In Vitro Maturation of Mammalian Oocytes

John J. Bromfield, Katie L. Jones, and David F. Albertini

Abstract Efforts to optimize oocyte quality as a result of *in vitro* maturation (IVM) are critical to achieving patient-specific success in assisted reproductive techniques. Traditional approaches to human IVM have been replaced by methodologies aimed at recapitulating the changing milieu of the ovulatory follicle following reception of signals initiated by LH. These include the deployment of sequential media changes that more accurately reflect the temporal shift in balance between biosynthetic and metabolic alterations that occur within the cumulus cells and their enclosed oocytes. Distinctions in cumulus cell physiology and gene expression in an *in vivo* and *in vitro* context are likely to serve as useful noninvasive biomarkers for the developmental potential of human oocytes that complete nuclear and cytoplasmic maturation under ex vivo conditions.

Keywords Oocyte · Germinal vesicle · Metabolic cooperation · Junctions · Cumulus oocyte complex cytoskeleton · Meiotic spindle · Chromosomes · Cell cycle

1 Introduction

The link between oocyte quality and the developmental capability of an embryo has long been appreciated. But exactly what properties in the oocyte, both prior to and following maturation, confer developmental competence to the conceptus has evaded rigorous definition until recently. What has become clear is that a protracted series of molecular and cellular modifications must occur during both the growth and maturation stages of oogenesis in mammals to realize successful preimplantation embryogenesis (1). While achieving ex vivo oogenesis is a novel and much sought after paradigm within emerging assisted reproductive techniques (ART), manipulating the final stages of oocyte maturation for purposes of embryo production has been at the heart of contemporary ART, originating with the classical studies of Edwards (2, 3). His work foresaw the need to develop and optimize cell culture techniques that would sustain oocyte maturation, and heralded 40 years of active research in animal and human systems. As the clinical application of *in vitro* maturation in human oocytes has entered the mainstream of ART, so too has the need to better understand the *in vivo* conditions that stimulate oocyte maturation during ovulation. Thus the purpose of this review is to outline recent evidence based on the physiology of ovulation and pose a series of questions that would allow for comparison of the quality of oocytes produced under *in vivo* and *in vitro* conditions. It is hoped that the concepts put forth here will stimulate further research designed to improve *in vitro* maturation for clinical applications.

1.1 Historical Considerations

Even before the classical studies of Edwards (2), there was ample evidence demonstrating the feasibility of *in vitro* maturation in mammalian oocytes based on the pioneering efforts of Pincus and his colleagues (4) (Table 1). Pincus' success in documenting the spontaneous maturation of rabbit oocytes from the germinal vesicle to metaphase-II stage of meiosis in culture

J.J. Bromfield, K.L. Jones, and D.F. Albertini (✉)
Center for Reproductive Sciences, Kansas University Medical Center, 3901 Rainbow Boulevard, Kansas City, KS, USA
e-mail: dalbertini@kumc.edu

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Table 1 Historical milestones in development of IVM

Author/Year	Observation	Ref.
Pincus and Enzmann, 1935	Spontaneous maturation of rabbit oocytes	(4)
Rock and Menkin, 1944	Spontaneous maturation of human oocytes	(5)
Edwards, 1965	Spontaneous maturation is common amongst mammals	(3)
Biggers et al., 1967	Oocyte metabolism depends on follicle cells	(6)
Cho et al., 1974	cAMP blocks meiotic resumption	(7)
Anderson and Albertini, 1976	Gap junctions connect oocyte and granulose cells in mammals	(8)
Schroeder and Eppig, 1984	IVM in mice allows birth of live young	(9)
Downs et al. 1988	Oocyte maturation can be induced by FSH and EGF	(10)

prompted one of the first documented efforts to achieve IVM in the human. In 1944, Menkin and Rock (5) published what was debated to be the first "successful" IVF study in humans using aspirated follicular oocytes from the ovaries of women judged to be peri-ovulatory. They reported that in excess of 800 human oocytes were retrieved between 1938 and 1944 and were "bathed in Locke's solution and incubated in the patient's blood serum for 24 h to bring them to maturity." While many of these oocytes were observed to have matured based on the extrusion of a first polar body, it remains speculation as to whether or not any of these patients would have undergone an endogenous gonadotropin surge to influence these earliest results. Nevertheless, the concept that human oocytes could undergo spontaneous IVM was established.

From the outset, there was little doubt that traditional culture conditions would support at least nuclear maturation but short of carrying out IVF and embryo culture, evidence was lacking to support the idea that IVM oocytes from any animal species had acquired the necessary cytoplasmic properties that would support embryonic development. This barrier to further advances was in part dictated by the lack of information on the basic metabolism of the mammalian oocyte. Biggers et al. (6) were the first to study this problem and in the mouse, showed that metabolism of the mouse embryo was comparable to that of the oocytes in terms of using pyruvate as a preferred energy substrate, and more importantly, identified the source of this substrate as the follicle cells attached to the oocyte. This fundamental observation has withstood the test of time and laid the foundation for appreciating the extreme dependence of the embryo's metabolism upon energy resources derived from the companion cells of the cumulus oophorus. Following this work, a series of discoveries was made over the next 20 years in animal models that would set the stage for using human IVM (Table 1). These included identification of cAMP as a meiosis arresting agent (7), the discovery of gap junctions between oocytes and granulosa cells (8), the demonstration that mouse oocytes matured *in vitro* could develop to blastocysts and yield live young after IVF and embryo transfer (9), and the finding that even under conditions of meiotic arrest, FSH or EGF could promote IVM in mice with the resulting oocytes exhibiting higher developmental potential than oocytes matured in the absence of cumulus cells or hormonal supplements (10). Collectively, this body of knowledge established the notion that factors within the ovarian follicle suppressed spontaneous maturation using metabolic cooperation between the oocyte and granulosa cells, and that hormonal signals relieved this inhibition, supporting IVM in a fashion consistent with achieving conceptus developmental competence.

Concurrent with these substantive advances were initial (11, 12) and then later (13–15) attempts to undertake human IVM using explanted follicular oocytes cultured in a single medium for variable amounts of time. In 1997, Bavister and Boatman working with the rhesus monkey introduced the notion of deploying a sequential medium postulating that the needs and requirements for the oocyte and cumulus cells changed at successive stages of the maturation process (16). As will be discussed further, new information on the cascade of signals generated during ovulation suggests that indeed the microenvironment in which the oocyte undergoes final maturation is a changing one and will need to be tightly regulated under *in vitro* conditions to consistently and efficiently achieve full nuclear and cytoplasmic maturation (Fig. 1).

2 In Vivo Vs. In Vitro Maturation of Oocytes

2.1 A Revised Physiological Perspective of the Follicular Milieu

Over the past 10 years, collective evidence from primarily rodent model systems has added complexity to our understanding of the intrafollicular sequence of

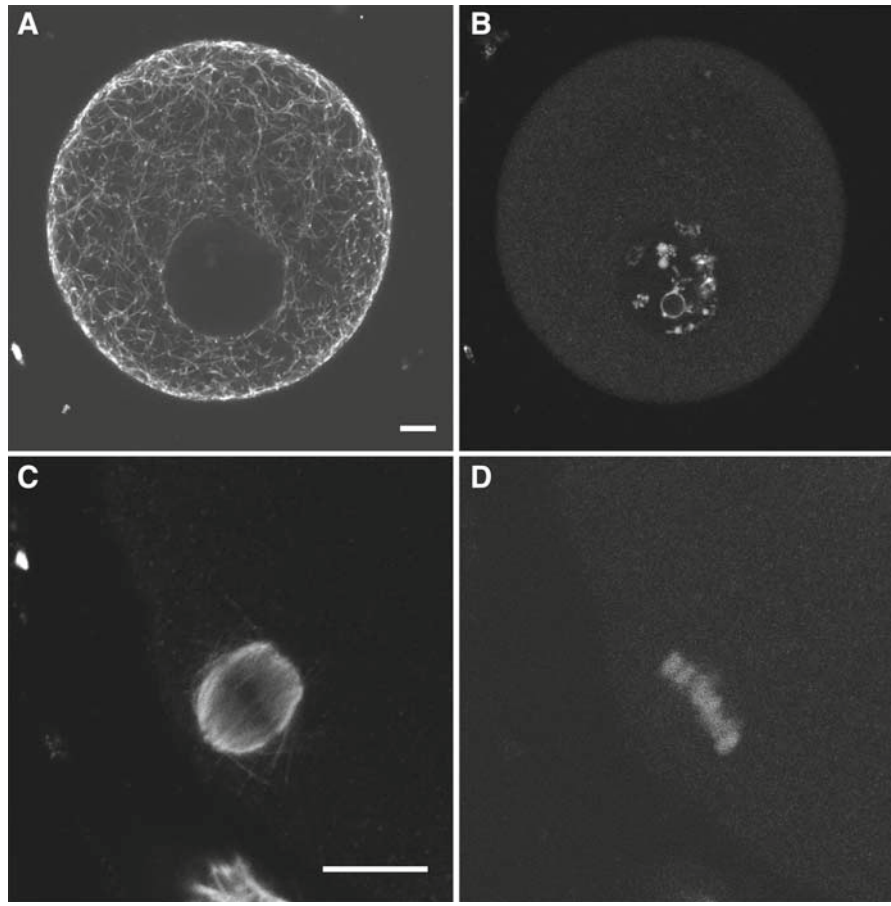

Fig. 1 Configuration of immature germinal vesicle and MII stage human oocytes. Confocal scanning microscopy of human oocytes at the germinal vesicle (A, B) and MII (C, D) stage. Germinal vesicle oocytes show an extensive tubulin network throughout the entire oocyte (**A**) and condensed chromatin within the germinal vesicle with a distinctive ring surrounding the nucleolus (**B**). MII stage oocytes display a well organized meiotic spindle with microtubules meeting at flattened poles adjacent to the oocyte cortex (**C**) with aligned chromatids along an equatorial plate (**D**). *Scale bars* represent 10 μm

events that are elicited by the LH surge during ovulation. Central to this new paradigm is the notion that a cascade of signaling steps brings about significant changes in the transcriptional activity of granulosa cells resulting in the synthesis and secretion of EGF-like molecules whose primary target is the cumulus oophorus (17, 18). Buttressed by the long-standing idea that the cumulus–oocyte complex is a highly integrated heterocellular syncytium, two key questions have remained that would mechanistically contribute to the problem of oocyte *in vitro* maturation. First, how is the switch from diplotene arrest into meiotic maturation triggered at the level of the oocyte? Second, how do cumulus cells participate in the resumption of meiosis and what role does this level of communication have in conferring cytoplasmic competency to the mature oocyte? These two questions lie at the heart of the technical and conceptual problems presently impeding optimization of IVM for human ARTs.

By tradition, and for lack of better alternatives, the inclusion of gonadotropins, steroids, growth factors, serum, and antioxidants has been used in the design of culture media to mimic what has been postulated to be an ex vivo environment within which immature oocytes could proceed through meiosis-I and progress through to metaphase of meiosis-II. However, it is not clear that persistent exposure to gonadotropins is beneficial in the context of cumulus–oocyte interactions during meiotic resumption or at later transition stages of oocyte maturation. In fact, many of the genes that direct the pathway of luteinization become transcriptionally activated within hours of LH treatment in mice

and these seem sufficient to direct terminal differentiation of the mural cell compartment. Although we know little about the lifespan of ligand–receptor activation and stability during ovulation, persistent exposure in a culture setting is likely to bring about inappropriate signaling of a prolonged duration that interferes with the maintenance of structural integrity within the cumulus oophorus. In animal models, this condition directly impacts cell cycle progression during IVM and further results in a failure to maintain meiotic arrest at metaphase-II (19). Moreover, both clinical and research programs have now reached a consensus that excess gonadotropin brings about a reduction in oocyte quality further emphasizing the need to use hormone supplements judiciously in designing IVM protocols that would better mimic follicular physiology in the context of ovulation.

Thus, mapping of the transcriptional requirements for oocyte and follicular maturation *in vivo* has already identified a triggering rather than constitutive role for the peri-ovulatory gonadotropin and pinpointed as an intermediary in this process, the generation of EGF-like molecules as pertinent to the microenvironment that sustains oocyte maturation (17, 18). It follows then that temporally limiting exposure to gonadotropins in combination with supplements conducive to both cumulus expansion and oocyte maturation provide logical first steps in the development of efficacious IVM protocols.

2.2 Signaling Meiotic Resumption

It has long been appreciated that coordination of nuclear maturation with that of the cytoplasm leads to oocytes exhibiting good developmental potential. How such coordination is achieved is a more perplexing problem but in general, the assumption that these events are temporally, if not spatially, synergized has provided a backdrop for studies on oocyte IVM. With respect to nuclear maturation, the first overt sign that diplotene arrest has been overcome and that there is a commitment to proceed through meiosis-I, is the process of germinal vesicle breakdown. While commitment to engage in M-phase progression is tantamount to the activation of the cdk/cyclin/kinase complex, an upstream signal releasing a state of cell cycle arrest is pivotal to initiating meiotic maturation. Recent evidence has shed important new light on how this critical early step is regulated in the rodent.

Within 1–3 h of receptor activation by LH in mural granulosa cells, EGF-like proteins including amphiregulin and epiregulin are synthesized from newly transcribed mRNAs (17, 18). These factors can elicit oocyte maturation as long as granulosa cells are present, again indicating that the signal to commence maturation is transduced through the cumulus oophorus and is not the direct result of LH per se. As discussed earlier, cAMP is believed to be a central regulator of meiotic resumption due to the ability of oocytes to generate this factor which exerts a direct PKA-mediated negative effect on the MPF activator CDC25 (20). What therefore regulates cAMP levels in the oocyte? In the mouse, this pivotal function appears to rely upon a Gs-coupled receptor (GPR3) located in the oocyte plasma membrane. Studies by Mehlmann et al. (21) have shown that when GPR3 is genetically depleted from mice, oocytes undergo precocious maturation in the follicle, and meiotic arrest can be restored by replenishing the mRNA for GPR3 in oocytes from the mutant mice. Thus the long-standing model invoking cAMP metabolism in maintenance of meiotic arrest finds support from these recent studies and has been used clinically to synchronize immature human oocytes prior to IVM (22).

Clearly, the ability to regulate the onset and progression of meiotic maturation in mammalian oocytes are important factors to consider in designing clinically appropriate protocols for human oocyte IVM. At least in the case of releasing meiotic arrest, pharmacological agents such as phosphodiesterase inhibitors have been used to block the precocious advancement of the oocyte cell cycle (22). It will be necessary to identify agents that delay or impede cumulus cell responses to EGF to synchronize the metabolism of the cumulus with that of the oocyte upon release from meiotic arrest. The more pressing challenge will be to determine the causes of cytoplasmic maturation and the role of cumulus cell integrity on this developmentally relevant aspect of oocyte quality (Table 2).

2.3 Linking Cumulus Oocyte Integrity to Cytoplasmic Maturation

The most obvious changes that distinguish oocyte maturation *in vivo* from those which occur *in vitro* relate to the state of cell interactions within the cumulus–oocyte complex (Table 2). Specifically, little is known about the short or long term effects of follicular disruption

Table 2 Mechanisms integrating COC metabolism

Interface	Transfer mode	Substrate or signal propagated
Granulosa-Granulosa	Gap junction	ATP, GSH, cAMP, Ca
	Adhesion junctions	Receptor tyrosine kinases
Granulosa-Zona pellucida	Integrins, proteases, crosslinkers, hyaluronic acid matrix binding	EGF, IL-6, GDF-9, BMP-15
Granulosa-Oocyte	Gap junction	ATP, GSH, cAMP, Ca
	Adherens junctions	RTKs, scaffold proteins
	Lipid exchange	Cholesterol, phosphoinositides
	Local endocytosis and exocytosis	Proteolytic cleavage products

induced during the process of ovum retrieval. For achieving IVM, it is known that the presence of cumulus cells provides a physical basis for integrating both nutrient supply and signal conveyance if the contacts between the oocyte and cumulus cells are retained. But again, the dynamics of the cumulus–oocyte-complex (COC) are subject to progressive change due to the process of cumulus expansion and the resulting subdivision of labor between those cumulus cells that retain adhesive contacts with the zona pellucida and those that assume more distal locations as cumulus expansion progresses. Models of these interactions have been proposed but most tend to ignore the consequences of diluting COCs into relatively large volumes of medium that would irreversibly modify both the structure and contents of the extracellular matrix enveloping the oocyte (23). The importance of this interaction, and a major reason to think that it must be sustained throughout the course of meiotic maturation, is that after signaling to resume meiosis, major changes in oocyte structure and metabolism occur that are linked to cytoplasmic maturation (Table 2).

Amongst the more recent principles governing cellular regulation in many systems is the notion that mRNAs are localized within the cytoplasm to perform site specific functions, once activated for translation to occur. Thus, both maintenance of appropriate levels of mRNAs by establishing a means for preventing degradation, and assignment to correct locations, synergize to produce robust responses controlling cell cycle progression and timing, and organelle positioning and activation (24). These processes are hallmarks of oocyte maturation. Correct readout of stored maternal mRNAs, positioning of mitochondria and the meiotic spindle, and timely initiation of anaphase onset at both meiosis-1 and 2 are vital to ensure the synchronous maturation of the nucleus and cytoplasm. Notably, none of these processes would involve transcription, thereby emphasizing the need to focus on posttranslational dynamics in the case of the oocyte itself.

In contrast, transcriptional regulation at the level of the cumulus cells is characteristic of their function both prior to and following the LH surge, as noted above. Here, several aspects of cumulus cell function are subject to regulation not only via the activation of LH or FSH receptors but also by the system of TGF beta molecules derived from the oocyte itself (1). For example, the inability of the oocyte to undergo glycolysis and derive energy substrates like pyruvate is compensated for by the metabolism of the granulosa cells to which it is attached. Recent evidence in the mouse now shows that BMP-15 and FGF-8 are made in the oocyte, and their secreted products stimulate glycolysis in the cumulus cells (25). Moreover, in the mouse, GDF-9 and BMP-15 also seem to influence the delivery of cholesterol to the oocyte after it is synthesized in the cumulus cells (26). There is then precedence for so-called metabolic cooperation at many stages in the process that regulates meiotic maturation beginning with energy substrate provision, and ending with the loading of important molecules like glutathione and ATP. The common feature that links cumulus integrity with achieving cytoplasmic maturation appears then, to be based in an architecture that satisfies a symbiotic relationship between oocyte and cumulus cell, as summarized in Table 2. Defining this architecture and understanding how it changes in space and time during ovulation will set the stage for improvements in human IVM.

The metabolic demands during meiotic maturation are formidable (27). A constant energy supply is required to sustain ATP-consuming kinases that, in turn, drive entry into metaphase-I and maintain metaphase-II arrest. The intrinsic lack of a glutathione generating capacity in the oocyte means that the only source of this essential redox regulator is from the surrounding cumulus cells (28). Stored maternal proteins are needed to generate the meiotic spindle and, in murine models, this has been shown to be directly influenced by IVM conditions. Specifically, the localization and assembly of the meiotic spindle *in vivo* involves a spatial restriction that limits the amount of tubulin that is effectively integrated during progression of meiosis (29); *in vitro* maturation under conditions that compromise cumulus cell attachment leads to excess tubulin recruitment into spindles and a loss of

this maternal protein into the polar bodies. It was recently shown that spindle enlargement is due to the failure to retain gamma-tubulin containing organizing centers in the oocyte cortex, again due to a loss in cumulus cell contact (30). Similar forces acting to stabilize the oocyte cortex during maturation are likely to influence the location and integrity of other organelles required during preimplantation development. This aspect of IVM needs to be better characterized in human oocytes especially with reference to the nature of oocyte granulosa cell interactions (31).

3 Lessons Learned

Optimizing conditions for human IVM has, under certain circumstances, drawn upon discoveries made with animal models. These animal systems offer abundant material, the ability to manipulate gene and protein actions, and more importantly, in the case of the mouse and cow, the ability to translate IVM conditions to a clinical outcome measure whether it be pregnancy establishment or term birth. On the other hand, without a clear picture of normo-ovulatory processes in humans and nonhuman primates there are likely to be additional or different factors that are called into play to achieve oocyte maturation *in vivo* or *in vitro*. While this is an unrealistic research scenario for studies on humans, future work in this area can be guided by the principles of ovulatory physiology gleaned from animal studies and suggest that the lessons learned in these models will find application in the clinic (Table 3).

One set of lessons can be viewed from the perspective of oversimplification. Just as the facility to study gene transcription set the stage for deducing many of the gene networks involved in murine ovulation, so too will the need to map the spatial and temporal aspects of oocyte proteins that underlie developmental competencies. Given the oocyte's transcriptionally dormant state and its reliance on the cumulus cells for fundamental aspects of metabolism, it will be essential to achieve effective support of mRNA processing and the lifetimes for specific proteins in cell cycle control. This will ultimately require definition of the protein factors that drive chromosome alignment and segregation at both meiotic anaphases, and those maternally inherited proteins that support the fidelity of cell division in the early conceptus. What sets the primate oocyte apart from other mammals in this regard remains a perplexing problem, given the high incidence of aneuploidy that is known to compromise human oocyte health.

Another example of oversimplification derives from the microenvironment that the oocyte finds itself in both prior to, and following, cumulus expansion. Viscous hyaluronate-rich gels provide physical rigidity and biochemical accessibility for growth factors in many developmental systems and this should not be overlooked in the case of the COC. If nothing else, mounting evidence for the dualistic functions of both granulosa and oocyte secreted proteins argues strongly that any enzymatic or dilution effect imposed on the COC is likely to alter the immediate interfaces being used to initiate or sustain signaling cascades as oocyte maturation progresses. Likewise the basic culture conditions now employed are also apt to generate stress responses in both cumulus cells and oocytes due to the heightened metabolism of the former, and the protracted dependence of the latter, on the energy requirements for both the oocyte and zygote. Addressing both the sources of and ways to micromanage reactive oxygen species will measurably

Table 3 Physicochemical factors required for IVM optimization

Factors	Consequence	Remediation prospect
Matrix stability	Loss of growth factors	Substitution of enriched artificial matrices
Diffusion	Reversal of local metabolite and protein gradients	Limit vessel volume and number of medium exchanges
Culture stress	ROS generation, metabolic diversion	Medium quenching, directed metabolism
Enhanced catabolism	Precocious depletion of maternal mRNAs, proteins	Medium conditioners to adjust protein phosphatase, proteosome, and RNA degrading machinery
Cell contact interactions	Loss of metabolic cooperation	Identify and overexpress/stabilize junctional complexes
Oolemma stability	Modification of oocyte domain structure and linkage to cytoskeleton initiates macromolecular turnover	Identify cumulus/zona (extrinsic) and oocyte (intrinsic) molecules that regulate membrane stability using pharmacological agents

serve to protect both long lived and rapidly turning over proteins that determine oocyte quality. And finally, taking the dimensions of time and space and putting them into the context of the changing demands on the oocyte while cumulus expansion proceeds, will necessitate the adoption of microfluidic technology to effect the *in vivo* situation. While some of these modifications from existing clinical approaches are underway and promising, the majority of clinics retain standards for IVM that require updating in an effort to improve the efficiency and safety of this form of ART.

4 Clinical Applications Summary

Human oocyte IVM will continue to be used for clinical embryo production. This need will be driven by benefits to patients that include lessening the risk of ovarian hyperstimulation syndrome (OHSS), especially in cases of polycystic ovary syndrome (PCOS), minimizing both costs and adverse oocyte quality derivative from excessive follicular stimulation, and offering in general a more patient-friendly experience. This potentially major role that human IVM may play in current ART practices is likely to grow as more suitable conditions are defined for manipulating both oocyte and cumulus cell physiology with respect to the array of factors yet to be discovered that contribute to establishing consistent and high levels of oocyte quality. One of the more relevant questions at this point then pertains to identifying biomarkers that directly or indirectly reflect cytoplasmic maturation without compromising the functionality of the cumulus–oocyte complex. New assays are under development that will allow for rapid detection of mRNAs from cumulus cells that might have biomarker potential for assessing oocyte quality. Alternatively, using microassays and sensitive detection strategies to monitor COC metabolism based on catabolism and metabolite secretion might afford the opportunity to profile a given COCs metabolome that could in practice serve as a quality indicator. While all of these approaches offer some merit for clinical application, predictors of oocyte quality will ultimately depend on demonstration of the features of oocytes that best dictate the initiation and persistence of an otherwise error-prone program for pre and post implantation development. Thus, any summary of the state of human IVM must look beyond currently accepted practices, such as whether to use hCG priming or not, and introduce practical modifications in technology that are based on our best guess, drawn from results with animal models such as nonhuman primates.

An impending opportunity for carrying out studies on human IVM should derive from the introduction of fertility preservation programs. Specifically, the use of oocytes isolated from grafted pieces of ovarian cortex and grown to maturity will expand research-oriented clinical programs to analyze the developmental program for human oogenesis at a level of detail not previously appreciated. Moreover, the growth of cryopreservation options for both immature and mature human oocytes will similarly stimulate more study into the staging of oocyte maturation in a context that will bear fruits consistent with improved clinical outcome. As always, the burden of proof in any new procedure will be enhancement of pregnancy rates and birth of healthy offspring in large numbers; data we await from the initial phase of human IVM.

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