IN-VITRO INDUCTION OF MEIOTIC DIVISION IN FOLLICLE-ENCLOSED RAT OOCYTES BY LH, CYCLIC AMP AND PROSTAGLANDIN E₂

A. TSAFRIRI, H. R. LINDNER, U. ZOR AND S. A. LAMPRECHT

Department of Biodynamics, The Weizmann Institute of Science, Rehovot, Israel

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Summary. Isolated Graafian follicles cultured intact were used as a test-system for the meiosis-inducing action of hormonal preparations and for analysing the mediation of this hormone effect.

When enlarged follicles were explanted from rats on the day of pro-oestrus before 14.00 hours, i.e. before the preovulatory LH-surge, the oocytes remained in the dictyate state of meiosis throughout an 18-hr culture period. Completion of the first meiotic division could be induced by addition of LH or prostaglandin E₂ to the culture medium, or by microinjection of dibutyryl cyclic AMP into the antrum of the cultured follicle. Addition of HCG or FSH to the culture medium was also effective, though possibly due to LH-like activity present in these preparations. Prostaglandin F_{2α}, at 2.8×10^{-5} M, was only partly effective; prolactin, progesterone, 20 α -dihydroprogesterone, oestradiol-17 β , linolenic acid and adenosine-5'-monophosphate were completely ineffective. The maturation-inducing action of LH was not blocked by cyanoketone, an inhibitor of steroid synthesis.

Addition of LH to the culture medium stimulated the formation of cyclic AMP by the isolated follicles. Exogenous cyclic AMP enhanced protein kinase activity in the supernatant fraction of follicular homogenates.

It is proposed that the action of LH on oocyte maturation involves the mediation of the adenyl cyclase/cyclic AMP system and possibly of the prostaglandins. An action of steroids could not thus far be implicated. The experimental model described permits study of the mechanism of the meiosis-inducing action of LH under controlled conditions *in vitro*.

INTRODUCTION

Oocytes of many mammalian species, irrespective of their stage of development, will mature in hormone-free media *in vitro* if artificially released from their follicles (Pincus & Enzmann, 1935; reviewed by Schuetz, 1969). Although of potential practical utility (Edwards, 1965; Edwards, Bavister & Steptoe, 1969), this model can hardly represent the physiological events, since ovum maturation *in vivo* is clearly dependent on the release of hypophysial gonadotrophin(s) and precedes the rupture of the follicular wall.

It is not clear which cellular component of the follicle is the primary target of the maturation-triggering action of gonadotrophin, nor what biochemical mechanism underlies this action. In some lower forms, e.g. in echinoderms and amphibia, an extraovarian hormone (radial nerve factor and hypophysial gonadotrophin, respectively) acts on the follicular cells, and these respond by releasing a substance that stimulates the oocyte to resume meiosis: 1-methyladenine in the starfish (Kanatani, 1969) and possibly progesterone in *Rana pipiens* (Smith & Ecker, 1970). Moreover, maturation of mammalian oocytes *in vitro* seems to be favoured by retention of the corona and cumulus cells (Robertson & Baker, 1969; Cross & Brinster, 1970), again suggesting an influence of these follicular cells on the behaviour of the oocyte.

The stimulatory action of LH on ovarian steroidogenesis appears to be mediated by cyclic adenosine 3',5'-monophosphate (cAMP) and is also mimicked by prostaglandins (Robison, Butcher & Sutherland, 1968; Marsh, 1970; Lamprecht, Zor, Tsafiriri & Lindner, 1971). There is no information on whether the adenyl cyclase-cAMP system or the prostaglandins are also implicated in the mechanism by which gonadotrophins effect ovum maturation.

We therefore studied the effect of LH, and of substances known to mimic some of its actions, on the resumption of the meiotic process by rat oocytes cultured while maintaining their normal relationship to the follicular apparatus.

MATERIALS AND METHODS

Animals

Three-month-old Wistar-derived rats of the Biodynamics colony were used, that had shown at least two normal 4-day cycles, as determined by daily vaginal smears, immediately before the start of the experiment. The animals were housed in air-conditioned quarters illuminated between 05.00 and 19.00 hours. They were offered Purina Lab chow (Ralston Purina Co.) and water without restriction.

Culture of follicle-enclosed oocytes

Animals were killed by cervical dislocation either between 08.00 and 12.30 hours or between 19.00 and 20.00 hours on the day of pro-oestrus. The ovaries were collected at once and placed in Petri dishes containing Eagle's medium. Large follicles (about 1 mm in diameter) protruding from the surface of the ovaries were excised under a dissecting microscope (five- to tenfold magnification), using iris forceps and scissors. The follicles from each pair of ovaries (ten to fourteen) were placed on the stainless steel grid of a Falcon organ culture dish (60 × 15 mm diameter, Pl. 1, Fig. 1a) containing in its central compartment the medium, with or without addition of a hormone or other substance to be tested (see below), and in its outer compartment a water-soaked paper ring. The culture dishes were transferred into an incubator maintained at $37.5 \pm 0.5^\circ \text{C}$ and flushed continuously with a mixture of O_2 (66%) and N_2 (34%) saturated with water.

Eagle's medium (Gibco) supplemented with 20% fetal calf serum (Gibco) was routinely used. In one experimental series, serum from rats bled on the

morning of pro-oestrus was substituted for the fetal calf serum. The medium was adjusted to pH 7.3 with 2.383 g/litre HEPES (*N*-2 hydroxyethylpiperazine-*N'*-2'-ethanesulphonic acid, Calbiochem), 0.5 ml/litre of a 7.5% solution of sodium bicarbonate and an appropriate amount of 1 N-NaOH. The buffered medium was sterilized by filtration through a cellulose acetate membrane of 0.22- μ m pore size (Millipore Corp.). Unless indicated in the text, the osmolality of this medium (280 mosmol) was reduced to 210 mosmol by addition of double-distilled sterile water. Penicillin G (Assia, 100 units/ml), dihydrostreptomycin (Assia, 100 μ g/ml) and fungizone (Squibb, 2.5 μ g/ml) were added to the medium. All additions of substances to be tested were made in a volume of 5 μ l/ml medium. Steroid hormones and prostaglandins were first dissolved in absolute ethanol and diluted with water; the final concentration of ethanol in the medium never exceeded 1%. Insulin (Calbiochem; bovine recrystallized, B grade) was dissolved in a minimal volume of 0.01 N-HCl and added to the medium at a concentration of 5 μ g/ml, unless otherwise stated. The other hormone preparations used were LH (NIH-LH-s15), FSH (NIH-FSH-s8), HCG (Organon), progesterone (Ikapharm), oestradiol-17 β (Organon) and 20 α -dihydroprogesterone (Ikapharm). Prostaglandins were the product of Upjohn Company (Kalamazoo, Michigan) and cyclic AMP and its dibutyryl derivative of Boehringer and Söhne (Mannheim). In control incubations, an equal volume of the corresponding vehicle was added to the incubation medium.

After 15 to 18 hr incubation, the walls of the follicles were incised and their contents aspirated with a micropipette and transferred to a depression slide. The ova were then picked up under a stereomicroscope and examined by Nomarski interference contrast microscopy. Ova showing no germinal vesicles and nucleolus were then treated according to the method of Tarkowski (1966) to render the chromosomes visible and examined under phase-contrast with an oil immersion lens.

Microinjection into the follicular antrum in vitro

Isolated follicles mounted on the steel grid of a Falcon organ culture dish were injected with various test substances in a volume of approximately 0.35 μ l/follicle, using a microsyringe (Hamilton Company, Inc.) with a removable needle hub. A glass needle of fine bore (approximately 50 μ m outer diameter) was prepared with the aid of a pipette drawer (E. Leitz, G.m.b.H., Wetzlar) and fitted to the syringe with a Teflon adaptor. A small amount of follicular fluid usually escaped from the puncture hole on withdrawal of the needle so that a small portion of the injected drug was probably lost. Adenosine-5'-monophosphate used in control injections was the product of Sigma (derived from equine muscle).

Production of cAMP

Follicles were incubated for 3 hr with [3 H]adenine (25 μ Ci/2 ml; 2.02 Ci/mM) at 37° C to label the endogenous ATP pool. The tissue was washed free of radioactive adenine and re-incubated for 20 min in the presence of theophylline with added LH or prostaglandin, or in a control medium. The 3 H-labelled cAMP so formed was purified and determined as described by Kaneko, Zor &

Field (1970). Alternatively, the level of cyclic AMP in the tissue was determined by the competitive protein-binding assay of Gilman (1970), following incubation of the follicles with or without added LH or prostaglandin E₂ for 15 min.

Activation of protein kinase

The 27,000 g supernatant of a homogenate of isolated Graafian follicles was incubated for 10 min with or without the addition of cAMP (5×10^{-5} M) in the presence of histone and ³²P-γ-labelled ATP, and net incorporation of ³²P into histone was determined by the method of Miyamoto, Kuo & Greengard (1969) as modified by Gilman (1970).

RESULTS

Action of gonadotrophins on the follicle in vitro

Behaviour of explanted follicles in hormone-free media. The fate of the oocytes in follicles cultured intact depended on the time of their collection. Those explanted on the day of pro-oestrus before 13.00 hours, that is, before the critical period (Everett, 1961; Tsafiriri & Kraicer, 1972) and the onset of the preovulatory LH-surge in our colony (Ayalon, Tsafiriri, Lindner, Cordova & Harell, 1972), retained their germinal vesicle, with few exceptions (about 4%), and did not show any signs of maturation when cultured in the absence of LH (Text-fig. 1). By contrast, 87% of oocytes in follicles collected during the evening on the day of pro-oestrus (19.00 to 20.00 hours) and explanted into hormone-free media had completed meiosis when examined after 12 hr culture. Hence, follicles explanted during the morning of pro-oestrus were used in all subsequent experiments designed to detect the meiosis-inducing action of LH or other agents.

The criteria for resumption of meiosis by the cultured oocytes were (i) dissolution of the germinal vesicle, and particularly the disappearance of its nucleolus; (ii) formation of a perivitelline space; (iii) extrusion of the first polar body—only rarely demonstrable because of its instability in the rat; and (iv) condensation of the chromosomes, as revealed by cytological examination of stained air-dried preparations, and replacement of the tetrads by dyads. (Many of the oocytes collected after 19.00 hours—about 75%—had already lost their germinal vesicle and nucleolus *in vivo*.)

When using media considered to be isotonic (280 mosmol), many of the oocytes collected on the morning of pro-oestrus and not exposed to LH *in vitro* exhibited cytoplasmic shrinkage, with retraction of the egg membrane from the zona pellucida, or signs of degeneration (Text-fig. 1). These effects on the control oocytes could be avoided by lowering the osmolality of the medium to 210 mosmol, and this procedure was routinely adopted. However, it was later found that oocytes in follicles cultured in LH-enriched media (see below) showed no adverse effects when the tonicity of the medium was increased to 280 mosmol (Text-fig. 1).

Induction of meiosis in vitro by LH. Oocytes in follicles explanted during the morning of pro-oestrus could be induced to resume meiosis in culture by the addition of LH to the medium.

FIG. 1. Culture of intact follicles. (a) Explanted follicles on stainless steel grid (mean pore diameter, 0.3 mm). (b) and (c) Chromosomes of oocytes from follicles cultured in medium containing LH (5 $\mu\text{g}/\text{ml}$): (b) normal second metaphase (21 dyads); (c) abnormal number of dyads (42) due to failure to form first polar body.

(Facing p. 42)

Concentrations in the medium of $0.1 \mu\text{g LH/ml}$ or less had no significant effect on the oocytes. Levels between 0.5 and $1.0 \mu\text{g LH/ml}$ were sufficient to bring about maturation changes in 82% (55/67) of the oocytes, including dissolution of the germinal vesicle, appearance of tetrads and dissociation of the bivalents, but in the majority of oocytes (61%), the process was arrested at this stage and chromosome preparations revealed the presence of 42 dyads (Pl. 1, Fig. 1c), i.e. a tetraploid complement of chromatids. This indicated a disturbance in the separation of the anaphase chromosomes during the first meiotic division and

TEXT-FIG. 1. Effect of culture conditions on LH-induced ovum maturation. The number of oocytes recovered after culture is indicated in parentheses on top of columns. The number of chromosome counts carried out is indicated at side of columns. F.C.S. = fetal calf serum.

failure to extrude the first polar body. Better results (84 to 95% responding oocytes) were obtained with 5 to $10 \mu\text{g LH/ml}$ in the medium and a concentration of $5 \mu\text{g/ml}$ was routinely adopted (Text-fig. 1). Even under these conditions, some of the responding oocytes retained 42 chromosomes. Addition of insulin ($5 \mu\text{g/ml}$) to the culture medium slightly but consistently reduced the incidence of this abnormality and was routinely adopted. Thus, in the presence of LH ($5 \mu\text{g/ml}$) and insulin ($5 \mu\text{g/ml}$) the majority of the responding oocytes (72 to 79%, Text-fig. 1) were found at the end of the culture period to be in second metaphase (containing twenty-one dyads, Pl. 1, Fig. 1b), and morphologically resembled normal fertilizable oocytes.

Substitution of rat serum (see 'Materials and Methods') for fetal calf serum in the medium did not significantly improve the results (Text-fig. 1).

Other gonadotrophic preparations. Of other preparations tested, HCG (5 to 10 i.u./ml medium) and FSH (10 to 20 $\mu\text{g}/\text{ml}$) were also effective in inducing re-sumption of meiosis in the cultured oocytes, but ovine prolactin (1 to 25 $\mu\text{g}/\text{ml}$) was inactive (Text-fig. 2). The FSH preparation used was stated to contain a small amount of LH-like activity (equivalent to 15 μg NIH-LH-s15/mg by an ascorbic acid depletion assay). The minimal effective concentration of FSH in the medium (10 $\mu\text{g}/\text{ml}$) was thus equivalent to 0.15 $\mu\text{g}/\text{ml}$ of LH-like activity in terms of NIH-LH-s15. Though this concentration of LH in itself falls just short of the level required to induce meiosis in our system (0.5 $\mu\text{g}/\text{ml}$), it is possible that FSH and LH act synergistically.

TEXT-FIG. 2. Hormonal induction of ovum maturation *in vitro*. The number of oocytes recovered is indicated in parentheses on top of columns. The number of chromosome counts carried out is indicated within the columns. For description of hormonal preparations used and of experimental conditions, see 'Materials and Methods' section.

Rôle of ovarian steroids. Progesterone (1.6×10^{-5} to 1.6×10^{-4} M), 20 α -dihydroprogesterone (1.6×10^{-5} to 1.6×10^{-4} M) and oestradiol-17 β (10^{-9} to 10^{-15} M) were all completely ineffective in triggering meiotic changes. Furthermore, in a preliminary experiment, the addition of 2 α -cyano-4,4,17 α -trimethyl-androst-5-en-17 β -ol-3-one (cyanoketone; 3×10^{-5} M) to the medium did not prevent the *in-vitro* initiation of oocyte maturation by LH: 79% (30/38) of the explanted oocytes lost their germinal vesicle, though an unusually high percentage of these (68%) failed to extrude the first polar body, as indicated by the retention of 42 chromosomes. Neither oestradiol-17 β (10^{-9} to 10^{-15} M) nor progesterone (3×10^{-6} M) inhibited the meiosis-inducing action of LH.

Rôle of cyclic AMP. Luteinizing hormone markedly stimulated production of cyclic AMP by the isolated rat follicle, whether this was assessed by the rate of incorporation of [^3H]adenine into cyclic AMP (Text-fig. 3), or by determination of the cAMP level in the tissue according to Gilman (1970). In the latter experiments, LH caused an increase (Mean $\pm$ S.E.) in cAMP level from 9.0 ± 0.84 to 216.0 ± 7.6 pmol/mg tissue during 20 min incubation. Cyclic AMP, in turn, enhanced protein kinase activity in the supernatant fraction of homogenates from such follicles: ^{32}P -incorporation into histone increased from a

TEXT-FIG. 3. Effect of LH and prostaglandin E_2 on synthesis of cyclic AMP by isolated Graafian follicles. Isolated Graafian follicles were incubated for 3 hr in a medium containing [^3H]adenine, washed and re-incubated for 20 min in a medium containing theophylline and the substance to be tested; [^3H]cyclic AMP formation was determined as described in the 'Materials and Methods' section.

control level of 8200 counts/min/mg tissue to 29,700 counts/min/mg in the presence of cAMP (5×10^{-5} M). Nevertheless, our attempts to induce ovum maturation *in vitro* by adding cAMP (2.9×10^{-4} to 1.4×10^{-3} M) or its dibutyryl derivative (DBC, 2×10^{-5} to 10^{-3} M) with or without theophylline (5×10^{-3} M) to the medium yielded negative results.

Since the failure of cAMP to elicit a meiotic response when added to the culture medium might be attributable to a permeability barrier, we injected the

dibutyryl derivative of this nucleotide, in another series of experiments, directly into the follicular antrum. The results are shown in Text-fig. 4. When

TEXT-FIG. 4. Induction of ovum maturation *in vitro* by injection of dibutyryl cyclic AMP into the follicular antrum. DBC, dibutyryl cyclic AMP. (The numbers appearing on the columns are explained in the legend to Text-fig. 2.)

TEXT-FIG. 5. Induction of ovum maturation *in vitro* by prostaglandins. PG, prostaglandin. (The numbers appearing on the columns are explained in the legend to Text-fig. 2.)

DBC was applied at the dose level of 6 μg per follicle (equivalent to 4 μg of the free nucleotide), 76% of the oocytes resumed meiosis. Control injections of an

equal volume of the medium or of adenosine-5'-monophosphate (3 to 6 μg) caused only 3 to 19% of the oocytes to lose their germinal vesicle.

Induction of meiosis by prostaglandins. Prostaglandin E_2 mimicked the action of LH in enhancing cyclic AMP formation in the isolated rat ovarian follicles (Text-fig. 3). This finding was corroborated by determining the level of cAMP in the follicles using the method of Gilman (1970). Furthermore, addition of prostaglandin E_2 to the medium (1.4×10^{-6} to 2.8×10^{-5} M) induced completion of the first maturation division in cultured follicles. The higher dose levels (1.4 to 2.8×10^{-5} M) were as effective as the optimal dose of LH (Text-fig. 5). Prostaglandin $\text{F}_{2\alpha}$ was ineffective at 1.4×10^{-5} M and only partly effective even at 2.8×10^{-5} M, and linolenic acid (serving as a control) was completely ineffective. Only three rats were used at each dose-level of prostaglandin $\text{F}_{2\alpha}$, and furthermore, a still higher dose of this compound might well have induced ovum maturation. Nevertheless, it appears that prostaglandin $\text{F}_{2\alpha}$ is less effective in this respect than its E_2 congener.

Behaviour of theca and granulosa cells in cultured follicles

Whether oocyte maturation was induced by LH or prostaglandin E_2 , none of the follicles ruptured during the culture period, even if this was prolonged to 48 hr, and none of the oocytes was spontaneously released. On the other hand, the follicles showed signs of luteinization when culture was continued for 48 hr in the presence of LH, after removal of the oocyte.

DISCUSSION

The work described establishes that follicle-enclosed rat oocytes explanted before the preovulatory LH surge into a hormone-free medium will remain in the dictyate state, but can be induced to resume meiosis by adding LH to the medium. This finding makes it possible, for the first time, to explore the mechanism of the meiosis-inducing action of LH in an *in-vitro* system. A previous attempt (Foote & Thibault, 1969) to induce maturation of mammalian oocytes (cow and sow) in intact follicles *in vitro* by addition of LH or FSH, or by co-culture with a pituitary fragment, failed. The discrepancy may be due to the developmental stage of the follicles explanted, differences in culture conditions, or to interspecific differences.

Ovum maturation could also be induced, in our experiments, by addition of HCG or FSH to the culture medium. Both preparations contained LH-like activity, but the amount of LH present in the meiosis-inducing dose of FSH was probably too low (0.15 $\mu\text{g}/\text{ml}$ medium) to account, in itself, for the effect observed. Harrington, Bex, Elton & Roach (1970) induced ovulation in chlorpromazine-blocked rats with chymotrypsin-treated FSH, almost devoid of detectable LH-like activity, but a participation of the animals' pituitaries in the response cannot entirely be ruled out in their experiments. Definitive proof of an independent action of FSH on oocyte maturation *in vitro* would require the demonstration of its efficacy in the presence of anti-LH antibodies, or the use of a more highly purified FSH preparation.

The majority of the oocytes matured under the culture conditions described,

in the presence of an adequate concentration of LH, reached a stage morphologically indistinguishable from the second metaphase of meiosis. The ultimate test for the normality of such secondary oocytes would be the demonstration of their ability to be fertilized and subsequently to develop normally. This has not yet been attempted.

A proportion of the follicle-enclosed oocytes matured *in vitro* showed a disturbance in anaphase separation of the chromosomes during the first meiotic division, resulting in the retention of a tetraploid chromatid complement. The incidence of this abnormality was increased by suboptimal gonadotrophin concentration in the medium, and reduced by inclusion of insulin in the medium. The reason for this insulin effect is not clear, particularly since follicle-enclosed oocytes explanted after having been exposed to endogenous LH showed no deviation from normal chromosome number even when cultured in insulin-free media. Perhaps insulin assists the initial interaction of LH with its target cell.

Amphibian eggs can be induced to resume meiosis by treatment with progesterone (Smith & Ecker, 1970). Rondell (1970), studying porcine ovaries, proposed that LH induces changes *in vitro* in the distensibility of the follicular wall conducive to ovulation, and that these changes are mediated by progesterone. Moreover, Lipner & Greep (1971) found that ovulation could be prevented in immature PMSG- and LH-treated rats by administration of inhibitors of steroidogenesis, including cyanoketone, within 4 to 6 hr of the LH injection. By contrast, we failed to induce maturation changes in cultured follicles by addition of progesterone, 20 α -dihydroprogesterone or oestradiol-17 β to the medium; nor was the action of LH on the cultured follicles blocked by the addition of cyanoketone to the medium. None of the steroids tested inhibited the action of LH. We have not yet examined, however, whether exogenous progestagens or cyanoketone actually penetrate the follicle.

Cyclic AMP has been implicated in the steroidogenic action of LH in the ovary (Savard, Marsh & Rice, 1965; Channing, 1970). Nevertheless, our attempts to induce ovum maturation *in vitro* by adding cAMP or its dibutyryl derivative to the culture medium were unsuccessful. Direct injection of dibutyryl cAMP into the follicular antrum, however, brought about completion of the first meiotic division, suggesting that entrance of exogenous nucleotide into the follicle may be impeded by the thecal layer(s). These findings, together with the demonstration that LH enhances adenyl cyclase activity in isolated follicles (Text-fig. 3), support the hypothesis that the action of LH on ovum maturation is mediated by cyclic AMP. Furthermore, our experiments showed that addition of exogenous cAMP to a supernatant fraction from homogenized follicles markedly stimulated histone phosphorylation. It is widely held that many of the biological actions of cAMP are mediated by an activation of protein kinase(s) (Gill & Garren, 1971; Reimann, Brostrom, Corbin, King & Krebs, 1971), though it is too early to say whether protein kinases have any rôle in the regulation of ovum maturation.

Endogenous cAMP production by the follicle was also stimulated by prostaglandin E₂, at least to the same extent as by LH. This agent, too, proved capable of inducing ovum maturation *in vitro* with high efficiency. The phenomenon exhibited some degree of specificity in that prostaglandin F_{2 α} was much less

effective per unit weight, and linolenic acid proved inert. Kuehl, Humes, Tarnoff & Ham (1970) proposed that the prostaglandins constitute an essential link in the stimulatory action of LH on cAMP formation and steroidogenesis, but this view has been disputed (Marsh, 1971; Zor, Lamprecht, Kaneko, Schneider, McCann, Field, Tsafiriri & Lindner, 1972). Further work is clearly required to clarify whether the observed effect of prostaglandin E₂ on the follicle is merely a pharmacological one, or whether the prostaglandins have indeed a physiological rôle in the mediation of LH action on the oocyte. The assay of endogenous prostaglandins in the ovary, and the use of inhibitors of prostaglandin synthesis or of prostaglandin action, in conjunction with LH, should be informative in this respect.

The fact that free oocytes complete meiosis spontaneously when cultured may suggest that the LH removes an inhibitory influence exerted on the oocyte by the granulosa cells (Pincus & Enzmann, 1935; Foote & Thibault, 1969); alternatively, the hormone may induce the elaboration and release of a positive signal by the follicular cells, which may be a prostaglandin and/or cyclic AMP, or even a steroid. Finally, LH may merely alter the permeability of the follicular wall to respiratory gases or to a critical metabolite. While many of these issues remain unresolved, the experimental model described seems to offer a chance to analyse in greater detail the rôle of the different cellular and subcellular components of the follicular apparatus in the action of LH on ovum maturation.

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