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Ovarian follicle culture

Ovarian follicle culture: advances and challenges for human and nonhuman primates

Evelyn E. Telfer, Ph.D., F.S.B.,[a,b] and Mary B. Zelinski, Ph.D.[c,d]

[a] Institute of Cell Biology, and [b] Centre for Integrative Physiology, University of Edinburgh, Edinburgh, United Kingdom;
[c] Division of Reproductive and Developmental Sciences, Oregon National Primate Research Center, Beaverton; and
[d] Department of Obstetrics and Gynecology, Oregon Health and Science University, Portland, Oregon

The removal and cryostorage of ovarian cortical biopsies is now offered as a fertility preservation option for young women. The only available option to restore fertility using this tissue is by transplantation, which may not be possible for all patients. The full potential of this tissue to restore fertility could be achieved by the development of in vitro systems that support oocyte development from the most immature stages to maturation. The techniques of in vitro growth (IVG) combined with in vitro maturation (IVM) are being developed with human tissue, but comparing different systems has been difficult because of the scarcity of tissue so nonhuman primates are being used as model systems. There are many challenges to developing a complete culture system that would support human oocyte development, and this review outlines the approaches being taken by several groups using tissue from women and nonhuman primate models to support each of the stages of oocyte development. (Fertil Steril® 2013;99:1523–33. ©2013 by American Society for Reproductive Medicine.)
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The development of culture systems with the aim of growing oocytes from the earliest stage of follicle through to maturity for in vitro fertilization (IVF) could have a lasting impact on clinically assisted reproduction, particularly for the growing number of women who are surviving cancer only to face infertility as a result of the gametotoxic effects of cancer therapies (1). Recent success with ovarian tissue transplantation (2–4) has expanded interest in and efforts to cryopreserve and store ovarian tissue for future fertility.

Ovarian tissue cryopreservation is the only option available for young patients without partners or who cannot undergo ovarian stimulation for oocyte/embryo cryopreservation and for patients for whom the risk for reintroduction of malignant cells precludes transplantation. Thus, the need for follicle culture systems that can efficiently use all classes of ovarian follicles, derived from clinically cryopreserved ovarian tissue, as sources of gametes would maximize reproductive potential for future fertility. However, complete

in vitro growth (IVG) from immature primordial stages with subsequent IVF of oocytes followed by embryo transfer and production of live offspring has, so far, been achieved only in mice (5, 6). Several groups have focused on culturing later stages of follicle development from rodents and have produced developmentally competent oocytes and viable offspring (7–11). The success of these techniques has encouraged the demanding challenge of adapting them for humans and other primates.

Although these techniques have been used to study the regulation of follicle development, the ultimate aim of follicle culture is to increase the availability of developmentally competent oocytes from what would be available from conventional methods, and there is still much to do before follicle culture can be used as a strategy for obtaining competent oocytes. In recent years a great deal of progress has been made in developing culture techniques

for humans and nonhuman primates, and in this review we describe the technologies and discuss the prospects as well as the problems of applying them clinically.

FOLLICULAR DEVELOPMENT

Female reproductive function requires cyclic development and maturation of ovarian follicles on a background of continuous activation from the pool of primordial follicles. Primordial follicles are formed before birth and represent a population of germ cells from which recruitment for growth occurs throughout the woman's reproductive life. Follicular growth and development involves a series of complex and precisely regulated events: 1) initiation of primordial follicle growth and development to the preantral follicle stage; 2) formation of antral follicles where expansion to the preovulatory or graafian follicle is associated with granulosa cell proliferation and antral fluid accumulation within the basement membrane; and 3) rupture of the graafian follicle, releasing a cumulus-oocyte complex at ovulation in response to the midcycle LH surge (Fig. 1A).

As the oocyte grows within the follicle it is held in meiotic arrest at the dictyate stage of prophase I, but during development within the follicle it must acquire the ability to resume meiosis (meiotic competence) and the ability to support fertilization and embryonic development (developmental competence). Thus the oocyte depends on the local environment within the follicle for subsequent function as a gamete, and the formation and maintenance of connections facilitating bidirectional communication between the oocyte and granulosa cells are key to oocyte development in all species.

The development of culture conditions for immature germ cells (both eggs and sperm) is one of the greatest technical challenges of reproductive technology. An understanding of the physiologic requirements of the oocyte, granulosa, theca, and perhaps even the stromal cells is needed. These requirements are complex and change during growth, so a major consideration is the starting point of the culture system, i.e., which stage of follicle to start with. The majority of follicles within the ovary in all young mammalian females are at the primordial stage of development, and those follicles are continually being used during reproductive life (12). We do not know if this pool represents a homogeneous population but it is thought that at this stage follicles have not yet been exposed to selection processes that lead to follicle degeneration (13, 14). Although rodents are excellent models for pioneering technologies, intermediate species are needed to test feasibility for human applications. Follicles of some domestic animals (cows, sheep, and goats) can resemble those of humans regarding growth rates and size, but the protracted length of folliculogenesis in vivo, estimated in women to be 90 days from the entrance of a preantral follicle into the growing pool to preovulatory follicles (15), and the long length of the follicular phase of the spontaneous menstrual cycle in nonhuman primates (~2 weeks compared with a few days in domestic mammals) more closely reflect that of women. Therefore, nonhuman primates are emerging as an important translational model to advance technologic developments in follicle culture.

DEVELOPMENT OF FOLLICLES IN VITRO

Several approaches have been taken to develop human follicles in vitro with the use of fresh (16, 17) and thawed cryopreserved (17, 18) human cortical tissue. It is now clear that if we are to achieve complete development of human oocytes, a dynamic multistep culture system is required to support each of the transitional stages (16, 19–21). The first step is to support the initiation of primordial follicle development and early growth, the second stage is to optimize the growth of follicles from preantral to antral stages, with the completion of oocyte growth being achieved during the third stage. The focus should be primarily on oocyte development, which may not require the development of large follicular structures but rather the maintenance of differentiated somatic cells in contact with the developing oocyte. A multistep system for IVG follicles has been proposed (22, 23) to produce competent oocytes from human ovarian cortical tissue. The multistep approach needs to support the changing requirements of the developing oocyte and its surrounding somatic (granulosa) cells with the main focus being on maintaining oocyte–somatic cell interactions. Several groups have worked on each of the steps required to support human oocyte development in vitro: 1) activation of primordial follicles through culturing ovarian cortex (16, 17); 2) isolation and culture of growing preantral follicles to achieve oocyte growth and development (16, 24–31); and 3) aspiration and maturation of oocyte-cumulus complexes (32, 33). The aim of ongoing research in this field has been to combine each of these steps to achieve complete development of human oocytes (16). Progress in achieving this goal and the use of nonhuman primate models will be reviewed.

Activation of Primordial Follicles

The majority of follicles within ovarian cortical tissue are quiescent primordial, so the first consideration of an IVG system should be to optimize initiation of primordial follicles in vitro and support early follicle development. The factors regulating follicle initiation and early growth are still not well defined, but the process requires a combination of inhibitory, stimulatory, and maintenance factors (34). Studies using knockout mouse models have demonstrated the importance of the phosphatidylinositol-3′-kinase (PI3K)–Akt signaling pathway within the oocyte in regulating follicle activation (35). The phosphatase and tensin homologue deleted on chromosome ten (PTEN) acts as a negative regulator of this pathway and suppresses initiation of follicle development (35). The transcription factor FOXO3a is a downstream effector of this pathway and acts to inhibit follicle recruitment (36). However, primordial follicles of fetal and juvenile macaque ovaries lack FOXO3a expression, suggesting that alternative transcription factors may mediate follicle activation in primates (37). Other components of this pathway depend on the mammalian target of rapamycin complex 1 (mTORC1), a serine/threonine kinase that regulates cell growth and proliferation in response to growth factors and nutrients and regulates primordial follicle activation (38). How these pathways regulate human follicle

FIGURE 1
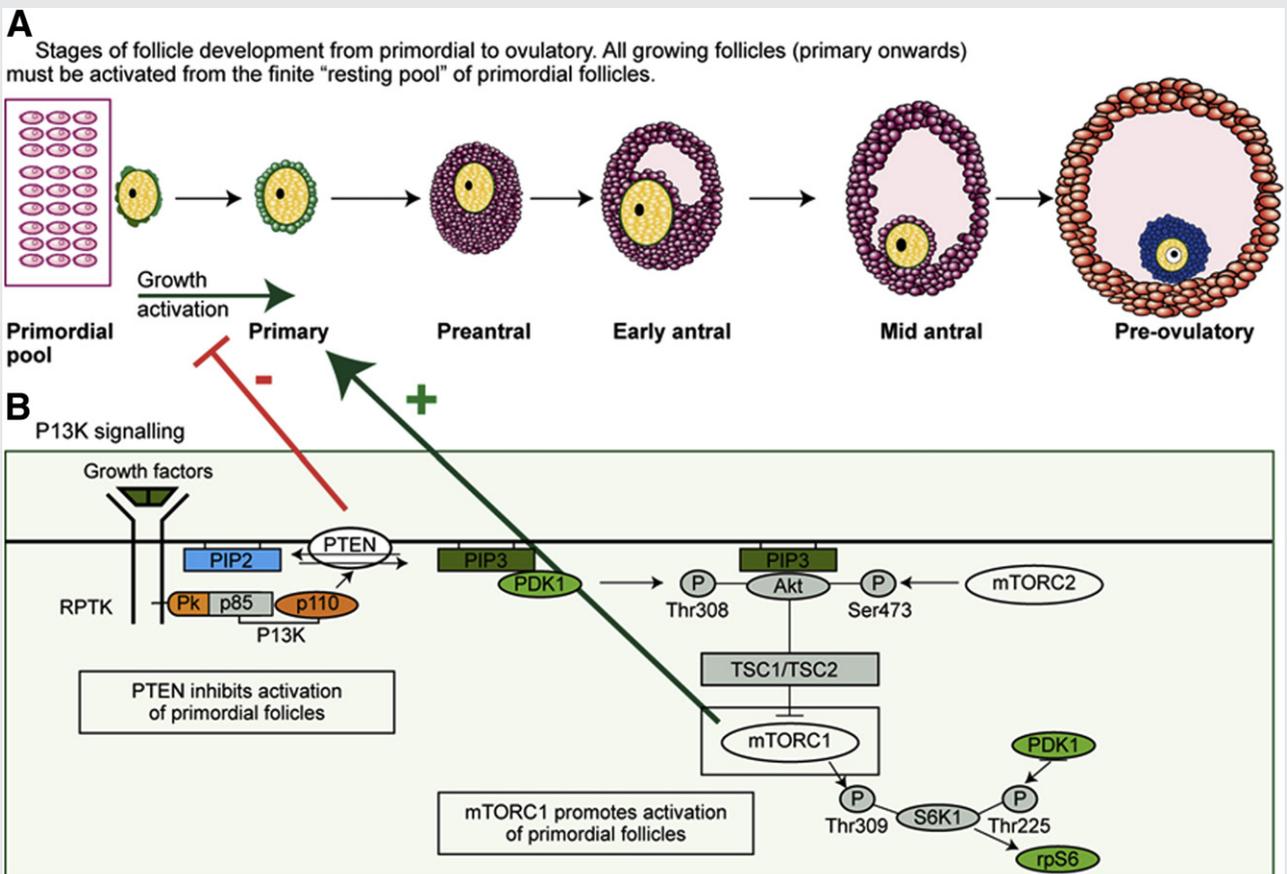
(A) Diagrammatic representation of follicle growth from the nonproliferating pool of primordial follicles. Primordial follicles are continuously activated into the growing population, where they become primary follicles consisting of an oocyte arrested at the dictyate stage of prophase I of meiosis (*yellow*) surrounded by granulosa cells (*green*). Primary follicles undergo oocyte growth and granulosa cell proliferation and differentiation (*purple*), when they form an antral cavity. Antral follicles continue to grow, and granulosa cells differentiate into two subpopulations of cells: 1) cumulus surrounding the oocyte (*blue*); and 2) mural lining the wall of the follicle (*orange*). Exact timings for this developmental sequence in humans are not known; estimations suggest several months, but it is not known whether the growth profile is continuous or whether there are "resting" phases through follicle development. (B) Simplified version of the phosphatidylinositol-3'-kinase (PI3K) pathway. The factors initiating this process are largely unknown, but a body of evidence is emerging to show that the PI3K-Akt signaling pathway is a major regulator of early follicle/oocyte development and that components of this pathway are involved in controlling the rate of activation from the nongrowing population of follicles. The phosphatase PTEN converts phosphatidylinositol phosphate (PIP) 3 to PIP2, which negatively regulates PI3K activity. Signaling mediated by PI3Ks converge at phosphatidylinositol phosphate dependent kinase-1 (PDK1). Phosphatidylinositol-dependent kinase (PDK) 1 phosphorylates Akt and activates it. Akt can phosphorylate and inactivate tuberous sclerosis complex 2 (TSC2, or tuberin), which leads to the activation of mammalian target of rapamycin complex 1 (mTORC1). mTORC1 can phosphorylate (activate) S6 kinase 1 (S6K1). S6K1 subsequently phosphorylates and activates ribosomal protein S6 (rpS6), which enhances protein translation that is needed for cell growth. mTORC1 can be inhibited pharmacologically with rapamycin and stimulated by leucine. The manipulation of this pathway could have important clinical applications in the field of fertility preservation. RPTK = receptor protein tyrosine kinase.

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development is unclear, but culture models facilitate the study of these processes. Using pharmacologic inhibitors of PTEN in vitro, increased activation of presumably human primordial follicles has been demonstrated after xenotransplantation of ovarian cortical strips into immunodeficient mice (39). A recent study using a human culture model has also demonstrated that treatment with rapamycin (an inhibitor of mTORC1) results in decreased activation of primordial follicles as well as oocyte loss in growing follicles (40). Figure 1B illustrates components of the PI3K pathway and their influence on follicle activation.

One limitation of determining primordial follicle activation with the use of cultured human ovarian cortex includes difficulty in assessing the follicular cohort within the starting material. In human (41) and nonhuman primate (42) ovaries, the distribution of follicles within the ovarian cortex is heterogeneous. It is important to note that follicular density can vary more than two orders of magnitude in pieces of human cortical tissues from within the same ovary (41). Each cortical piece has variation in number and stage distribution of follicles, so it is difficult to compare numbers of follicles in a piece of control starting tissue with those in pieces

subjected to different treatments in vitro (43, 44). Recently, a procedure using the vital dye neutral red was developed for visualizing preantral follicles within ovine cortical pieces and successfully applied to determining follicular density in human cortical tissue (45, 46). Whether primordial follicles can develop normally within the human or nonhuman primate ovarian cortex during in vitro culture after exposure to neutral red is not known, but if so, this could prove to be a useful tool for identifying pieces of cortex containing viable primordial follicles for experiments on follicle activation, as well as for screening pieces of tissue before ovarian transplantation.

Human primordial follicles can be activated to grow and develop within mechanically loosened cortical pieces. Multilaminar preantral (secondary) stage follicles can be detected in this system after 6 days of culture (16). This system differs from those described in other studies (17, 26) because the culture medium is serum free and no supporting matrix is present. The vital step in this process lies in the preparation of tissue which involves the removal of most of the underlying stromal tissue and any growing follicles (Fig. 2). When these small fragments of human ovarian cortex are cultured, there is a significant shift of follicles from the quiescent to the growing pool over short culture periods of 6–10 days (16), an observation repeated in cows, where extensive primordial activation has been reported within 2 days in vitro (22, 47, 48), indicating that activation results from a release from intraovarian factors that act to inhibit the initiation of follicle growth (49) (Fig. 2). It is clear that tissue shape and stromal density are important factors that contribute to the regulation of follicle growth initiation in vitro, because solid cubes or strips (1 mm thick) of human cortical tissue show less growth initiation (17) and a high proportion of atretic follicles (50) compared with cortex cultured as flattened "sheets" where much of the underlying stroma is removed (16). Likewise, relatively thin pieces of macaque cortical tissue ($0.5 \times 1 \times 1$ mm) exhibit atretic oocytes and follicles within contracted nonviable tissue by 7 days of culture (Ting, personal communication). The physical environment of the follicles within the cortical tissue affects their response to stimulatory and inhibitory factors and thus influences their ability to grow (49).

Primordial follicles isolated enzymatically from nonhuman primate (42) and human (50–52) ovarian tissue also spontaneously grow in vitro. Remarkably, both human and macaque primordial follicles initiate their growth at similar sizes ($\sim$30–40 μm) after isolation and grow to identical diameters (50–58 μm) after 1 week in culture. In both species, concomitant with the small increase in diameter, granulosa cells surrounding the oocyte apparently proliferate in a subset of these follicles and can be morphologically identified after fixation for hematoxylin and eosin staining (42) or transmission electron microscopy with toliduine blue staining (52), as depicted in Figure 3. Human primordial follicles were reported to reach secondary stages, based on the presence of more than one layer of granulosa cells (52), despite their diameters being much less than that typical for secondary follicles (150–225 μm) isolated from human (29) and macaque (27, 28) ovarian cortex. The discrepancy may be attributed to the lack of appreciable oocyte growth, as reported for macaque primordial follicles (42), which is believed to occur in primates concomitant with the rapid proliferation of granulosa cells associated with the secondary stage (15). Therefore, the multilayer "secondary" morphology may actually represent an earlier developmental stage when the oocyte is just initiating its growth. In these studies, groups of primate primordial follicles were cultured within alginate matrices, in which inhibitory interactions may be more localized, although recent studies in mice suggest that group culture of primordial follicles in alginate confers an advantage to subsequent growth via paracrine mechanisms (53). Until primate primordial follicle culture is extended to intervals longer than 1 week, it is not known whether currently reported culture conditions can support further growth of both follicles and oocytes beyond "early" secondary stages.

Once follicle growth has been initiated within cortical tissue, human follicles can develop to multilaminar stages. Large multilaminar follicles do not survive well within the cortical environment, and it appears to be inhibitory to further growth, resulting in a loss of follicle integrity and oocyte survival (16, 26). Therefore, to support further development, it appears that multilaminar follicles need to be released from the cortical stromal environment and cultured individually to limit the effect of follicle interactions (16, 22) and/or oxidative damage and nutrient diffusion into the tissue in vitro (Fig. 2).

Early Follicle Development In Vitro

Because of the limited amount of human tissue available for clinical use and research, as well as declining follicle numbers in specimens obtained from older versus younger patients, optimizing the yield of good-quality follicles is important. Isolation of preantral follicles from cortical tissue after culture can be achieved by mechanical dissection, enzymatic isolation, or a combination of both. Although enzymatic isolation with the use of collagenase and DNase to remove preantral follicles from stromal tissue results in more follicles than by mechanical dissection (54, 55), it method can cause damage that leads to poor survival of follicles. Growing follicles need theca layers to retain their structure and to survive the second stage of IVG, and collagenase treatment may compromise those layers (56, 57). Some of this damage may be avoided by using new purified enzyme preparations, including liberase (42, 43, 52, 58). Careful mechanical isolation with the use of fine needles has the advantage of preserving follicular integrity by maintaining the basal lamina and theca layers of the follicle, but the yield is low and the procedure slow because of the dense fibrous cortical tissue in human ovaries where follicles are embedded and relatively inaccessible. In contrast, hundreds of secondary follicles can be isolated from the similarly dense and fibrous ovarian cortex in a pair of rhesus macaque ovaries (27, 28), and this is also age dependent as tissue from older primates yields fewer follicles (27).

When culturing large mammalian follicles, the use of V-shaped microwell plates has allowed maintenance of

FIGURE 2
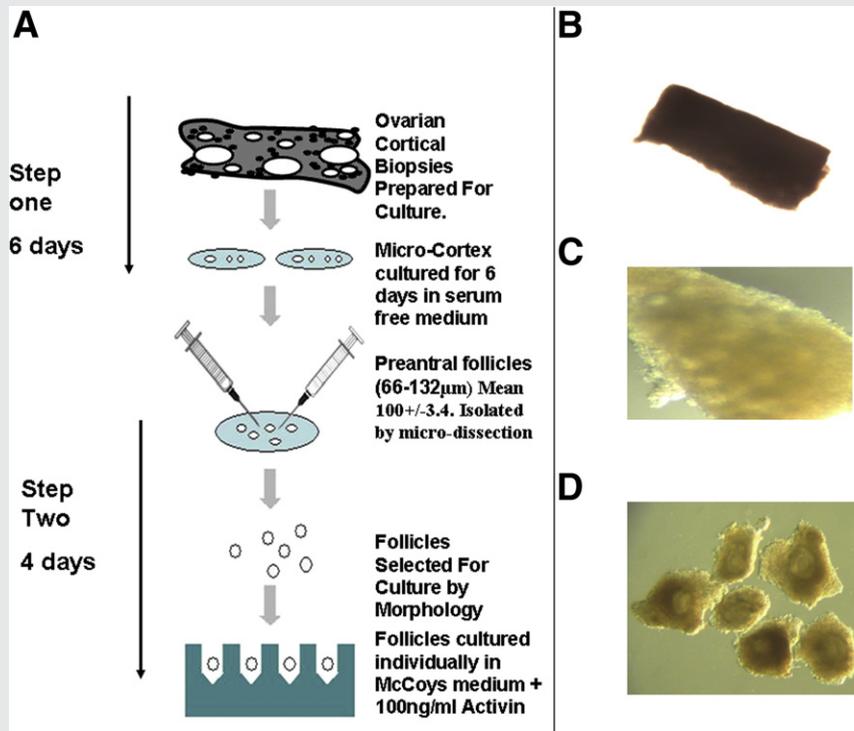
Two-step culture system to obtain growing human follicles. (A) Outline of the preparation of human ovarian cortical biopsies to initiate development of primordial follicles (step 1) and after 6 days in serum-free medium dissect out multilaminar growing follicles and grow these individually (16). Photomicrograph of freshly prepared microcortex (B) before culture and (C) after 6 days of culture followed by (D) dissection to isolate multilaminar follicles with theca cells attached. Isolated follicles can be cultured individually as outlined in A, and antral formation occurs during a further 4-day culture period.
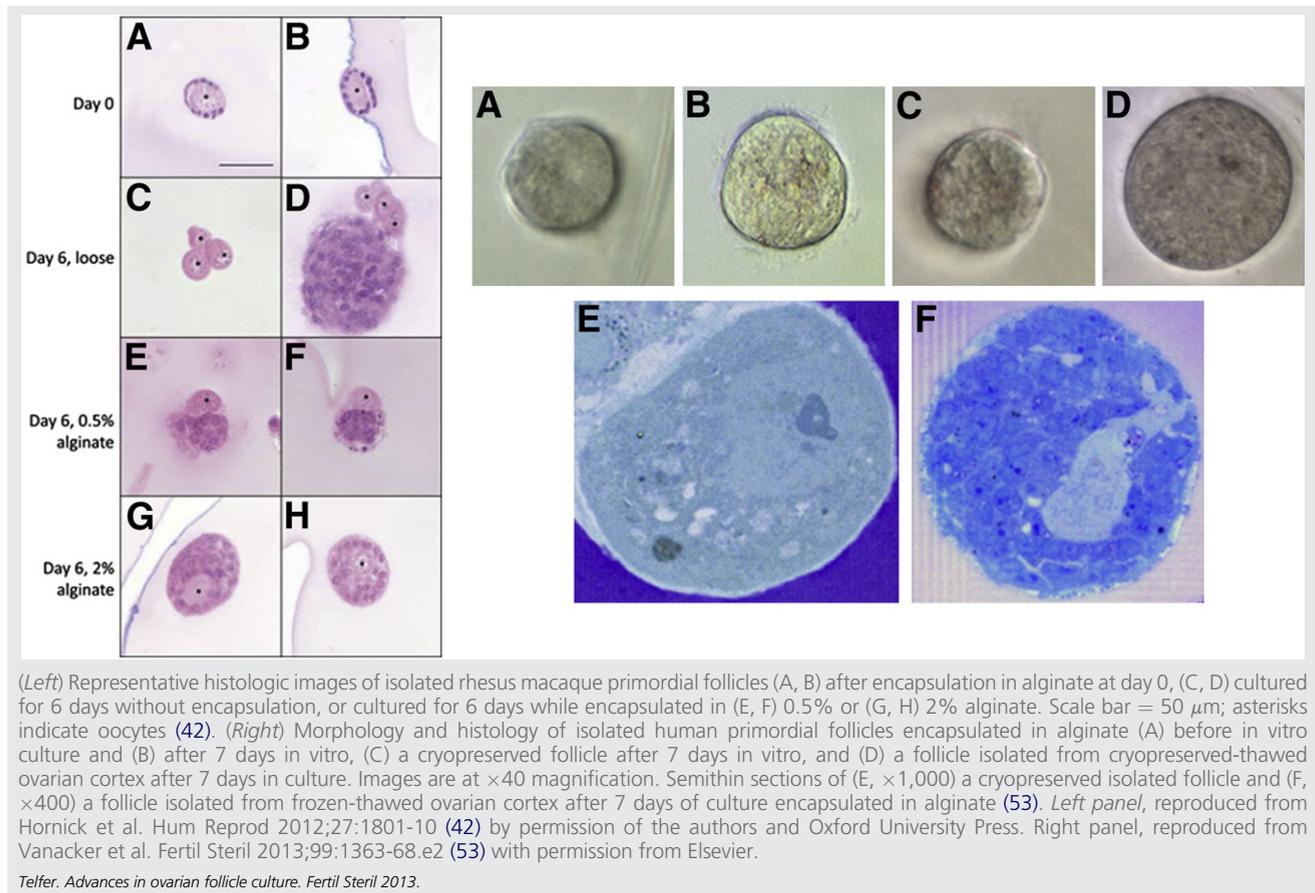
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three-dimensional (3D) follicular architecture in vitro while promoting growth and differentiation in cow (59–61) and human (16) follicles, with antral formation occurring within 10 days. In addition to V-shaped microwell culture plates, follicle encapsulation in alginate hydrogels has been used to support secondary human (29), rhesus monkey (27, 28, 30), and baboon (31) follicle growth in vitro. Alginate encapsulation is thought to mimic the extracellular matrix in vivo regarding its ability to facilitate nutrient, oxygen, hormone, and growth factor exchange between the follicle and the culture medium. Furthermore, its flexibility can accommodate cell proliferation, but its rigidity prevents dissociation of the 3D follicular unit. The rigidity of the alginate capsule affects follicle development, because inhibition of growth and reduced steroidogenesis have been reported in mouse follicles embedded in 1% alginate gels (62), whereas fully grown human (29) and macaque (27, 28, 30) oocytes have been produced with the use of 0.5% and 0.25% gels, respectively. Indeed, a more rigid environment of 2% alginate supported macaque primordial follicle growth in vitro (42).

The progression of human follicles after isolation from the cortex is remarkable. In the presence of FSH, enzymatically isolated secondary human follicles can differentiate, become steroidogenically active, and complete oocyte growth in 30 days (29) (Fig. 4). Quiescent follicles activated to grow within cultured fragments of cortex and mechanically isolated as secondary follicles become steroidogenic and undergo differentiation after a 10-day in vitro period with or without activin (16). These observations confirm that local ovarian factors inhibit follicle development in vivo; however, the question remains whether the growth rate observed in vitro is accelerated or whether it represents growth without the brakes that are required in vivo to regulate follicle development within the context of the reproductive cycle. Likewise, recent advancements in understanding primate folliculogenesis have been modeled in nonhuman primates, where preantral follicle growth in vitro is initiated at the secondary stage. By this stage, early follicles have undergone activation and selection through two steps of folliculogenesis in vivo. Macaque secondary follicles (125–225 μm diameter) isolated mechanically and encapsulated in alginate can survive ($\sim$50% of the total isolated) and routinely achieve growth to the small antral stage (1 mm diameter) in 4–5 weeks under defined culture conditions (27, 28) (Fig. 4). Follicle survival and growth in vitro is a function of stage of the menstrual cycle, ovarian age, oxygen concentration during culture, and inclusion of fetuin in the medium (27, 28, 30).

FIGURE 3

(*Left*) Representative histologic images of isolated rhesus macaque primordial follicles (A, B) after encapsulation in alginate at day 0, (C, D) cultured for 6 days without encapsulation, or cultured for 6 days while encapsulated in (E, F) 0.5% or (G, H) 2% alginate. Scale bar = 50 μm; asterisks indicate oocytes (42). (*Right*) Morphology and histology of isolated human primordial follicles encapsulated in alginate (A) before in vitro culture and (B) after 7 days in vitro, (C) a cryopreserved follicle after 7 days in vitro, and (D) a follicle isolated from cryopreserved-thawed ovarian cortex after 7 days in culture. Images are at ×40 magnification. Semithin sections of (E, ×1,000) a cryopreserved isolated follicle and (F, ×400) a follicle isolated from frozen-thawed ovarian cortex after 7 days of culture encapsulated in alginate (53). *Left panel*, reproduced from Hornick et al. Hum Reprod 2012;27:1801-10 (42) by permission of the authors and Oxford University Press. Right panel, reproduced from Vanacker et al. Fertil Steril 2013;99:1363-68.e2 (53) with permission from Elsevier.

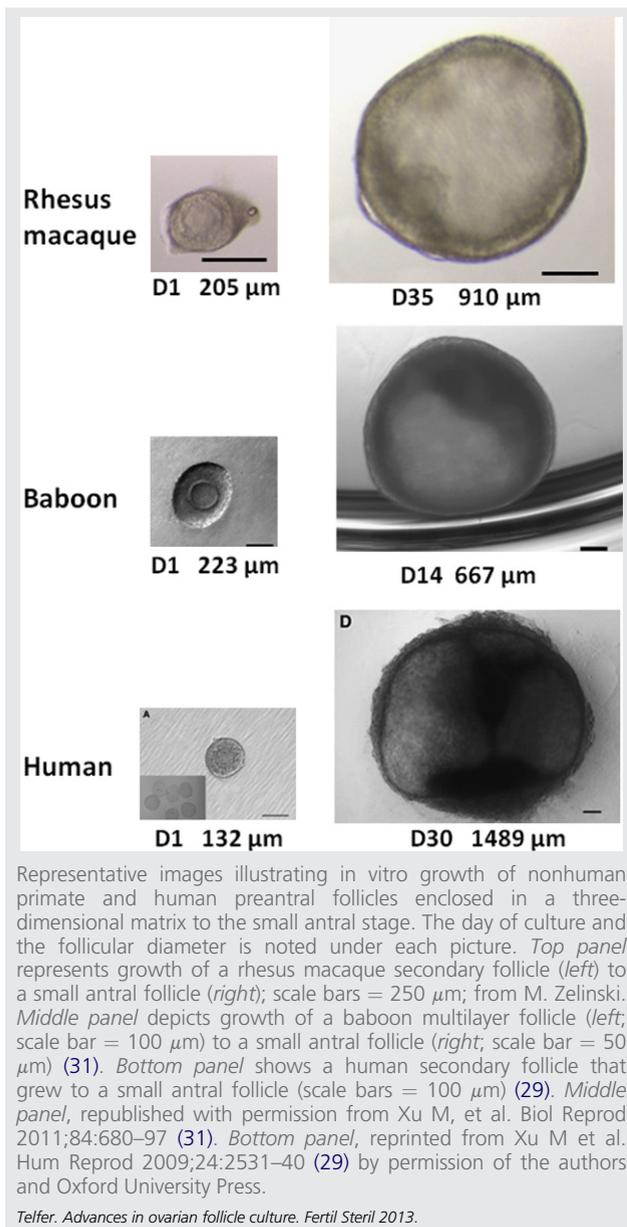*Telfer. Advances in ovarian follicle culture. Fertil Steril 2013.*

A semidegradable matrix containing fibrin, alginate, and Matrigel also facilitated baboon follicle expansion and antrum formation in vitro (31) (Fig. 4).

Notably, surviving secondary follicles from macaques were heterogeneous regarding their growth rates in vitro when encapsulated in alginate as single follicles, a consistent feature of secondary follicles in this system in every animal studied to date. Three distinct cohorts were observed based on their diameters at week 5 (27, 28). The cohort that does not significantly change in diameter (<250 μm) is termed "no-grow" follicles. "Slow-grow" follicles represent the cohort that doubles their diameters (250–500 μm). And the group that increases their diameters at least threefold (>500 μm, in some instances >1 mm) are designated "fast-grow" follicles. All slow- and fast-grow follicles exhibit an antral cavity within 3–4 weeks of culture. Differences in follicle growth rate of single human secondary follicles encapsulated in alginate were less obvious, though it was noted that the majority of follicles developed antral cavities, whereas others remained at the multilayer stage through 4 weeks of culture (29). Thus, the population of secondary follicles in the primate ovary at early follicular phase of the cycle is heterogeneous in the capacity to grow in vitro. Growth rates may differ owing to follicle response to FSH or other hormones (27, 28), or they may respond to other local factors that regulate follicular growth. The ability of human (29) and macaque (27, 28) follicles to

produce steroid hormones is also a function of follicle growth. Only growing follicles synthesized increasing levels of steroids beginning at antrum formation, with levels of E_2, androstenedione, and P greater in fast- than in slow-grow follicles. Vascular endothelial growth factor (VEGF) production also parallels steroidogenesis in growing follicles (27) and may reflect a requirement for vascularization by the small antral stage follicle to achieve further development in vivo with additional substrates and release of hormones. In addition to an angiogenic action, VEGF may protect antral follicles from apoptosis and atresia. Interestingly, antimüllerian hormone (AMH) production by growing follicles exhibited a transient elevation at weeks 3–4 of culture; levels were greater in fast- than in slow-grow follicles (28). These data suggest that AMH may be a biomarker for early preantral follicle growth, in addition to being an important local regulator of folliculogenesis in primates. Direct actions of AMH on primate preantral follicles await demonstration.

Xu et al. (63) recently developed a serum-free alginate-encapsulated culture system for isolated primary follicles from rhesus macaques. A number of unique features were noted regarding secondary follicle culture, in that primary follicles: 1) required 13 weeks to achieve the small antral stage in vitro; 2) displayed only two growth patterns in vitro, no-grow and fast-grow; 3) grew to secondary follicles within 4 weeks, at which time they produced AMH; and 4) did not

FIGURE 4

Representative images illustrating in vitro growth of nonhuman primate and human preantral follicles enclosed in a three-dimensional matrix to the small antral stage. The day of culture and the follicular diameter is noted under each picture. *Top panel* represents growth of a rhesus macaque secondary follicle (*left*) to a small antral follicle (*right*); scale bars = 250 μm; from M. Zelinski. *Middle panel* depicts growth of a baboon multilayer follicle (*left*; scale bar = 100 μm) to a small antral follicle (*right*; scale bar = 50 μm) (31). *Bottom panel* shows a human secondary follicle that grew to a small antral follicle (scale bars = 100 μm) (29). *Middle panel*, republished with permission from Xu M, et al. Biol Reprod 2011;84:680–97 (31). *Bottom panel*, reprinted from Xu M et al. Hum Reprod 2009;24:2531–40 (29) by permission of the authors and Oxford University Press.

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produce steroids or VEGF until they began to rise with the initiation of antrum formation at 9 weeks in vitro. Thus, macaque primary follicles encapsulated in alginate grow very slowly and appear to be quiescent for a month regarding production of somatic cell–derived hormones and factors until granulosa cells proliferate with the beginning of secondary follicle formation in vitro. For the first time, a mature oocyte was produced by an isolated macaque primary follicle in 3D culture, but it developed only to a zygote after insemination in vitro. Because primary follicles are more abundant within the ovarian cortex than secondary follicles, they can provide an additional cohort of growing preantral follicles for potential production of mature gametes in vitro.

A question arises as to just how large primate antral follicles need to progress to produce a competent oocyte.

The typical diameter of a preovulatory follicle in macaques is 5–6 mm, whereas it is much larger, 15–20 mm, in humans. This poses a technical challenge for growing preantral follicles to preovulatory diameters in vitro. Recent data provide evidence that perhaps achieving the preovulatory size is not necessary, and that preantral follicles can be grown to much smaller antral sizes compatible with oocyte growth, maturation, and competence. In macaques, small antral follicles can be derived from the medulla of ovaries during early follicular phase. This pool of follicles (0.5–2 mm diameter) represents the cohort from which the single dominant preovulatory follicle is selected. Developmental competence of macaque oocytes to the blastocyst stage was possible when oocytes matured in vitro were derived from 1–2-mm small antral follicles (64). Definitive confirmation of oocyte developmental competence from follicles smaller than preovulatory diameters was recently provided by Guzman et al. (65), who reported live births from in vitro matured (IVM) human oocytes retrieved from antral follicles ≤6 mm.

Table 1 summarizes the growth characteristics and oocyte outcomes of growing primate follicles encapsulated in varying alginate matrices. Collectively, these studies represent the most recent advances in 3D primate follicle culture and demonstrate the principle that all classes of growing preantral follicles can contribute to the reproductive potential of women. Furthermore, these studies provide a glimpse into the follicle selection process, in that cohorts of primordial, primary, and secondary follicles isolated from the ovarian cortex are not homogeneous regarding their survival, growth rates, and endocrine/paracrine functions in vitro. With the use of a 3D supportive matrix, primary and secondary follicles maintained bidirectional communication between somatic cells and the germ cell, creating an environment conducive to oocyte growth and steroid production. Clearly, current culture systems can support somatic cell functions of the preantral follicular unit. The next crucial step is to demonstrate whether the oocytes produced in these systems are capable of IVM and to determine whether the growth pattern in vitro is deleterious to oocyte function, epigenetic changes, and health.

Development of Fully Grown Oocytes

The ultimate aim of a follicle culture system is to obtain developmentally competent oocytes; therefore, these oocytes need to be matured in vitro. IVM of oocytes already exists as a separate strategy and is used routinely in human assisted reproductive technology processes with varying degrees of success (66). As discussed earlier, achieving and sustaining oocyte growth is the major objective of any complete in vitro development system, because this is a size-specific indicator of the oocyte's ability to resume meiosis (67, 68). The system must also be capable of supporting nuclear maturation and cytoplasmic differentiation of oocytes in vitro (69).

It is widely accepted that although 40%–80% of immature human oocytes can successfully complete IMV and IVF giving rise to live births, the rate of maturation of immature oocytes is still well below that of oocytes harvested from stimulated ovaries, indicating that the protocols are suboptimal or that many of the harvested oocytes are intrinsically unable to

TABLE 1

Growth characteristics and oocyte outcome of nonhuman primate and human growing follicles in 3-dimensional culture.

Species	Ref.	3D matrix	Initial follicle stage (diameter, μm)	Days to antrum formation	Days to antral stages ($\geq$500 μm)	IFM or IVM	Oocyte nuclear stages reported at recovery	Fertilization and embryo development
Rhesus	27, 28	Alginate	Secondary (125–225)	21	35–40	IFM	GV, MII, degenerating	ICSI, early cleavage
Rhesus	63	Alginate ± fibrin	Primary (80–120)	63	91	IFM	GV, MII, degenerating	ICSI, zygote
Baboon	31	FAM	Multilayer (250)	10	≥14	IVM	GV, IVM to MI and MII	Not inseminated
Human	29	Alginate ± Matrigel	Secondary (125–225)	15	≥35	IFM	GV	Not inseminated

Note: FAM = semidegradable fibrin, alginate, Matrigel matrix; ICSI = intracytoplasmic sperm injection; IFM = in-follicle maturation (rhesus: 1,000 IU r-hCG in medium, oocytes harvested and inseminated 34 h after hCG; human: hCG was not administered); GV = germinal vesicle (intact oocyte); IVM = in vitro maturation (cumulus-oocyte complex removed from antral follicle, FSH/LH/hCG/epidermal growth factor in medium, oocytes denuded and inseminated 42–46 h later); MI = metaphase I oocyte; MII = metaphase II oocyte.

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undergo maturation (66). IVG oocytes may require a further period of growth within the cumulus complex before maturation (22). One way to achieve this final growth phase of the oocyte is removal of the oocyte–cumulus cell unit from the antral follicle. Maintaining oocyte–somatic cell interactions and cytoskeleton stability is important at this stage, and in cow follicles it has been demonstrated that the correct balance of activin and FSH in vitro affects these processes (22).

Oocyte growth within human complexes has been demonstrated in vitro (33), and live births have been reported with the use of cow oocyte–granulosa cell complexes that were aspirated from immature follicles and grown for 14 days until the oocyte was large enough to be matured in vitro (70). Compact cumulus-oocyte complexes isolated from IVG baboon antral follicles underwent IVM to yield oocytes that reinitiated meiosis to the metaphase II stage, with a normal appearing spindle structure (31). Fertilization was not attempted in that study. These results give encouragement that a similar system could be applied to human cumulus-oocyte complexes aspirated from IVG follicles to achieve oocyte diameters suitable to undergo IVM.

Alternatively, in vitro developed follicles that reach the antral stage can also be treated with recombinant hCG in the medium to examine reinitiation of meiotic maturation of the oocyte within the follicle (27, 28, 63). Healthy and degenerating oocytes are obtained from small antral stage (0.5–1 mm diameter) IVG macaque follicles. The majority of healthy oocytes ($\sim$100 μm diameter) remain at the germinal vesicle stage. However, metaphase II–stage oocytes ($>$110 μm) within an expanded cumulus matrix have been retrieved from hCG-treated macaque antral follicles after growth from both primary and secondary follicles under chemically defined conditions. Fertilization can occur after insemination by intracytoplasmic sperm injection, but to date only the resulting zygotes from secondary follicle culture cleave, and embryonic development is arrested within 3 days in vitro. Thus, the few oocytes obtained from IVG primary and secondary macaque follicles with the use of the alginate encapsulated culture system can achieve nuclear maturation and fertilization, but they do not complete the cytoplasmic maturation necessary for embryonic development.

Evidence of normal offspring derived from IVG of primordial (5, 6) and secondary follicles both with (11) and without (7, 71) alginate in mice provides an impetus for considering the feasibility of follicle culture to someday achieve births in nonhuman primates, followed by clinical translation to humans. A realistic concern remains whether culture systems would confer interference at the epigenetic level and perhaps alter imprinting patterns in oocytes and the maintenance of genomic imprinting required for subsequent normal embryonic development. Although only a few investigations have been made to date, correct DNA methylation establishment has been observed in mouse and sheep oocytes derived from IVG follicles (72). Whether normal imprinting establishment and maintenance would be sustained in primate oocytes and embryos derived from follicles grown in defined culture conditions is currently unknown.

Because only a small proportion of preantral follicles reaching the antral state in vitro enclose fully grown oocytes surrounded by cumulus cells, current culture systems favor somatic cell function perhaps at the expense of the oocyte, as they fall short of supporting oocyte-granulosa cell communication, leading to oocyte competence. Oocyte-derived factors, such as growth/differentiation factor 9 and bone morphogenetic protein 15, secreted during their characteristic stages of folliculogenesis would be logical biomarkers of oocyte function to assess during in vitro follicle culture (20). However, reagents for noninvasive assays of oocyte-specific factors to evaluate oocyte function while enclosed in preantral follicles during their growth and development in vitro are not yet available for routine use. Furthermore, if oocytes were healthy and functioning normally within the cultured follicles, there should be no need to add exogenous oocyte-specific factors; however, these experiments have not yet been performed with primate follicles. Thus, manipulation of current follicle culture systems (i.e., media components) to optimize oocyte–somatic cell communication throughout growth to the antral stage in vitro are limited to morphologic measurements and hormone production of somatic cells, with invasive removal of cumulus-oocyte complexes at the end of culture to assess oocyte viability and competence.

CONCLUSION

Major hurdles remain for improving culture systems to promote coordinated development of the cumulus-oocyte complex to achieve oocyte growth and the competence to produce embryos, and to attain live offspring as the ultimate clinical end point. Current yields of mature oocytes from IVG follicles are low; therefore, a concomitant goal is to increase the efficiency and reproducibility of producing mature competent oocytes. This is no small task considering the complex systems that regulate oocyte development and ultimate function (73). A looming challenge remains in evaluating the utility of current culture systems to support the growth and function of cryopreserved-thawed follicles for clinical use in patients who have banked ovarian tissue. Early investigations are yielding promising results. The survival and growth of preantral follicles from frozen-thawed human (51) and vitrified-thawed macaque (44, 74, 75) ovarian tissue has been recently demonstrated. Continued growth of alginate-encapsulated secondary follicles from vitrified ovarian tissue to the antral stage in macaques is accompanied by steroidogenesis in vitro (44, 74, 75). Individually cryopreserved macaque preantral follicles (76) and human follicles enclosed in biomatrices before cryopreservation (53) can also survive and grow in vitro after thawing.

Advances in bioengineering novel matrices that support follicle survival, growth, somatic cell differentiation, and germ cell maturation, as historically and elegantly demonstrated in the mouse model, are critical for further progress in human follicle culture. For example, biodegradable matrices with interpenetrating networks (IPN) of fibrin (77) can be engineered to link proteins within the matrix allowing local and sustained delivery of growth factors. Indeed, a novel VEGF-containing IPN matrix supported folliculogenesis and

oocyte maturation, resulting in live offspring when transplanted to the ovarian bursa in mice (78). These new matrices will allow studies of individual or combinations of growth factors to improve folliculogenesis in vitro that supports the coordinated development of somatic and germ cells necessary for embryonic competence and production of live offspring. Emerging design of hydrogels containing cross-linked peptides that degrade in response to proteases secreted by the follicle can locally allow follicle expansion while maintaining the integrity of the hydrogel essential for supporting the 3D architecture of the follicle in vitro that is essential for supporting oocyte growth and competence (79). In contrast to alginate, which is not degradable, these synthetic hydrogel systems with "tunable" properties allow the follicle to influence its own local environment. These systems will enhance the ability of human follicles to volumetrically expand beyond the 1-mm diameter currently achieved with the alginate matrix to diameters (i.e., 5 mm in women) that represent those capable of producing competent oocyte and live births in vivo.

Technologies for human follicle culture today are comparable to the early beginnings of human IVF. Consistent and careful research in human and nonhuman primate models will focus on optimizing culture conditions that favor efficient production of meiotically and developmentally competent oocytes from fresh or cryopreserved ovarian tissue that can be performed in a clinical infertility setting (21). While aiming toward the lofty goal of fertility preservation, the amazing recent advancements in the culture of individual or groups of primate follicles in vitro is also providing insights into the basic biology of primate folliculogenesis that have never before been possible. Whether these systems ever reach clinical application or not, they are still invaluable for providing insight into the follicular system.

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