live births, both due to natural conception and IVF [41]. The length of time that menstrual cycling returns is generally thought to be directly related to the number of primordial follicles delivered in the graft(s). This first strategy can be executed while also using pharmacological agents shown to induce the growth activation of primordial follicles (see below). A second, less mature strategy for the use of patient ovarian cortex avoids the need for surgical re-delivery of tissue and instead, relies on *in vitro* tissue and follicle culture.

In vitro: Cortex removal followed by tissue and/or follicle culture

Individual primordial and other small growing follicles can be isolated from fresh or frozen-and-thawed pieces of human ovarian cortex. A recent study reported what appears to be the highly efficient recovery of small follicles from cortical pieces $60 - 300$ mm^3 in volume. Treatment with liberase and DNase I enzymes in combination with mechanical filtration of the disrupted cortex was undertaken [42]. Compared to an earlier method reported by the same group [43], the newer improved method [42] increased both the number of isolated intact follicles (e.g., follicles with central, non-extruding oocytes), and the viability of isolated follicles (96%). Following isolation, some follicles were embedded within fibrin clots -- a biological matrix the authors had previously shown to be compatible with follicle survival and growth [44] In this case, clots were grafted under the ovarian bursa of immunocompromised mice and examples of surviving human follicles were noted 7 days after transplantation. Primordial follicles were shown to maintain growth arrest after survival in the grafts, showing that the technique can maintain the follicles' physiological state of

either growth or arrest, even 7 days after isolation, handling, and grafting into the host animals [42]. This means that primordial follicles can withstand impressive isolation and re-location while maintaining their growth arrest and potential to support ovarian function over extended time periods (see below).

Several other biomaterials have also been shown to be compatible with follicle survival *in vitro*. Specifically, three-dimensional collagen [45–47], hyaluronan [48], or alginate [49,50] matrices have each been used to house follicles as they grow *in vitro*; these matrices can improve morphological features of follicle growth relative to those grown directly in culture dishes. For instance, alginate has been used to grow rhesus monkey follicles successfully [51,52]. Beyond providing scaffolding for follicles to grow within, these biomaterials can be modified in terms of their concentration (and thus permeability), as well as mechanical rigidity to improve follicle survival and proper development [51]. Further, different extracellular matrix or other macromolecular components might be mixed within these materials in ways that might enhance follicle development and improve the chances of producing mature eggs [53], an area warranting further investigation. Overall, however, the field has advanced to a point where attempts are being made at generating "artificial ovary" tissues [42,52] where many isolated human follicles can be aggregated within a biomaterial scaffold. With the intent of increasing the number of follicles available to support ovarian function, and possibly, fertility, such aggregates might be re-introduced *in vivo* to the surface of a dormant ovary lacking significant immature follicles (unlike cortex removal, preservation, and replacement; see above).

A second technique for the *in vitro* culture of follicles is referred to as a 'two-step' approach. [33,38]. First, intact pieces of ovarian cortex are cultured until a single growing follicle of sufficient size is noted. Subsequently, the growing follicle can be isolated from the surrounding cortical tissue for further culture, possibly within one of the biomaterial matrices mentioned above. One study showed that such a two-step procedure could result in the generation of large antral follicles [33,38]. Specifically, this technique has the benefit of allowing follicles to initially develop in their native ovarian tissue, in some ways mimicking *in vivo* conditions. However, a large number of immature follicles are lost to atresia during the first stage of cortical strip culture [33]. It remains to be seen whether the relative advantages and disadvantages of culturing follicles singly or using the two-step approach ultimately impact the quality of mature eggs produced. Regardless of the culture method used, inducing the growth and development of primordial follicles seems likely to open an 'untapped reserve' of high quality mature eggs for patients, and this is an area that merits further investigation.

Mechanistic Control of Primordial Follicle Growth Activation

Primordial follicle growth activation (PFGA) is the process by which individual primordial follicles leave their dormant phase and enter a growth phase that can culminate in the development of a mature peri-ovulatory follicle containing a nearly mature egg [28]. As mentioned, clear mechanistic information is available on how primordial follicles stay in this growth-arrested state for as long as 35 years within the ovary, and even remaining growth-arrested when placed into *in vitro* cultures. A "summary" model of the signal transduction

and gene expression pathways regulating PFGA, in conjunction with a table of additional factors shown to impact PFGA, are illustrated in Figure 2.

c-Kit/Kit Ligand (KL) signaling was one of the first pathways identified as a PFGA regulatory mechanism. C-Kit is a receptor tyrosine kinase, activated by its ligand KL [54]. Disruption of this pathway using the *Steel panda* mutant mouse that expresses low levels of Kit ligand [55], or, *via* use of a c-Kit blocking antibody [56], each were found to result in enhanced arrest of primordial follicles and a reduction in the number of small growing follicles [55,56]. A second key regulatory molecule was discovered some time later, where forkhead transcription factor *FoxO3* knockout mice were shown to exhibit the opposite effect: a total commitment of primordial follicles to growth (depletion of the entire primordial follicle population) by 2–3 months of age was detected in histomorphometric studies, while in contrast, wild-type controls maintained ovarian function for the normal duration [57]. This suggested that FoxO3 was responsible --in large part -- for keeping primordial follicles growth-arrested [57].

Other central regulators of PFGA include the lipid phosphatase Phosphatase and tensin homolog deleted on chromosome ten (Pten), and its downstream proximal substrate, protein kinase Akt [28,60]. Pten and Akt are well-known for their role(s) in controlling cell growth and survival during normal cell and tissue function and as dysregulated in cancers [58]. Their action relies upon downstream regulation of mechanistic Target of Rapamycin (mTOR) activity (Figure 2, blue boxes), and also upon the regulation of nucleo-cytoplasmic FoxO3 transport. High Pten activity downregulates Akt due to action upon Pi3 kinase (Pi3k), resulting in low downstream mTOR activity. This culminates in blocked cell cycle progression (*via* p70s6 Kinase [p70s6k]) and limits upon protein translation (*via* Eukaryotic translation initiation factor 4E-binding protein [4E-BP]). High Pten activity also corresponds to hypo-phosphorylation and nuclear localization of FoxO3, where it can activate the transcription of genes consistent with cell cycle arrest, including *Cyclin dependent kinase inhibitor 1b* (*Cdkn1b/p27Kip1*) [59]. Conversely, low Pten activity results in upregulated mTOR and hyper-phosphorylation and nuclear export of FoxO3, relieving post-translational (mTOR) and transcriptional (FoxO3) enforcement of growth inhibition and allowing PFGA. [59] Accordingly, blocking PTEN activity and/or activating AKT have been shown to increase growth activation of primordial follicles both experimentally and in the clinic (see below).

Pharmacological inhibition of the Pten enzyme using the Pten-specific inhibitor (and indirect Akt activator) bisperoxovanadium bpV(HOpic) (bpV(pic)) during *in vitro* mouse primordial follicle and ovary cultures [60], and separately, oocyte-specific genetic loss-of-function *Pten* deficient mice[61–63], each have been shown to result in significantly increased PFGA, producing more growing follicles than (and reduced numbers of primordial follicles compared to) corresponding controls. Translating these findings to human primordial follicles, including an attempt at the treatment of infertility followed soon thereafter.

As seen in the mouse models, it was possible to induce growth activation of primordial follicles in human frozen-and-thawed ovarian cortical tissue [64,65]. In this experimental treatment paradigm, PTEN inhibition/AKT activation using bpV(HOpic) was combined with

an ovarian cortex fragmentation approach to offset the growth-inhibitory Hippo signaling pathway [65]. A cohort of 27 amenorrheic patients was selected that had a mean duration of amenorrhea of nearly 7 years. Isolated cryopreserved-and-thawed ovarian cortex from these patients was cut into small cubes and treated with AKT activators and 740YP *in vitro*. Cubes of tissue were then surgically auto-transplanted back to the Fallopian tube serosa of patients, and the graft sites were monitored for follicle growth *via* ultrasound. Eggs were successfully retrieved from five patients, and following fertilization by intracytoplasmic sperm injection (ICSI), one live birth was achieved [65].

This remarkable finding suggests that indeed, inducing PFGA in primordial follicles that would otherwise remain dormant in women with POI may be a plausible strategy to increase the chances of a successful pregnancy and live birth. Despite this outcome, a cautionary set of data was published soon afterwards that showed that inhibition of PTEN by bpV(HOpic) can result in poorer survival of immature follicles in human ovarian cortex cultured *in vitro* [66]. The live birth outcome for even one of these previously amenorrheic patients, however, seems to justify a larger clinical trial of this strategy on pregnancy outcomes.

It is also worthwhile to consider additional secreted and other extracellular factors that have been shown to impact PFGA. These might be added to culture systems or measured in patients to determine how their concentration(s) might impact the accessibility of primordial follicles. A recent hypothesis article that evaluated and interpreted the action of AMH [67], a list of activators [55,56,68–82] and repressors [67, 83–86] of PFGA was provided. Figure 2 extends this informative list, organizing effectors based on available experimental evidence for their action on PFGA— either genetic (usually loss-of-function) models, *in vitro* culture experiments, or both.

The Physiological Clock that Controls PFGA

As described, much is now known of key signals that enforce the physiological growth-arrest of primordial follicles. Loss-of-function of negative regulators such as *Pten* [60–62], *FoxO3A* [57], and the mTOR protein effector *Tuberous sclerosis 2 Tsc2* [87] in mouse models results in vastly accelerated PFGA and exhaustion of the primordial pool of follicles early in life. However, identifying these negative regulators has only provided clues about how the steady rate of PFGA is achieved during the normal physiological lifespan. To us, this is the major remaining question in the field. How can seemingly equivalent primordial follicles behave so differently from one another in humans, where growth-activation in some follicles can occur as far as 35 years apart (see Outstanding Questions)? In a thought experiment, might isolated human primordial follicles placed into tissue culture last years without growth-activation, finally undergoing PFGA according to an internal "clock?"

As discussed, external physiological factors can impact the likelihood of growth activation of primordial factors. One such molecule is AMH, the same molecule used as a serum measurement to indirectly estimate the ovarian reserve [67,83]. *Amh* knockout mice [83] show an acceleration of PFGA, characterized by a decline in primordial follicle number and an increase in growing follicles at different postnatal time points. While these data point towards a definite impact of Amh upon mouse PFGA, AMH's mechanism of action upon

primordial follicles is not entirely clear at this time, as while the AMH receptor AMHR2 can be detected in human primordial follicles [88], the orthologous receptor has been shown to be lacking in rat primordial follicles [89]. This suggests that AMH might partially suppress PFGA indirectly in some species but not others.

Four additional models where the duration of ovarian function is significantly extended have been established in the mouse. Dietary supplementation with omega-3 fatty acids [90] or coenzyme Q10 [91], or alternatively, systemic (intraperitoneal) delivery of the mTOR inhibitor Rapamycin [92,93] have all been shown to extend ovarian lifespan in mice. In a transgenic mouse model (*Fshb*$^{-/-}$ *HSFHB*^{Mut}) where FSH is genetically "rerouted" in such a way that its expression is pulsatile due to fusion with a carboxyterminal Luteinizing Hormone motif, ovarian lifespan also appears to be significantly extended relative to controls [94]. In all four distinct models, the extension of ovarian function appears to result from a slower rate of PFGA and not a reduction in follicle atresia. Whether or not these models involve regulation of a molecular "clock" that can control the rate of PFGA over time, these are valuable lessons when considering how to regulate the process in patients in the future.

Concluding Remarks

Reproducible, near-term strategies for increasing the number of healthy offspring-competent eggs in model systems have been discussed. While some of these strategies have been successfully translated to human interventions, there is much room for improvement. When considering future perspectives for the treatment of human patients, initial attempts should be made to improve the quality of eggs produced during current stimulation-and-retrieval protocols. Because only one (or possibly, a few) high quality eggs are necessary per IVF cycle to produce an offspring, improving egg quality so that those few high-quality eggs are produced instead of *none* is the first priority.

A new strategy consists of the inclusion of inhibin-blocking antisera in ovarian stimulation entry (during stimulation cycles). In mice, this strategy vastly increases offspring-competent oocyte yield due to the prevention of ovary-derived inhibin and its negative feedback on pituitary FSH production [95,96]. Separately, the same mouse model of pulsatile FSH expression shown to extend ovarian lifespan (above, [94]) also exhibited significantly improved oocyte yield after gonadotropin stimulation. It is not yet known whether either blocking inhibin or delivering FSH in a pulsatile fashion can similarly result in increased oocyte yield in human patients with a normal ovarian reserve, let alone a limited reserve of follicles. Thus, we return to consider the primordial follicles found in human ovarian cortex and how this reserve might be exploited.

Given increased mastery of ovarian cortex cryopreservation and handling, it should be possible to more precisely control PFGA, follicle survival, and mature egg production from frozen-and-thawed tissue. Technical hurdles remain, the first of which is the attrition of immature follicles after only a few days in culture in two-step culture protocols [33,97]. How such attrition might be overcome is presently unclear. Isolating viable immature follicles from ovarian cortical tissue is still technically challenging, but improving methods

[42,97] might be bringing this closer to routine clinical performance. Some combination of cortical tissue culture followed by dissociation, isolation of individual follicles, and subsequent single follicle culture might eventually contribute to increasing the number of available mature eggs. Basic mechanistic information gained from these studies should also shed light on how the rate of PFGA is regulated over the adult reproductive lifespan of humans.

Beyond the technical challenges of tissue and follicle handling, a critical question to address is that of intrinsic egg quality (See "Outstanding Questions", box 1, and also [30]). Can oocyte quality be compromised even as far back as the primordial follicle? In the short term, it may be that no intervention designed to isolate mature eggs can overcome what is a fixed poor quality of eggs within certain, and potentially all, follicles [29]. Because some women are never able to produce healthy offspring, and other women lose their ability to do so with age, overcoming this problem may require early intervention, perhaps by manipulating the eggs within immature follicles.

The time that follicles are placed within *in vitro* culture can be exploited for intervention using powerful new techniques that modify the genetic and epigenetic state(s) of eggs. Injection of the egg within the growing mouse follicle has been successfully performed in multiple laboratories [98,99], showing that macromolecules can be delivered in a way that is compatible with egg and follicle survival. It is a short leap to the delivery of CRISPR/Cas9 and guide RNAs [100], or, modified enzymes that target DNA methylation enzymatic activity to the genome at precise sequence-directed location(s) [101–103]. Proof-of-concept delivery of CRISPR/Cas9 reagents into human one-cell embryos (post-fertilization), or, mature eggs (injected with the sperm cell during ICSI) has been demonstrated with detailed information available regarding embryo viability, targeting efficiency, and embryonic mosaicism where only a subset of embryonic cells harbor the genomic alteration or epigenetic mark [100]. One might anticipate delivery of these reagents to eggs inside follicles grown *in vitro* with the aim of improving the likelihood of survival and production of an offspring-competent egg, perhaps being able to correct specific genetic abnormalities found to compromise egg development (Figure 1).

Follicles cultured *in vitro* to produce mature eggs starting with the primordial stage could therefore offer a broad window of access and control, improving fertility and also reducing disease in offspring. In this way, the limitation of the number of eggs available for conception should be possible to overcome for all but those patients with the fewest remaining primordial follicles. For even those patients with zero available primordial follicles, options may lie on the more distant horizon. For example, the production of mature, fertilization-competent eggs entirely *in vitro* using embryonic stem cells or induced pluripotent cells (iPSC) cells, has been achieved in mouse models [104]. A careful stepwise process of iPSC generation from somatic cells, followed by *in vitro* stem cell differentiation, *in vitro* growth (of cells specified as oocytes), and *in vitro* oocyte maturation, resulted in the production of normal metaphase II eggs capable of giving rise to offspring [104]. Generation of patient-matched, high quality mature eggs for human infertility treatment using iPSC is daunting, but should be plausible given time and attention from the field.

While all of the possible future directions mentioned here have varying degrees of difficulty, most are likely to be reached in the next several years given the demand-driven, rapid pace of progress in reproductive and molecular genetic techniques. The rate-limiting step(s) appear to reside at the level of tissue handling and possible, inherent biological limitations of the primordial oocytes themselves, rather than the availability of molecular tools and creative clinical/translational researchers working in this exciting area.

Acknowledgments

Glossary

Amenorrheic (amenorrhea)
The absence of menstruation for one or more months, indicative of the loss of reproductive cycling.

Antimüllerian hormone (AMH)
(or Müllerian Inhibitory Substance (MIS)) is a TGFβ family peptide hormone produced by small growing mammalian ovarian follicles to repress primordial follicle growth activation. Serum AMH concentration is used clinically as an indirect measure of the ovarian reserve, where < 1 ng/ml is viewed as indicative of **Diminished Ovarian Reserve**.

Antral follicles
Ovarian follicle whose growth and development has resulted in the acquisition of a fluid-filled cavity termed the antrum. Antral follicles contain multiple layers of cuboidal, proliferative granulosa cells and a growing, maturing oocyte.

Atresia
death and involution of the ovarian follicle structure in a process that includes hallmarks of granulosa cell and oocyte apoptosis. The vast majority of ovarian follicles will die *via* atresia, while only a small fraction survive such that a single egg is ovulated each menstrual cycle.

Egg Retrieval
The clinical procedure whereby human eggs (within cumulus granulosa cells) are collected. Egg retrievals usually occur as outpatient procedures, using local anaesthetic/analgesic treatment to manage the ultrasound-guided transvaginal puncture of follicles and suction at the surface of the ovary.

Embryonic Mosaicism
Often referring to the blastocyst stage, refers to an embryo composed of cells of differing genotypes. The unique genotypes often consist of cells whose chromosomes have been segregated correctly to daughter cells after cell division (euploid cells) and cells whose chromosomes have been segregated inappropriately/unequally (aneuploid cells). In the case

of genomic editing of embryos, embryonic mosaicism can occur when only a subset of cells carry the desired alteration.

Diminished Ovarian Reserve (DOR)

A clinical diagnosis characterized by evidence of advanced ovarian aging independent of chronological age, including elevated FSH and reduced AMH relative to same-age fertile women. Women with DOR often produce low numbers of eggs during IVF cycles.

Fertility Preservation/Oncofertility

Egg and embryo freezing, as well as ovarian cortex freezing are all core strategies for female fertility preservation. After thawing, each can be used in later attempts at conception.

Gonadotoxic Therapies

Therapeutic treatments with an inordinate impact on gonadal function, often due to germ cell toxicity. Both female and male germ cells are known to be sensitive to radiotherapy and specific chemotherapeutic agents. If the standard-of-care or preferred treatment (for example, for a life-threatening disease) for a condition is known to place a patient at risk for the loss of ovarian function, Fertility Preservation/Oncofertility strategies can be considered.

Growing Follicle

an ovarian follicle that has committed to a growth phase consisting of an increase in oocyte volume and an increase in the surrounding population of granulosa cells due to proliferation. Growing ovarian follicles eventually acquire a fluid-filled cavity termed the antrum as they approach ovulatory size.

Ovarian hyperstimulation (Syndrome - OHSS)

A condition that occurs almost exclusively after fertility treatments (most often after IVF) when the ovaries get markedly enlarged and produce fluid shifts from the intravascular space to the abdominal cavity (so called "third space").

Ovarian Stimulation

the standard-of-care for *in vitro* fertilization cycles, where gonadotropin hormones (Follicle Stimulating Hormone [FSH] and Luteinizing Hormone [LH]or analogues in various formulations) support the growth and survival, then recovery of a cohort of growing ovarian follicles. Inclusion of **Inhibin blocking antisera** in mouse stimulation protocols ("superovulation") abrogates endogenous suppression of FSH by ovarian inhibin, resulting in significantly increased oocyte yield.

Primordial Follicle

a dormant ovarian follicle consisting of an oocyte surrounded by a few non-proliferative pre-granulosa cells. Compare to "**Growing Follicle**."

Primordial Follicle Growth Activation (PFGA)

The primordial follicle consists of a few squamous ("flat") pregranulosa cells surrounding a primordial oocyte. Upon PFGA, the pregranulosa cells change to a cuboidal appearance and begin to proliferate, and the oocyte begins to grow and mature.

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Highlights

- Women that are currently considered infertile due to poor egg quality or are at risk of ovarian failure may have new options. Ovarian cortex can be isolated, cryopreserved and re-delivered to the ovary whereupon ovarian function might resume.

- The ovarian cortex tissue is also currently being used in attempts to produce mature fertilizable eggs *in vitro*.

- Mechanisms that control primordial follicle growth activation (PFGA) have been identified and used to induce follicle growth in mouse models (genetic and *in vitro* follicle cultures) and also in experimental human clinical interventions.

- *In vitro* follicle culture offers control over follicle development (and access to the follicles themselves from early developmental stages) so that modern genetic/epigenetic tools might be used to improve egg quality. For example, genome editing (e.g via CRISPR/Cas9) reagents might be injected into growing follicles to correct defects compromising egg quality. This type of technology also aims to correct disease states in offspring; however, these newer approaches in reproductive medicine, although promising, are still in their infancy.

Box 1

Clinician's Corner:

- During the normal menstrual cycle, small cohorts of "resting" primordial follicles are recruited into a growth cycle; most are destined for atresia while typically only a single dominant follicle survives to ovulation

- Oocyte quality and quantity declines precipitously with age; women of advancing age are at risk of "diminished ovarian reserve" (DOR), with decreasing chances of conception and live birth over time

- Certain cancer treatments and other treatments for non-malignant conditions (often medical or radiological) can damage the reserve of primordial follicles within the ovary, accelerating ovarian demise. Fertility preservation/ oncofertility options are becoming more widespread for patients faced with these treatments so that attempts at conception can occur after patient recovery. Eggs, embryos, and/or ovarian cortex can be cryopreserved for fertility preservation purposes.

- Actionable information about the physiological control of primordial follicle growth activation, or, "recruitment" is available from studies of model systems and human ovarian cortex. Translational studies seeking to support fertility by activating dormant primordial follicles are being performed

- The population of dormant follicles may represent an important target for experimental clinical strategies designed to increase the quantity of oocytes available for use in assisted reproduction treatments

Outstanding Questions

- **What regulates the "clock" that controls regular PFGA during the human/mammalian reproductive lifespan?** Despite an improved understanding of the mechanisms that can either activate or repress PFGA, little is known about the regulation of PFGA rate time during reproductive maturity. Experimental models that extend the ovarian lifespan are beginning to provide clues in this area.

- **Is poor egg quality established as early as the primordial follicle, or can it be corrected?** Because some women produce only poor-quality eggs that may be incompatible with offspring production, it is currently unclear whether any intervention can improve their situation. With the assumption that it may be possible to gain control of the continuum of follicle development, enabling the growth and maturation of primordial follicles, we may potentially overcome the problem of poor egg quality, provided that oocytes are not already compromised beyond "correction."
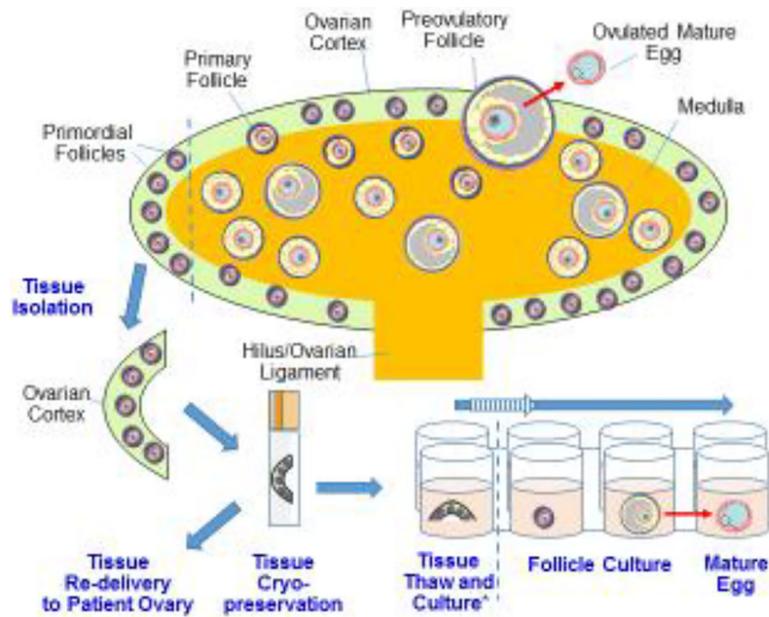
Figure 1. Human Ovarian anatomy and tissue handling for fertility support and preservation.
A cartoon representation of the human ovary, its organization, and its constituent follicles is shown. The exterior cortex (green) is the location of most if not all primordial follicles, while growing follicles are found more centrally within the medulla (orange). Once per cycle, a follicle survives to ovulate (top, red arrow). Clinical access to the primordial reserve of follicles can be gained by surgical removal of "strips" of ovarian cortex (left, TISSUE ISOLATION). Cortical strips can be cryopreserved, and optionally thawed and re-grafted to a patient's ovary, whereupon follicle growth can resume and conception might follow [33–36]. Alternatively, tissue can be thawed and placed into a tissue culture scheme (bottom right). In a 'one-step' system, follicles are immediately isolated and cultured singly. In a 'two-step' system (*), cortex strips are first cultured until a growing follicle is visible, after which the growing follicle is mechanically isolated for further culture. Ideally, optimization of follicle culture will result in the production of a mature, offspring competent egg (bottom right, red arrow). Note that for each stage of tissue handling and culture *in vitro*, follicle-enclosed eggs are accessible for treatment, including the possible injection of reagents designed to improve egg quality.
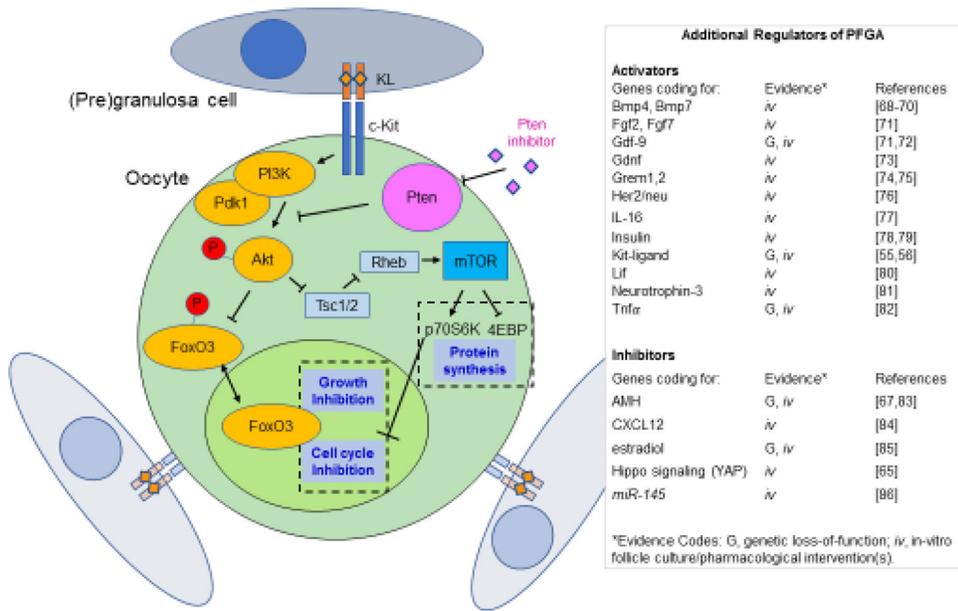
Figure 2. Molecular regulation of PFGA in Mice.
The cartoon on the left depicts primordial follicles consisting of a primordial oocyte and a few pregranulosa cells stay dormant due to the negative regulation of cell growth and the cell cycle by transcription factor FoxO3, and negative regulation of the mTOR signaling pathway, both controlled by the upstream action of Pten [69,93,105]. Accordingly, pharmacological inhibition of Pten using molecules such as bpV(HOpic) can result in enhanced PFGA. FoxO3 has been shown to inhibit the cell cycle via regulation of Cyclin dependent kinase inhibitor 1b (Cdkn1b/p27). Oocytes with low mechanistic Target of Rapamycin (mTOR) activity are likely to be held in a state of low protein synthesis due to a lack of phosphorylation of the mTOR target Eukaryotic translation initiation factor 4E-binding protein (4-EBP), remaining out of an active cell cycle due to activity of P70S6-kinase (P70S6K), itself a positive regulator of cyclin D1, cyclin dependent kinase 4 (CDK4), and Retinoblastoma (Rb) proteins [106]. The table on the right presents an updated list of genes and factors shown to impact PFGA, with experimental evidence categorized by *in vitro* and/or *in vivo* genetic evidence. Cartoon adapted from [105].