Future potential of in vitro maturation including fertility preservation

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In several mammalian species, oocytes from small antral follicles after in vitro maturation (IVM) are successfully used for procreation. Humans are the exception, mainly because of limited access to immature oocytes and because oocyte maturation is uniquely regulated in women. With the introduction of cryopreservation of the ovarian cortex for fertility preservation, immature oocytes from small antral follicles in the medulla are now available for developing IVM on the basis of actual human studies. This review presents recent findings in favor of developing human IVM, including the oocyte diameter, follicle size from which the immature oocytes are collected, necessary level of follicle-stimulating hormone and luteinizing hormone to accelerate IVM, and secretion of factors from the cumulus-oocyte complex that affect the way oocyte maturation takes place. Furthermore, on the basis of studies in human granulosa cells and follicle fluid collected during the final maturation of follicles in vivo, a number of signal transduction pathways and hormone levels active during physiological conditions have been identified, providing new candidates and ways to improve the current IVM platform.

Furthermore, it is suggested that the small droplet of culture medium in which IVM is performed mimics the hormonal milieu within a follicle created by the somatic cells and oocyte in vivo and may be used to advance oocyte nuclear and cytoplasmic maturation.

Collectively, we envision that a continued research effort will develop a human IVM platform equally effective as for other mammalian species. (Fertil Steril® 2023;119:550–9. ©2023 by American Society for Reproductive Medicine.)

Key Words: Human oocyte maturation, IVM, small antral follicles, fertility preservation, recreating follicle environment in vitro

In response to the midcycle surge of gonadotropins, final oocyte maturation takes place in preovulatory follicles, which leads to the ovulation of a fully mature oocyte capable of sustaining further development. The transition of oocytes to the fully mature stage includes the following: nuclear maturation, from the prophase of the first meiotic division (i.e., the germinal vesicle [GV] stage) to the metaphase of the second meiotic division (i.e., metaphase II [MII] stage), and cytoplasmic maturation, which encompasses changes in the oocyte cytoplasm required to sustain chromosomal rearrangements, epigenetic modifications, and fertilization as a whole.

For several mammalian species except humans, assisted reproduction is performed by aspirating immature GV oocytes from small antral follicles (SAFs), often ex vivo, which have received no ovarian stimulation with exogenous hormones. Robust protocols for in vitro maturation (IVM) of immature oocytes with subsequent in vitro fertilization (IVF) treatment have now resulted in high-efficacy methods with healthy offspring. This reflects many years of studying the regulation of oocyte maturation in easily accessible oocytes from rodents and larger domestic species (1–3).

Only recently have human immature oocytes become available for such studies, where the introduction of cryopreservation of the ovarian cortex from young women for fertility preservation has increased the accessibility of human immature oocytes. Frequently, 1 of the 2 ovaries is surgically removed, and only the primordial follicles, primarily positioned in the cortex, are procured for freezing, whereas the surplus medulla, which is normally discarded, now forms the basis for collecting immature oocytes from SAFs with a diameter mainly ranging from 0.5 to 3 mm. In addition, immature oocytes are aspirated from SAFs with a diameter ranging from 3 to 8 mm. This has allowed a new approach to human IVM, which resembles that of other species and initiated the development of a more scientifically based platform for human IVM.

The aim of this review is to delineate the current knowledge on the

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regulation of human oocyte maturation from SAFs and present a rationale for a new human IVM platform that accounts for the accelerated maturation in vitro compared with in vivo. In our view, fertility preservation providing access to immature oocytes now allows human IVM to be studied in more detail and paves the way for the development of new strategies that will advance human IVM to become clinically applicable.

CLASSIFICATION OF HUMAN IMMATURE OOCYTES AND OOCYTE DIAMETER

To advance human IVM, parameters important for oocyte maturation need to be defined. Normally, human oocytes are classified as immature oocytes provided that they are in the GV stage (or are in the process of transition to the MII stage), irrespective of the diameter of the oocyte or the follicle from which the oocyte is derived.

During follicular development, the oocyte receives continuous support from the somatic cell compartment, and the developmental competence of oocytes increases in parallel with the follicular diameter. Thus, oocytes from follicles with a diameter of just a few millimeters would be expected to possess a reduced developmental potential compared with those derived from preovulatory follicles (e.g., as noted during IVF treatment) reflecting enhanced capacity to sustain both nuclear maturation and cytoplasmic maturation. Thus, the quality of immature oocytes is dependent on the diameter of the follicle from which they are derived. When collecting oocytes from the medulla in connection with fertility preservation, it is impossible to determine the diameter of the follicle from where the oocyte originates, and it is questionable whether the oocyte diameter reflects the follicle diameter.

In fact, in humans, the oocyte diameter is only weakly related to the follicular diameter in contrast to many other species (4–6). It appears that the human oocyte reaches its final diameter at the early antral stage. Human oocytes collected from SAFs with a diameter of approximately 0.5–3 mm have, on average, a diameter of 110–120 μm similar to that of fully grown oocytes collected during IVF treatment (6), confirming that in humans, there is asynchrony between the follicular and oocyte diameters.

Surprisingly, it has been shown that the human oocyte diameter is highly significant and positively associated with the ability to sustain an MII transition during IVM (6). This suggests that fully grown human immature oocytes from SAFs have the capacity to sustain nuclear maturation and provided that cytoplasmic maturation can also be advanced, arguing in favor of developing human IVM.

This notion is supported by detailed histologic studies on human ovaries performed by Gougeon (7), who found that human follicles with a diameter between 2 and 5 mm showed an atresia rate of between 58% and 77%, whereas smaller follicles with a diameter of 0.4–2 mm showed a reduced rate between 15% and 24%. The difference in the atresia rate between these follicle sizes was related to the intercycle follicle-stimulating hormone (FSH) drop, where follicles experience a period with reduced FSH levels. This suggests that oocytes are more frequently derived from healthy follicles when the diameter of the follicles is <2 mm. This actually coincides with most follicles, which become available from the medulla during fertility preservation. Thus, if immature oocytes from SAFs in vitro are exposed to conditions that attenuate atresia (e.g., by exposure to high FSH levels), oocyte viability may be rescued. This will obviously be dependent on the proper cumulus cell compartment through which the oocyte will be affected. However, this may also represent an opportunity to enhance the cytoplasmic maturation and, in this way, derive oocytes with a higher potential for clinical use.

IS CYCLIC ADENOSINE MONOPHOSPHATE THE MAIN REGULATOR OF OOCYTE MATURATION IN HUMANS?

In several mammalian species, oocyte maturation has been studied intensively, and high intraoocyte levels of cyclic adenosine monophosphate (cAMP) have repeatedly been shown, mainly in the mouse model, to be of utmost importance for controlling resumption of meiosis and oocyte maturation (1). Once oocytes have been isolated from the antral follicular compartment, it is mandatory to prevent cAMP from being degraded in the oocyte by, for instance, adding hypoxanthine (3 μM), 3-isobutyl-1-methylxanthine (5 μM), or dibutyryl cAMP (100 μM) to prevent oocytes from resuming meiosis. Once removed from such an environment, the rate of spontaneous maturation is close to 100%. This contrasts with immature human oocytes from SAFs, where the rate of spontaneous maturation remained at a modest 20%–30% in oocytes irrespective of whether they were surrounded by cumulus cells (cumulus-oocyte complexes [COCs]) or not (8–10), suggesting that the cAMP level is not as important for the regulation of oocyte maturation in humans as in rodents. Likewise, evidence in bovine indicates that cAMP acts in synergy with other signaling pathways during oocyte meiotic arrest and resumption (11). This is likely to reflect that the regulatory mechanisms involved in human oocyte maturation differ from those well characterized in rodents and bovine.

REGULATION OF OOCYTE MATURATION IN HUMANS IN VITRO

Previous reports in animal models have demonstrated that adding gonadotropins to IVM medium improves cumulus expansion, fertilization, and early embryo development in a dose-response manner (12–15). In connection with human IVM, the FSH level in IVM medium needs to exceed a threshold of 70–250 IU/L to significantly stimulate oocyte maturation in the COCs from SAFs (10). No effect of FSH was observed on naked immature oocytes, confirming the absence of FSH receptor (FSHR) in human oocytes. Although the FSHR expression remains high in cumulus cells without FSH added to maturation medium, it was significantly down-regulated in COCs exposed to FSH at levels of 70–250 IU/L (10). In contrast, the luteinizing hormone (LH) receptor (LHR) expression, in turn, became up-regulated in parallel to the FSHR down-regulation, which coincided with the MII transition. Therefore, when cumulus

cells from SAFs are exposed to the FSH levels exceeding 70 IU/L, the LHR expression is induced despite FSHR being expressed at relatively low levels (Fig. 1). In vitro maturation medium containing 100 IU/L of LH will signal through the newly expressed LHR leading to oocyte maturation.

It is noticeable that the addition of LH to maturation medium without FSH is unable to augment the maturation rate and demonstrates that FSH is required to secure the LHR expression, which then leads to oocyte maturation (10).

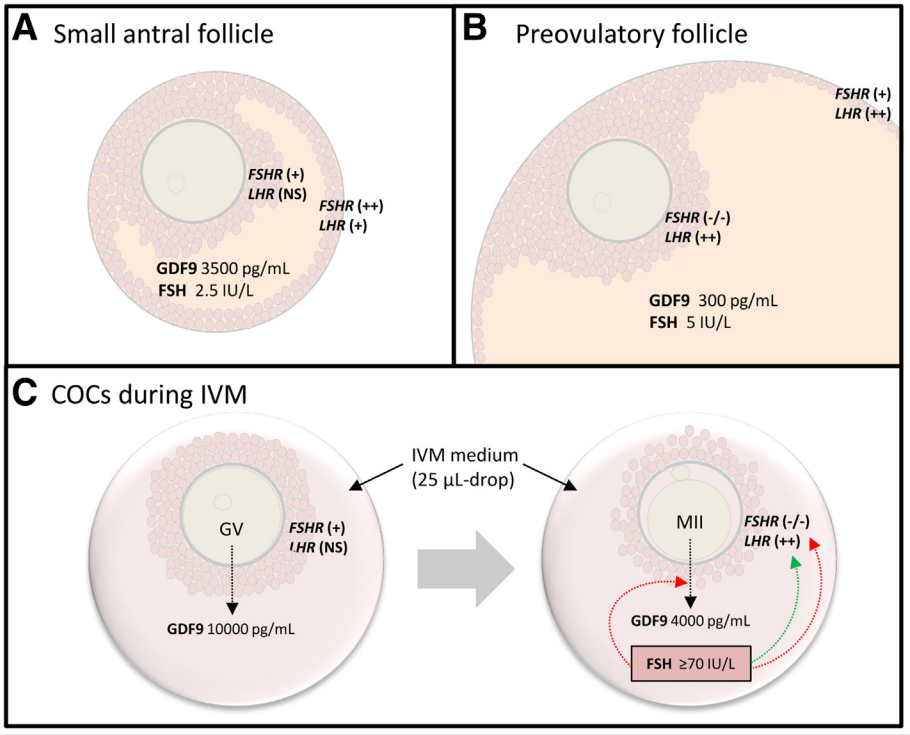
The importance of the LHR in the MII transition in human oocytes contrasts with the murine model, where FSH dose-dependently increases the MII rate in COCs exposed to 8–75 IU/L, whereas there was no effect of LH (even in levels up to 1,500 IU/L) (12). This has been confirmed in another study (13) and suggests that in rodents, FSH exerts an effect on cumulus cells that directly affects oocyte resumption of meiosis but this is not the case in human oocytes, where the LHR expression is required to be present.

The importance of FSH in IVM medium for human oocytes is well recognized. The addition of FSH associated with LH during human IVM has been shown to improve oocyte maturation (10, 16, 17), IVF (16), and embryonic development (18). However, the hormonal requirements of immature oocytes to sustain maturation and further development may depend on the diameter of follicles from which the oocytes are derived and illustrate that different hormonal environments may be needed to secure properly both nuclear maturation and cytoplasmic maturation between different classes of fully grown immature oocytes.

EFFECTS OF GROWTH DIFFERENTIATION FACTOR-9 AND BONE MORPHOGENETIC PROTEIN-15 ON OOCYTE MATURATION IN ADULT HUMAN OOCYTES

Growth differentiation factor-9 (GDF9) and bone morphogenetic protein-15 (BMP15) are almost exclusively produced by oocytes and influence granulosa and cumulus cell function, with downstream effects on oocyte maturation and follicle development (19–22). Both growth factors are synthesized as precursor proteins with prodomains and mature domains that are further processed to form homodimers or heterodimers (i.e., 1 molecule of GDF9 and 1 molecule of BMP15 called cumulin) that bind to specific serine-threonine transmembrane receptors, BMP receptor type II (BMPR2), which recruits the activin receptor-like kinase-5

FIGURE 1



Relative gene expression of the follicle-stimulating hormone (FSH) receptor (*FSHR*) and luteinizing hormone (LH) receptor (*LHR*) in cumulus and mural granulosa cells and the levels of FSH and growth differentiation factor-9 (GDF9) in follicular fluid and spent media after in vitro maturation (IVM). (**A**) Small antral follicle. (**B**) Preovulatory follicle. (**C**) Cumulus-oocyte complex (COC) during IVM. The "+" indicates expressed; "−/−", ≥10-fold down-regulated; and++, ≥10-fold up-regulated. The black dotted arrow indicates the oocyte's secretion of GDF9, the green dotted arrow indicates stimulation, and the red dotted arrow indicates inhibition. GV = germinal vesicle; MII = metaphase II; NS = not expressed in significant levels.

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TABLE 1

Mean GDF9 and BMP15 levels in follicular fluid and IVM spent media.

	Follicular fluid		IVM spent media	
Growth factor	**SAFs**	**Preovulatory follicles**	**GV oocytes**	**MII oocytes**
GDF9 (pg/mL)	3,498	307	10,144	4,126
BMP15 (pg/mL)	573	ND	931	430
Cumulin (AU/mL)	483	63	1,270	1,204

Note: Cumulin was a GDF9/BMP15 heterodimer. BMP15 = bone morphogenetic protein-15; GDF9 = growth differentiation factor-9; GV = germinal vesicle (oocytes after IVM); IVM = in vitro maturation; MII = metaphase II (oocytes after IVM); ND = not detected; SAF = small antral follicle.

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and activates the intracellular SMAD2/SMAD3 pathway (23, 24). In contrast, the mature form of BMP15 activates the BMPR2/activin receptor-like kinase-6 complex activates the SMAD1/SMAD5/SMAD8 pathway (25).

Cumulin has been shown to improve the efficacy of human IVM (26). Furthermore, the addition of pro-BMP15 or pro-GDF9 to IVM and embryo culture enhances oocyte quality and embryo formation in murine and bovine models and humans (27–29).

Recently, it has been measured to which extent these growth factors were secreted in spent medium during the IVM period (30). Surprisingly, high GDF9 and BMP15 levels were measured with GDF9 levels of approximately 10 ng/mL, whereas the BMP15 level was approximately 10 times lower at 1 ng/mL. The actual cumulin level could not be determined because the standard is currently only available in arbitrary units.

Both GDF9 and BMP15 levels were significantly reduced in spent media in which the oocyte advanced to the MII stage compared with oocytes that remained in the GV stage, whereas cumulin remained constant (Table 1). Although the synergistic actions of GDF9 and BMP15 on oocyte maturation have been widely debated (31, 32), the massive surplus of GDF9 suggests that the homodimer exerts functions on its own. On the other hand, cumulin has been shown to activate the SMAD2/SMAD3 pathway in mouse granulosa cells (33), stimulate the SMAD2/SMAD3 and SMAD1/SMAD5/SMAD8 pathways in a human granulosa cell line (COV434) (34), and augment embryo development after IVM in mouse and porcine oocytes (34, 35). However, similar cumulin levels were found in spent media from oocytes that remained in the GV stage compared with those from MII oocytes, suggesting that cumulin is not regulated or affected during human IVM.

The significantly up-regulated expression of both the receptor *BMPR2* and the intracellular downstream genes *SMAD3* and *SMAD5* in cumulus cells from MII oocytes indicated an enhanced activity from these 2 signaling pathways. With both signaling pathways open, the lower levels of GDF9 and BMP15 may be hypothesized to result from a consumption of the ligand in those COCs that reached the MII stage. An alternative explanation may involve the dose-dependent effect of GDF9 on reducing the LHR expression on cumulus cells and progesterone production, as described in rodents (19, 36). Thus, assuming that the LHR expression on human COCs is also negatively affected by GDF9, the stimulus for meiotic resumption will be attenuated and potentially lead to lower maturation rates. It is still not clarified which signal transduction pathways the LHR stimulation initiates in human COCs. This obviously represents an exciting new way of understanding the regulation of human oocyte maturation.

REGULATION OF HUMAN OOCYTES IN VIVO

A valuable starting point for improving human IVM is to understand in detail how the regulation of human oocyte maturation takes place in vivo. Recently, we conducted a study in which follicular fluid (FF) and granulosa cells were aspirated at 5 time points during the process of the final maturation of follicles in women undergoing IVF treatment (37). Samples were collected at the time of ovulation induction (T = 0 hours) and 12, 17, 32, and 36 hours after induction of the final maturation of follicles. A number of substances and signal transduction pathways reported to be active during human oocyte maturation in vivo were evaluated, including the effects of signaling from epidermal growth factor–related peptides comprising amphiregulin and epiregulin, C-type natriuretic peptide (CNP) pathway, inhibins and activins, FF meiosis-activating sterol (FF-MAS), GDF9, BMP15, and the growth factor midkine (37).

Some studies have shown that oocyte maturation in human preovulatory follicles is induced during the first half of the midcycle surge of gonadotropins (38, 39). The data from our study confirmed that amphiregulin was strongly up-regulated during the first 17 hours of ovulation induction—both gene transcription and secreted protein, increasing from being almost absent at T = 0 hours to protein levels reaching 200 ng/mL, 3 times higher than that previously reported (40). In contrast, the epiregulin protein levels were only expressed moderately, whereas the gene expression profile showed a similar pattern as that of amphiregulin (37). The epidermal growth factor receptor genes *EGFR*, *ERBB2*, *ERBB3*, and *ERBB4* were almost constantly expressed at relatively low levels during ovulation induction.

The CNP peptide has successfully been used during a prematuration step in human IVM with the aim to maintain elevated cyclic guanosine monophosphate (cGMP) levels in cumulus cells and the oocyte and, thereby, prevent meiotic

resumption (41–44). In contrast to previous reports, our studies were unable to confirm that the CNP became strongly regulated during the final maturation of follicles in vivo. In fact, both gene expression results and actual measurements of protein showed a constant low presence of this protein. In contrast, the CNP receptor, natriuretic peptide receptor (*NRP2*), was significantly down-regulated during the first 12 hours after induction of the final maturation of follicles, which potentially would result in a reduced cGMP level within oocytes. The down-regulation of the *NRP2* receptor suggests that CNP signaling pathways may be of importance during human oocyte maturation in vivo.

Studies more than 2 decades ago showed that both inhibins and activins at levels of 100 ng/mL improved and advanced meiotic resumption of human immature oocytes (45, 46). Our new studies now, for the first time, show that the levels of especially inhibins within the first 17 hours after ovulation induction reach unexpected collective levels of inhibin-A and inhibin-B of >600 ng/mL in FF (37). This suggests that the oocytes are exposed to much higher levels of inhibins in vivo than previously expected and warrants further studies on the effects of inhibins on oocyte maturation at physiological levels.

Although we have been unable to measure the FF-MAS levels in FF collected during the midcycle of gonadotropins, the gene expression profile of the enzyme catalyzing the conversion of lanosterol to FF-MAS (i.e., cytochrome P-450 14α demethylase [*CYP51*]) is significantly up-regulated from 0 to 12 hours after ovulation induction. Furthermore, the enzyme that advances downstream conversion of FF-MAS, Δ14-sterol reductase (which, in humans, is encoded by the *TM7SF2* gene [i.e., transmembrane 7 superfamily member 2 gene]), is significantly down-regulated. In addition, the gene expression of 24-dehydrocholesterol reductase (*DHCR24*), which also metabolizes FF-MAS to inactive metabolites in relation to oocyte maturation, is significantly reduced during the first 12 hours of the final maturation of follicles in humans. Collectively, this suggests the accumulation of FF-MAS during the early stages of the final maturation of follicles, potentially contributing to advance meiotic resumption.

The reported effect of GDF9, BMP15, and cumulin on oocyte maturation during in vivo conditions was probably modest because GDF9 and BMP15 were only detected in a small fraction of the FF, which contrasts to FF from SAFs (diameter, 3–12 mm) in which levels were at least 10 times higher (Table 1). Cumulin was better detected in the FF samples but without variability during the process of oocyte maturation. The mean level was approximately 7 times lower than that reported for SAFs (47), and a clear physiological function of the heterodimer is not apparent from these studies (37).

The growth factor midkine is expressed in most organs but is abundantly and with a clear maximum expressed in the ovaries (http://www.proteinatlas.org/ENSG00000110492-MDK/summary/rna). In COCs from SAFs, midkine improves oocyte maturation during human IVM (48), and in FF collected from SAFs (49) and preovulatory follicles (50), the level and gene expression of midkine appear to follow a pattern similar to that of amphiregulin with an increase during the first 17 hours although the differences were less pronounced compared with than of amphiregulin. A potential receptor for midkine is *IGTA6*, which significantly increases during the first 12 hours after induction of the final maturation of follicles and suggests that signal transduction initiated by midkine is taking place (37).

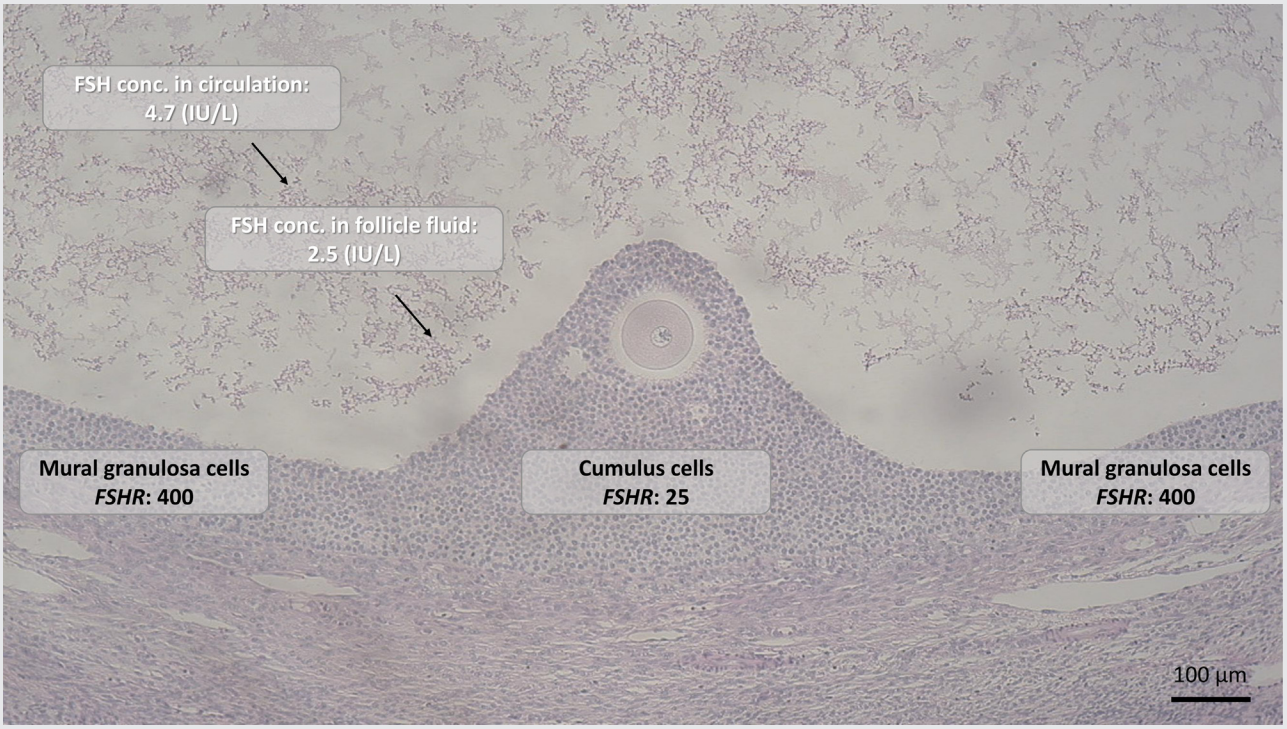
Collectively, these data suggest that a multitude of signaling pathways are in operation during the final maturation of human follicles in vivo. Expression of substances advancing oocyte maturation occurs quickly after the induction of the final maturation of follicles, during the first 12–17 hours and confirms that this process is regulated during the first half of the midcycle surge of gonadotropins. This study demonstrates strong up-regulation of substances that augment oocyte maturation, including amphiregulin, inhibin-A and inhibin-B, midkine, and potentially FF-MAS, whereas the effect of the CNP possibly occurs at the receptor level. A specific effect of GDF9 and BMP15 is less likely to be of importance during human oocyte maturation. This study provides new information for improving in vitro conditions during human IVM.

Invariably, IVM is performed using immature oocytes from follicles considerably smaller than the ones in which oocyte maturation naturally takes place, and although it may be argued that hormonal conditions that are required to stimulate MII transition in oocytes from smaller follicles will be different, it is still valuable to unravel the changes that lead to oocyte maturation in vivo. As outlined in the following, it appears that LHR signaling is an important component in both in vivo maturation and IVM of oocytes. One example of this could be that the GDF9 and BMP15 levels are approximately 10 times higher in FF from SAFs with a diameter of 3–6 mm than in the preovulatory follicles (37, 47). As part of physiology, the LHR expression in SAFs may be prevented by high GDF9 and BMP15 levels, whereas the LHR expression continuous to increase in parallel with decreasing GDF9 levels in larger follicles (Fig. 1).

IMITATE THE FOLLICULAR HORMONAL ENVIRONMENT IN CULTURE CONDITIONS FOR IMMATURE OOCYTES

The analysis of spent medium from IVM with COCs from SAFs demonstrated that GDF9 and BMP15 accumulated in levels significantly exceeding those of the FF in corresponding SAFs (Table 1). Every single COC recovered from SAFs was cultured in droplets of 25 μL under oil during IVM. This demonstrates that it is possible to create local hormonal conditions during culture, which are likely to be able to affect oocyte maturation, both nuclear and cytoplasmic, to enhance oocyte competence. In connection with the newly acquired knowledge on how oocyte maturation takes place during in vivo maturation (37), this provides cues on how to optimize conditions during IVM. Therefore, in essence, the small, confined environment of the droplet in which IVM is performed constitutes a way of mimicking the special and often unique hormonal environment that oocytes are exposed to within follicles and in which oocyte competence is acquired

FIGURE 2



Human small antral follicles. Relative gene expression of follicle-stimulating hormone (FSH) receptor (*FSHR*) (in relation to *GAPDH*X1000) in cumulus and mural granulosa cells (black) and the FSH level in follicle fluid from small antral follicles (IU/L) and in the corresponding circulation (white).

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guided by the surrounding somatic cells but also by secretions from the oocyte itself.

Collectively, combining locally produced and exogenous factors of importance for oocyte maturation within the confinements of isolated small droplets and better knowledge of the downstream effects of LH stimulation in connection with oocyte maturation, an improved platform for human IVM is likely to be developed.

THEORY FOR THE ACCELERATED OOCYTE MATURATION IN VITRO

In vitro maturation of human immature oocytes from SAFs requires the presence of FSH in levels exceeding 70 IU/L to significantly augment the maturation rates, whereas the FSH levels of 20–40 IU/L are less efficient or ineffective (10). The FSH levels exceeding 70 IU/L approach the menopausal levels and by far exceed the FSH levels that granulosa cells are exposed to in women during their reproductive years, even during ovarian stimulation with exogenous gonadotropins, in which the FSH levels normally will not surpass 20–25 IU/L. For FSH to reach the COC, it needs to diffuse across the basal membrane surrounding the follicle and pass through the granulosa cell layer to reach the FF and affect the COC. The granulosa cells express the FSHR and are likely to bind FSH and act as a deterrent for FSH to enter the follicle and reach the COCs (Fig. 2). Measurements of FSH in corresponding serum and FF samples from either SAFs or preovulatory follicles confirmed that the FSH level in FF is a fraction of what is observed in circulation, seldom exceeding 1-digit levels (Table 2) (51, 52). Thus, the COC is shielded from FSH in vivo and is exposed only to modest FSH levels. Furthermore, cumulus cells have an FSHR expression that is 5–10 times lower than in mural granulosa cells from SAFs and from larger follicles (10, 53, 54) (Fig. 2). Thus, it is not conceivable that FSH acting on COCs in vivo is the physiological signal for oocyte maturation; the FSH level is too low, and the FSHR expression is relatively low.

In line with the well-known positive effect of a human chorionic gonadotropin bolus trigger on oocyte maturation, the signal in vivo is more likely to be derived from the stimulation of the LHR on mural granulosa cells, on which the

TABLE 2

Mean follicle-stimulating hormone levels in plasma and follicular fluids.

	SAF		Preovulatory follicle	
Hormone	**Plasma**	**FF**	**Plasma**	**FF**
FSH (IU/L)	4.7	2.5	17.1	5.3

Note: FF = follicular fluid; FSH = follicle-stimulating hormone; SAF = small antral follicle.

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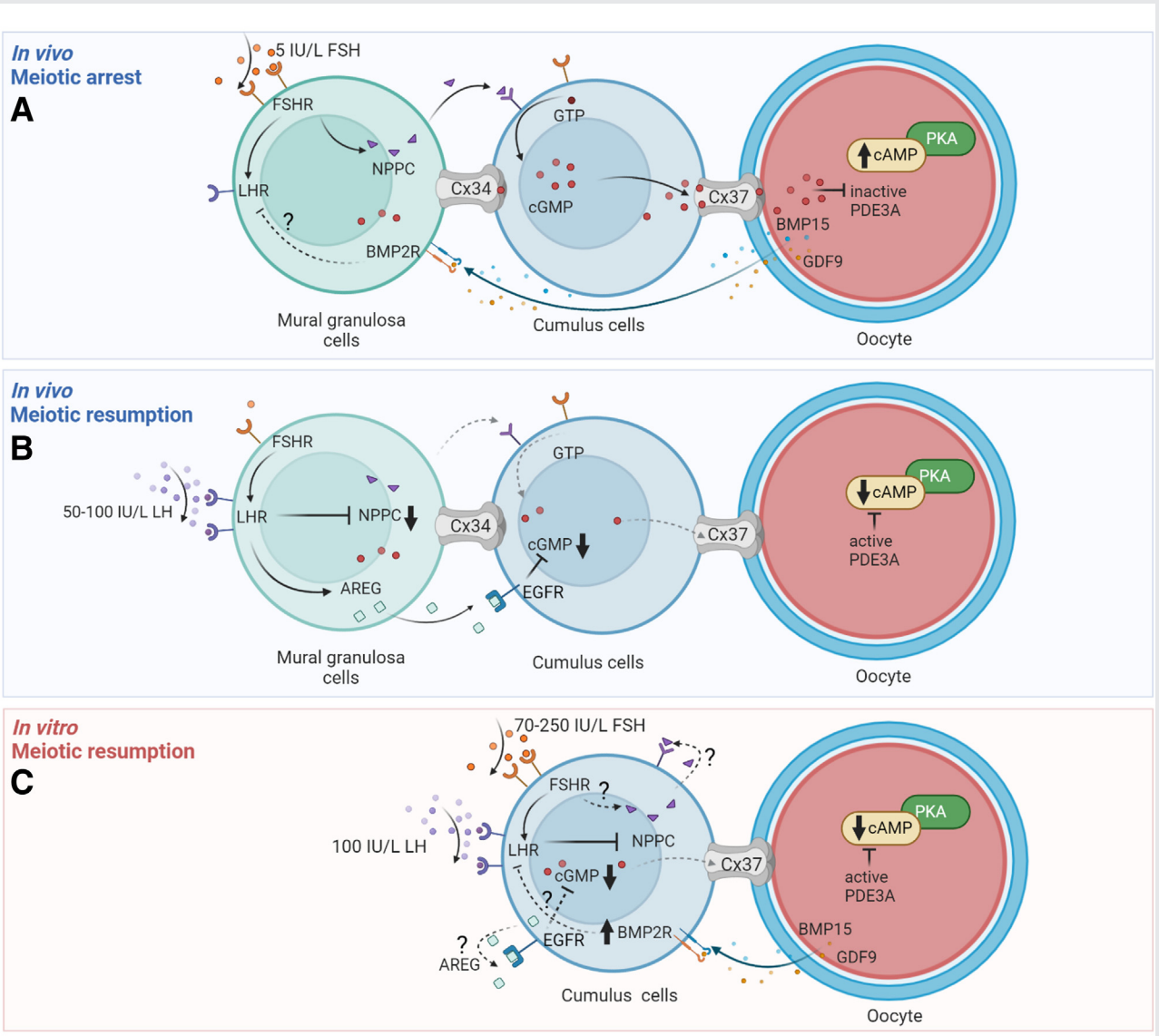
FIGURE 3



Schematic illustration of human meiotic arrest and resumption in vivo and in vitro. (**A**) Mural granulosa cells, cumulus cells, and the fully mature oocytes cooperated to synthesize high levels of cyclic adenosine monophosphate (cAMP) that ensured meiotic arrest in vivo. The follicle-stimulating hormone (FSH) bound to the FSH receptor (FSHR) on the mural granulosa cells, which promoted the expression of the luteinizing hormone (LH) receptor (*LHR*) and natriuretic peptide precursor type C (NPPC) production. The NPPC activated the NPPC receptor on the cumulus cells converting guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) that transferred to the oocyte via Cx37 gap junctions. High cGMP levels in the oocyte inhibited phosphodiesterase type 3A (PDE3A) activity, thereby preventing the degradation of cAMP. High cAMP levels activated protein kinase A (PKA), which, in turn, activated a cascade of mediators, preventing the activation of the arrested oocyte. High levels of bone morphogenetic protein 15 (BMP15) and growth and differentiation factor 9 (GDF9), growth factors exclusively secreted by the oocyte, which can activate the BMP2 receptor (BMP2R) on the mural granulosa cells. The BMP2R activity may have an inhibitory effect on the expression of LHR. (**B**) The preovulatory LH surge activated the LHR on the mural granulosa cells, which led to meiotic resumption in vivo. The LHR expression inhibited the NPPC production and promotes the synthesis of amphiregulin (AREG) that activated the epidermal growth factor receptors on the cumulus cells, which, in turn, inhibited the conversion of GTP to cGMP in the cumulus cells. In the oocyte, the low cGMP levels allowed PDE3A activity, which promoted cAMP degradation. Low levels of cAMP and PKA could no longer prevent meiotic resumption. (**C**) During human meiotic resumption in vitro, there were no mural granulosa cells present, and cell-cell communication was via the cumulus cells and the oocyte only. From culture media, the cumulus cells were exposed to high FSH and LH levels, and it is suggested that the cumulus can be pushed by these high levels to function as mural-like-granulosa cells. High FSH levels activated the FSHR, which, in turn, promoted LHR receptor expression and may induce the production of NPPC in the cumulus cells. The NPPC may exert some autocrine function via the NPPC receptor. However, the high LH levels inhibited NPPC production leading to an overall reduction of NPPC and cGMP in the cumulus cells. Amphiregulin was produced by the cumulus cell and may have autocrine activation on the epidermal growth factor receptors, which inhibited the conversion of GTP to cGMP. In the oocyte, the low cGMP levels allowed PDE3A activity, which promoted cAMP degradation. Low cAMP and PKA levels could no longer prevent meiotic resumption. Low levels of oocyte-produced BMP15 and GDF9 activated the BMP2R on the cumulus cells and may have an inhibitory effect on the LHR.

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LHR becomes up-regulated via FSH signaling (55). Hormones from the mural granulosa cells will, in turn, affect the cumulus cells or the oocyte directly, as described in the previous section.

Therefore, the induction of oocyte maturation in women is likely to follow a sequence of events including (but not limited to) FSH-induced LHR on mural granulosa cells, which, on stimulation with LH activity during the midcycle of gonadotropins or by the effect of an exogenous human chorionic gonadotropin bolus trigger, induces the secretion of a number of substances and activate signal transduction pathways that promote oocyte maturation leading to MII transition and an appropriate cytoplasmic maturation before ovulation (Fig. 3).

During IVM, the mural granulosa cells are missing, and only the cumulus cells surrounding the oocyte are included. Therefore, exposing the cumulus cells to FSH levels exceeding 70 IU/L is unphysiological. Although the FSHR expression in the cumulus cells is modest, high FSH levels induce the LHR expression in the cumulus cells, resembling expression levels as in mural granulosa cells of preovulatory follicles considered to be high (30, 53). The LH included in IVM medium (i.e., 100 IU/L) then acts via the LHR to either stimulate the oocyte directly to undertake oocyte maturation or via secretion of substances as described in the aforementioned section that will promote oocyte maturation (Fig. 3).

Thus, the in vivo and in vitro regulations of oocyte maturation in humans are fundamentally different because the mural granulosa cells are not included in the in vitro model. In vitro, accelerated expression of LHR on cumulus cells prematurely occurs, and strong stimulation of the LHR results in an enhanced maturation of oocytes compared with the in vivo situation.

IVM SAFETY, CLINICAL OUTCOMES, AND FUTURE PERSPECTIVES

The traditional approach for human IVM has focused on oocytes derived from follicles of at least 10 mm in diameter. Here, the results are now encouraging. Initially, an increased rate of abnormal meiotic spindle and chromosome anomalies was reported in IVM oocytes (56). However, there is growing evidence showing similar spindle conformation, ultrastructure, recurrence of aneuploidies, and imprinting mutations in both in vivo and in vitro matured oocytes (48, 57–59). In addition, embryos derived from human oocytes matured in vitro and in vivo present similar aneuploidy rates (60, 61). In 2013, it was estimated that more than 5,000 live births were derived from IVM oocytes worldwide (62). The American Society for Reproductive Medicine no longer considers IVM as experimental (63). However, in general, IVM protocols still generate lower cumulative live birth rates per cycle (approximately 40% vs. 50%) than conventional IVF in patients with a polycystic ovary syndrome (PCOS)–like condition and PCOS (64, 65). Hence, the main reason for the limited use of human IVM seems to be its lower rate of conception than that of classical IVF and not safety concerns (63).

Although IVM with human oocytes from SAFs used clinically is still in its infancy, it has been applied as a method to augment the fertility preservation potential in women receiving ovarian tissue freezing (10, 48, 66–69). Notably, this approach does not require stimulation with exogenous hormones, and on the basis of the immature oocytes aspirated from the ovary ex vivo or located in the surplus medulla tissue, a considerable number of MII oocytes can usually be derived. The aneuploidy rate in these oocytes has been reported to resemble that observed in oocytes derived from conventional IVF (48) and has, so far, proven to be safe with the birth of 5 healthy infants worldwide (66–68).

Therefore, the outlook for using IVM to secure fertility in women undergoing fertility preservation by having ovarian tissue is to become an important add-on method. The IVM procedure can augment the fertility potential without additional surgical interventions and with limited costs. In the future, the application of this method is likely to expand beyond patients with cancer. Other conditions to be included are genetic diseases such as thalassemia, sickle cell anemia, and Turner syndrome and ovarian cancer and other ovarian diseases because the oocyte itself does not harbor the malignancy. Furthermore, depending on its efficacy, the technique may be able to compete with traditional IVF treatment in the long run, especially in cases where several immature oocytes can be aspirated from SAFs, for instance, in patients with PCOS. To accomplish this, new aspiration techniques for the collection of oocytes from SAFs need to be further developed because there are no needles and equipment specifically designed for SAFs at the moment. The use of this method is already been taken up in several clinics and will most likely spread in the coming years. Therefore, there is a huge incitement to improve this technique further in the coming years.

In conclusion, the task for augmenting the usefulness of human IVM as a realistic clinical alternative is to combine an efficient protocol for nuclear maturation that also advances the cytoplasmic maturation to enable the oocyte to sustain further development. The following points now form the basis for the improvements to be developed:

- The human oocyte is fully grown already in SAFs, and its diameter is important for its ability to sustain the MII transition.
- The somatic cell compartment of the COCs is instrumental in accelerating oocyte maturation in vitro.
- The physical environment in the small droplet in which oocyte maturation takes place in vitro comprises an important hormonal milieu that can be manipulated to resemble the physiological conditions observed within the follicle because of the somatic cell compartment surrounding the oocyte.
- The FSH levels need to exceed 70 IU/L to stimulate the somatic cells of COCs, which possess a relatively low level of FSHR expression, sufficiently to secure LHR expression, thereby enabling oocytes to resume meiosis.
- Oocytes from COCs secreted unexpectedly high levels of especially GDF9, which in spent medium from human

IVM surpassed those of BMP15 by 10 times and more than double the levels detected in FF from SAFs.

- High GDF9 levels are associated with less frequent MII transitions that may reflect ligand consumption or perhaps prevention of LHR expression in the somatic cells of the COCs.
- In vivo, the mural granulosa cells shield the COC from high FSH levels, of which only a fraction of that noted in circulation enters the follicle, probably because of a relatively high FSHR expression on the mural granulosa cells.
- Recent observations in granulosa cells and follicle fluid collected during the final maturation of follicles in vivo in women have highlighted signal transduction pathways and hormone levels active in physiological conditions that are new candidates for improving the current IVM platform.

On the basis of these considerations and the recent information, several new studies to improve the human IVM platform can now be planned, generating new information that is likely to advance our understanding of the regulation of human oocyte maturation and eventually turn out to improve the efficacy of the procedure.

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