Hyaluronic Acid Synthesis by Mural Granulosa Cells and Cumulus Cells * in Vitro Is Selectively Stimulated by a Factor Produced by Oocytes and by Transforming Growth Factor-β*

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In ovarian antral follicles cumulus cells (~1,000/follicle) closely surround the oocyte, and mural granulosa cells (~50,000/follicle) are distributed at the periphery. Previous work (Salustri, A., Yanagishita, M., and Hascall, V. C. (1990) Dev. Biol. 138, 26-32) showed that oocytes produce a factor(s) which stimulates hyaluronic acid (HA) synthesis by mural cells during expansion of the cumulus cell-oocyte complex. We now show that mural granulosa cells also respond in vitro to the oocyte factor(s) with greatly increased HA synthesis. As with cumulus cells, a factor(s) present in fetal calf serum is required to retain newly synthesized HA in the extracellular matrix. Unlike cumulus cells, follicle-stimulating hormone (FSH) is not required for maximal stimulation, in part because mural granulosa cells synthesize prostaglandin E2 which can substitute for FSH in promoting cumulus cell-oocyte complex expansion. Several growth factors studied, only transforming growth factor-β1 (TGF-β1) stimulated HA synthesis in both cell types. However, the stimulation of HA synthesis by TGF-β1 was additive with that for the oocyte factor(s), and neutralizing antibodies to TGF-β did not inhibit the response to the oocyte factor(s). The results indicate that the oocyte factor(s) and TGF-β1 are not the same and that they operate through different receptors in stimulating HA synthesis. Epidermal growth factor was able to replace FSH in amplifying the response of cumulus cells to the oocyte factor(s) and in stimulating synthesis of dermatan sulfate proteoglycans.

The antral follicle contains two classes of somatic cells, mural granulosa cells and cumulus cells, which are both originally derived from the follicular cells of the primary follicle (1). The cumulus cells closely surround the oocyte while the mural granulosa cells are distributed at the periphery of the follicle, loosely surrounding the follicular fluid. During the preovulatory period, the cumulus cells synthesize and deposit an intercellular matrix enriched in hyaluronic acid (HA). This leads to expansion of the cumulus cell-oocyte complex (COC) (2, 3). Conversely, the mural granulosa cells do not elaborate a similar intercellular matrix. At the last stage of follicle maturation, as a consequence of this difference, the COC detaches from the follicle wall while the multilayer of mural granulosa cells remains adherent to the outer basement membrane. When COC expansion is nearly complete, ovulation occurs with the release of the COC through the ruptured follicle wall. Conversely, the mural granulosa cells remain in the follicle, contributing to the process of corpus luteum formation (4).

Expansion of compact mouse COCs can occur in vitro if the culture medium contains: 1) an appropriate hormone, such as follicle-stimulating hormone (FSH) or prostaglandin E2 (PGE2), or cAMP analogues to stimulate HA synthesis (5, 6); and 2) fetal calf serum (FCS) to retain the HA in the extracellular matrix (7, 8). However, we recently showed that the oocyte has a crucial role in the mucification process (9). Cumulus cells separated from the oocyte do not expand when they are cultured with FSH and FCS, but they do so when oocytes or oocyte-conditioned media are also added. The soluble factor(s) produced by the oocytes is required by the cumulus cells to initiate HA synthesis, and net synthesis is amplified ~10-fold when FSH is also present (9).

Studies to identify the oocyte and serum factors and to determine their biological roles are hindered because the cumulus cells are difficult to isolate and few in number (~1,000/follicle) (8). Conversely, the mural granulosa cells are more abundant (~50,000/follicle) (10) and easier to isolate. Because the paracrine action of the oocyte is essential to stimulate HA synthesis and induce expansion of the cumulus cell mass, it seemed possible that mural granulosa cells and cumulus cells differ in their ability to produce extracellular matrix in vivo because of their different proximities to the oocyte rather than from a specific differentiation of the two cell populations. The present study provides evidence that this is the case. The results demonstrate that mural granulosa cells obtained from antral mouse follicles synthesize and accumulate HA in vitro in response to the oocyte and serum factors in a manner similar to cumulus cells. For this reason, mural granulosa cell cultures can be used to facilitate studies of the oocyte and serum factors in vitro. Therefore, we have used mural granulosa cell cultures to investigate the possibility that the oocyte factor(s) could be a known growth factor.

**EXPERIMENTAL PROCEDURES**

Materials—The following were obtained from the indicated sources: female Swiss CD-1 mice from Charles River; Eagle's minimum essential medium (with Earle's salt), glutamine, HEPES, and FCS from Life Technologies/GIBCO; bovine serum albumin (BSA) from Miles; guanidine HCl from Life Technologies/Bethesda Research Laboratories; Triton X-100 from Pierce Chemical Co.; papain

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The abbreviations used are: HA, hyaluronic acid; COC, cumulus cell-oocyte complex; FCS, fetal calf serum; FSH, follicle-stimulating hormone; PGE2, prostaglandin E2; BSA, bovine serum albumin; FGF, epidermal growth factor; TGF, transforming growth factor; FGF, fibroblast growth factor; IGF-1, insulin-like growth factor 1; PMSG, pregnant mare's serum gonadotropin; DS, dermatan sulfate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PDGF, platelet-derived growth factor.

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from Boehringer Mannheim; chondroitinase ABC (Protein vulgaris) and chondroitin sulfate disaccharides from Sakaguchi Kogyo (Tokyo) through ICN Biotechnology Inc.; Sephadex G-50 (fine) from Pharmacia LKB Biotechnology Inc.; PGE,
indomethacin, pregnant mares' serum gonadotropin (PMSG), hyaluronic acid (from human umbilical cord), and dimethyl polysiloxane from Sigma; Partisol 5 PAC from Whatman; [35S]sulfate (43 Ci/mg) and [1-6-[3H]glucosamine from Du Pont-New England Nuclear. Recombinant basic fibroblast growth factor (EGF) from Collaborative Research; human platelet-derived growth factor (PDGF), human transforming growth factor-β1 (TGF-β1) and anti-TGF-β antibody with neutralizing activity against TGF-β1 and TGF-β2 from R & D Systems; human mare's serum gonadotropin (PMSG), hyaluronic acid (from human placenta), insulin-like growth factor 1 (IGF-1) from Amgen; highly purified FSH from the National Pituitary Agency, National Institute of Arthritis, Diabetes, and Kidney Diseases, and the National Hormone and Pituitary Program, University of Maryland School of Medicine.

Animals—Eighteen-day-old female Swiss CD-1 mice were injected with 5 IU of PMSG in 0.1 ml of physiological saline and put to death by cervical dislocation 44–48 h later. Ovaries were excised and placed in minimal essential medium buffered with 20 mM Hepes, pH 7.3, and supplemented with 1 mg/ml BSA.

Isolation and Culture of Cumulus Cells and Mural Granulosa Cells—COCs and mural granulosa cells were released into medium by puncturing large antral follicles. Cumulus cell cultures were prepared as described previously (9). Briefly, COCs were collected and transferrized to the bottom of a drop of culture medium covered with dimethyl polysiloxane to prevent evaporation. The COCs were then mechanically dissociated, the oocytes removed, and the cumulus cells cultured as described in the protocols. In all the experiments, cumulus cells obtained from 30 COCs (about 1000–1500 cells/COC) were cultured in 20-μl droplets of medium. In some experiments, cumulus cells were co-cultured in the same drops with the isolated oocytes or cultured as intact COCs.

In order to prepare mural granulosa cell cultures, COCs were removed and the mural granulosa cells transferred to a conical centrifuge tube. They were dispersed by gentle passage back and forth three times through an 18-gauge needle and pelleted by centrifugation for 5 min at approximately 20 × g. The supernatant was discarded and the cells resuspended in culture medium plus 5% FCS at a final concentration of ~20 × 10^6 cells/ml. For the experiment in which indomethacin was used, the mural granulosa cells were isolated as described above except that indomethacin (1 μg/ml) was included throughout. For all experiments, 5-μl aliquots of the cell suspension were added to 15-μl droplets of culture medium containing 5% FCS, isotopes, and other substances as described in the protocols.

Co-cultures were prepared by transferring freshly isolated oocytes in 1–2 μl of medium into mural granulosa cell cultures.

Oocyte-conditioned medium was obtained by culturing isolated oocytes (2 oocyte/μl unless specified in the text) in culture medium plus 5% FCS for 16 to 18 h (unless specified in the text). After this time period, the oocytes were removed and the conditioned medium was distributed in 10-μl droplets under dimethyl polysiloxane. To each droplet 5 μl of medium containing 5% FCS, isotopes, and test substances was added. For mural granulosa cell cultures, 5 μl of a mural granulosa cell suspension was added to each droplet. For cumulus cell cultures, 5 μl of culture medium with 5% FCS and 30 intact COCs were added and the oocytes rapidly dissected free of the cumulus cells and removed.

The culture medium was minimal essential medium supplemented with 0.25 mM pyruvate, 50 μg of gentamicin/ml, and 5% FCS. In the experiments in which the absence of serum was studied, FCS was replaced with 5% BSA. Other test substances such as FSH, PGE, growth factors, and antibodies were at the concentrations specified in the text. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. At least two different experiments were done for each protocol.

Metabolic Labeling of the Cells and Extraction of Radiolabeled Molecules—Oocyte-conditioned medium or media, [35S]sulfate (60 μCi/ml) and [1-6-3H]glucosamine (100 μCi/ml) were added to the culture medium and the cells incubated for 18 h. All manipulation steps of extraction were done under dimethyl polysiloxane. After labeling, media were carefully aspirated with a micropipette, and the cell layer was washed twice with 20 μl of medium. The conditioned medium, 20 μl of a papain solution (750 μIU/ml) in 0.1 M sodium acetate, 5 mM Na₂EDTA, 0.8% (w/v) cysteine HCl, pH 7.3, was added to each. After 1 h of incubation at 65 °C, the extraction was completed by adding 20 μl of 8 M guanidine HCl containing 4% (w/v) Triton X-100. Each cell layer extract was transferred to a tube and brought to 500 μl with 4 M guanidine HCl, 0.5% Triton X-100, 0.5% β-mercaptoethanol, pH 7.0. Media samples were submitted to the same extraction procedure. When total HA and dermatan sulfate (DS) production was analyzed, papain was added directly to the cell cultures. The digestion with papain prior to extraction with guanidine HCl and Triton X-100 increased the recovery of HA from the expanded COCs by about 50% (data not shown). All extracts were stored frozen until further analysis.

Quantitation of HA and DS—The extracts were analyzed for HA and DS contents as described elsewhere (8). Briefly, 100 μg of HA and 100 μg of BSA were added to each sample. They were then chromatographed on Sephadex G-50 columns (2-ml bed volume) equilibrated and eluted with 0.1 M sodium acetate, 0.1 M Tris, and 0.5% Triton X-100, pH 7.3, to remove unincorporated isotopes and guanidine HCl. The eluted macromolecules were digested with chondroitinase ABC (0.1 unit/ml) for 2 h at 37 °C. Aliquots of the digests were chromatographed on Sephadex G-50 columns (4-ml bed volume) equilibrated and eluted with 0.1 M sodium acetate, 0.1 M Tris, and 0.5% Triton X-100, pH 7.3. Fractions were analyzed to determine the proportion of radioactivity degraded by the enzyme and eluting in the included volume (HA and DS disaccharides). The other portions of each digest were analyzed for their relative proportions of HA and DS disaccharides by an high pressure liquid chromatography procedure using a Partisol 5 PAC column (11). The masses of HA and DS synthesized during the labeling period (in hexosamine equivalents) were then determined as described previously (12) by calculating the specific activity of the UDP-N-acetylglucosamine pools from the ratio of H to S in the monosulfated chondroitin-4-S disaccharide derived from the DS.

Radioactivities in samples were determined with a Beckman LS 5801 counter using Ready Safe scintillation fluid (Beckman).

Results

Effect of Oocytes, FSH, and FCS on HA and DS Synthesis by Mural Granulosa Cell Cultures—Mural granulosa cells were prepared as described under "Experimental Procedures" and co-cultivated with 18 with different numbers of isolated oocytes (from 0.5 to 4/μl) in the presence of FCS, FSH (100 ng/ml), and radiolabeled glycosaminoglycan precursors. After incubation, each medium was separated from its respective cell layer, and the contents of HA and DS in both were determined. The results (Fig. 1) show that total HA synthesis by mural granulosa cells increased with an increasing number of oocytes in the co-cultures, nearing a plateau at 2–4 oocytes/μl with a ~10-fold stimulation was obtained. In contrast, DS synthesis remained constant in all the cultures. With the exception of the cultures with no oocytes, which exhibited basal levels of HA synthesis, about 70% of the newly synthesized HA was retained in the cell layer.

Total HA synthesis was stimulated by the oocytes to essentially the same extent either in the presence or the absence of serum. However, there was a significant redistribution of HA; in the presence of FCS, ~65% was retained in the matrix whereas without FCS, only ~35% was retained (Fig. 2). The results indicate that a serum factor(s) is essential to retain HA; in the presence of FCS, ~65% was retained in the matrix whereas without FCS, only ~35% was retained (Fig. 2).

Unlike cumulus cells, however, mural granulosa cells do not require the presence of FSH to respond to the oocyte factor(s) effectively in vitro. When the same experimental protocol shown in Fig. 1 was done in the absence of FSH, nearly identical results were obtained (data not shown, see Fig. 6).
Oocyte Factor and TGF-β1 Stimulate HA Synthesis

**Fig. 1.** DS and HA synthesis by mural granulosa cells cocultured with oocytes. Different numbers of freshly isolated oocytes were added to mural granulosa cell cultures. All cultures contained FCS and FSH and were incubated for 18 h with radioisotopes. Media and cell layer extracts were prepared and analyzed for labeled DS (upper panel) and HA (lower panel) contents.

**Fig. 2.** Effect of FCS on the distribution of HA between medium and cell layer compartments in mural granulosa cell cultures. Cultures were incubated for 18 h with radioisotopes in the presence of BSA or FCS, with (+O) or without oocytes (1 oocyte/µl) as indicated. The amounts of labeled HA in the media and cell extracts were determined.

below). Previous studies have shown that PGE2 can replace FSH to induce COC expansion (7) and that mural granulosa cells secrete sufficient amounts of PGE2 in vitro to stimulate this process when COCs are co-cultured with them (14). Therefore, PGE2 may act as an autocrine factor and synergize with the oocyte factor to stimulate HA synthesis by mural granulosa cells. This was tested by co-culturing mural granulosa cells with isolated oocytes in the presence of 1 µg/ml indomethacin, an inhibitor of prostaglandin synthesis. In this case HA synthesis was significantly reduced, to ~45% of the fully stimulated cultures (Fig. 3). HA synthesis was restored when PGE2 (200 ng/ml) was simultaneously added with indomethacin to the culture. The residual HA synthetic activity in the presence of indomethacin was significantly greater than in the unstimulated (basal) cultures. Because the mural granulosa cells in this experiment were isolated and washed in the presence of indomethacin, it seems likely that this enhanced activity above basal is not related to residual PGE2.

**Effect of Preculture Time on Mural Granulosa Cell Response to Oocytes**—The stability of the response of the mural granulosa cells to the oocyte factor(s) during the culture time was assessed. Cultures were established (time 0), and freshly isolated oocytes were added together with the isotopes at different times of incubation. Total HA and DS contents were determined after 18 h of labeling in each case (Fig. 4). The response of the cells to the oocyte factor(s) decreases linearly with increasing preculture time from 10–15-fold stimulation of HA synthesis without preculture to 2–4-fold stimulation after 12 h. However, preculture time had no effect on basal HA production, i.e. without oocytes, and DS synthesis was the same in all culture conditions.

**Time Course of Oocyte Factor(s) Production**—The experimental protocol shown in Fig. 5A was designed to evaluate the time course of oocyte factor(s) production in vitro. In this protocol, every 3 h oocytes were freshly isolated and precultured in individual drops of medium for different times. At the end of the preculture time (time 0) each group of oocytes...
was transferred into a drop of fresh medium. Then, freshly isolated mural granulosa cells and radioisotopes were added to the oocyte-conditioned media (solid lines) and to the transferred oocytes (dashed lines). The incubation was then carried out for 18 h (labeling). This protocol ensures that each mural granulosa cell culture will have an equivalent capacity for stimulation of HA synthesis.

The effect of conditioning media with oocytes for different periods of time is shown in Fig. 5B. HA production increased when the mural granulosa cells were incubated with media conditioned by oocytes for longer times (solid line). This suggests that the oocytes continue to release the factor(s) throughout the 12-h preculture time. The precultured oocytes showed an inverse relationship; their ability to stimulate HA synthesis after transfer into mural granulosa cell cultures progressively decreased with the increase in their preculture time (dashed line). However, even after 12 h of preincubation, the transferred oocytes continue to produce sufficient amounts of the factor(s) to give ~50% of the maximal HA synthesis response obtained when oocytes were added directly without preincubation. Overall, these results suggest that the oocytes produce the factor(s) throughout the preculture period at a steadily decreasing rate. In all the following experiments, a conditioning time of 9 h was used to prepare oocyte-conditioned medium.

**Effect of Growth Factors on HA and DS Synthesis by Mural Granulosa Cells**—The effects of several growth factors on HA synthesis by mural granulosa cells were compared with that of the oocyte-conditioned medium. Cultures were incubated with oocyte-conditioned medium or with growth factors in the presence or absence of FSH (Fig. 6). The basal level of HA synthesis was not changed when FSH was added; however, DS synthesis was stimulated ~2-fold, as was shown previously (15, 16). Oocyte-conditioned medium (1 oocyte/μl for 9 h) stimulated HA synthesis to the same level with or without FSH, while DS synthesis showed only the FSH stimulatory effect. Among the growth factors tested only TGF-β1 (10 ng/ml) elicited a response similar to that of the oocyte-conditioned medium, namely a pronounced and FSH-independent stimulation of HA synthesis without any influence on DS synthesis. Although EGF did not induce any effect on HA production, it did stimulate DS synthesis more than 2-fold in the absence of FSH, and the addition of FSH failed to increase DS synthesis further.

The effect of TGF-β1 concentration on HA synthesis by mural granulosa cells was compared with their response to media conditioned with different numbers of oocytes (Fig. 7). The results indicate that TGF-β1 (solid circles) is able to stimulate HA synthesis to nearly the same magnitude achieved by oocyte-conditioned media (solid triangles). TGF-β1 gave a maximal stimulation at a concentration of 0.5 ng/ml with an ED50 of about 0.2 ng/ml while the oocyte-conditioned medium reached maximal stimulation at an equivalent concentration of ~1.5 oocytes/μl with an ED50 of ~0.8 oocytes/μl. More importantly, TGF-β1 and oocyte-conditioned medium were additive in their effects on HA synthesis (unfilled circles).

These observations suggest that the oocyte factor(s) is distinct from TGF-β1. This was further supported by the fact that the action of TGF-β1 was reversed in a dose-dependent manner by an anti-TGF-β antibody while the stimulatory effect of oocyte-conditioned medium was not (Fig. 8A). More...
TGF-β (ng/ml)

Fig. 7. Effects of TGF-β1 concentration, or media conditioned with different numbers of oocytes, or of a combination of both on DS and HA synthesis by mural granulosa cells. Mural granulosa cell cultures were incubated for 18 h with radio-isotopes in basal medium (unfilled triangle), with increasing concentrations of TGF-β1 (filled circles), or in oocyte-conditioned media (OCM) diluted to the specified concentrations (oocytes/μl) from an original concentrated OCM (4 oocytes/μl cultured for 9 h) (filled triangles), or in media with both TGF-β1 and oocyte-conditioned medium at the paired concentrations indicated on the abscissa in the abscissa (unfilled circles). Total labeled HA was determined in each culture. Error lines indicate the standard deviations of triplicate cultures.

Effect of TGF-β1 on HA Synthesis by Cumulus Cells—The following experiments were carried out to determine if TGF-β1 has similar effects on HA synthesis by cumulus cells. As shown previously, cumulus cells require FSH as well as the oocyte factor(s) for maximal stimulation of HA synthesis (Fig. 9, compare oocyte-conditioned medium with and without FSH) (9). Likewise TGF-β1 requires the presence of FSH to stimulate HA synthesis by cumulus cells (compare TGF-β1 with and without FSH). However, the anti-TGF-β antibody prevented stimulation of HA synthesis by TGF-β1 but did not prevent the stimulation induced by oocyte-conditioned medium (Fig. 9) as was observed with mural granulosa cells. Neither expansion (data not shown) nor HA synthesis (Fig. 9) were inhibited by the anti-TGF-β antibody in FSH-stimulated COCs. TGF-β1 had no effect on DS synthesis by the cumulus cells (data not shown) as was also observed for mural granulosa cells (Fig. 6).

Effect of EGF on HA and DS Synthesis by Cumulus Cells—EGF did not stimulate HA synthesis by cumulus cells, neither in the presence nor in the absence of FSH (Fig. 10) as was also observed with mural granulosa cells (Fig. 6). However, like FSH, EGF increased HA synthesis by cumulus cells when co-cultured with the isolated oocytes. DS synthesis was stimulated equally by FSH or EGF independent of the presence or absence of oocytes, and the combination of both failed to increase it further.

DISCUSSION

We previously showed that a soluble factor(s) produced by isolated oocytes is essential in combination with FSH for HA synthesis during expansion of cumulus cell-oocyte complexes in vitro (9). In vivo, during the preovulatory period the COC expands while the multilayer of mural granulosa cells does not (4). Thus, it has been thought that the two cell populations differed in their ability to synthesize and secrete HA. Our experiments demonstrate that mural granulosa cells can increase their synthesis of HA in response to the oocyte factor(s) (Fig. 1) and that most of the newly synthesized HA is retained in the extracellular matrix when FCS is present (Fig. 2). These results are the same as those previously observed for cumulus cells (8, 9). They also ruled out the possibility that mural granulosa and cumulus cells at the time of follicle maturation in vivo differ in HA synthesis because of inherent differences in their responses to the oocyte factor(s). More
likely the cells in closest proximity to the oocyte, i.e. the cumulus cells, bind and inactivate the oocyte factor as part of the biological response, thereby reducing the effective concentration of the factor at greater distances from the oocyte.

Cumulus cells require FSH in vitro to obtain maximal synthesis of HA in vitro (14). Direct evidence for this was provided by showing that indomethacin significantly inhibited the stimulation of HA synthesis by mural granulosa cells co-cultured with the oocytes and that exogenous PGE can overcome this inhibition (Fig. 3). However, the inability of indomethacin to reduce HA synthesis to basal levels suggests that mural granulosa cells may produce other factors which can enhance HA synthesis in the presence of the oocyte factor(s).

Some growth factors stimulate HA synthesis in cultures of several different cell types, including PDGF (17, 18), TGF-β1 (18, 19), EGF (18), and FGF (18) in human fibroblast cultures, IGF-1 in rabbit pericardial cell cultures (20), and TGF-β1 and TGF-α in rat liver fat-storing cell cultures (21). Moreover, indirect and direct evidence show that mural granulosa cells may synthesize several growth factors in vivo and in vitro, including IGF-1 (22, 23), TGF-β2 (24, 25), FGF (26), and EGF (27, 28) and that their metabolic functions can be affected in vitro by adding growth factors to the medium (29). In addition, growth factor gene expression, notably TGF-α and PDGF, has been detected in unfertilized oocytes (30). Therefore, the possibility that the oocyte factor(s) is a known growth factor or stimulates synthesis of such a growth factor by mural granulosa cells or cumulus cells was investigated. Mural granulosa cell cultures were tested with several growth factors to determine if any can stimulate HA synthesis in a fashion similar to the oocyte factor(s). Of those tested, only TGF-β1 stimulated HA synthesis above the basal levels (Fig. 6). The dose-response curves for TGF-β1 and oocyte-conditioned medium achieved nearly the same maximum stimulation of HA synthesis. However, the effects of TGF-β1 and oocyte-conditioned medium were additive. Moreover, TGF-β1 neutralizing antibody did not prevent stimulation of HA synthesis by the oocyte factor(s) in either mural granulosa cells or FSH-stimulated cumulus cells, nor did they inhibit expansion (data not shown) and HA synthesis by FSH-stimulated intact COCs. These results indicate that the oocyte factor(s) is not TGF-β1 and also that its action is not mediated by this growth factor.

A previous study showed that EGF, but not several other growth factors tested, can replace FSH to induce expansion of intact COCs in vitro (31). In agreement with this observation, we found that EGF, like FSH, amplifies the response of the cumulus cells to the oocyte factor(s) in stimulating HA synthesis. We also showed that EGF, like FSH, stimulates DS synthesis and that this activity is independent of the presence of the oocytes.

Interestingly, the mural granulosa cells increase synthesis of DS in response to FSH independent of pre-culture time whereas they gradually lose their ability to stimulate HA synthesis in response to the oocyte factor(s) (Fig. 4). This may explain in part the observation that COCs in vitro do not expand significantly if FSH (or luteinizing hormone) is added after at least 7 h of preculture (3, 32). In this case the cumulus cells may be losing their ability to synthesize HA in response to the oocyte factor(s) rather than losing their response to the gonadotropins. Thus, the rapid decrease in HA synthesis observed in FSH-stimulated COCs after 12 h in culture, which limits expansion (8), probably reflects both a desensitization of the cumulus cells to the oocyte factor(s) and a decline in production of the factor(s) by the oocyte after this time (Fig. 5B).

In conclusion, our novel finding that the mural granulosa cells induce synthesis of HA and retain it in the extracellular matrix in response to the oocyte and serum factors suggests that these cells can provide a more convenient model than cumulus cells for studying the mechanisms of action for these
factors. The utility of this approach was shown by its effectiveness in identifying TGF-β1 as a potent factor for inducing HA synthesis and defining its independence from the oocyte factor(s). Future studies are directed toward using this model to identify the various factors involved in COC expansion and to determine their modes of action.

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