Mouse Oocytes Regulate Metabolic Cooperativity Between Granulosa Cells and Oocytes: Amino Acid Transport

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Short title: Oocytes control amino acid uptake by cumulus cells

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ABSTRACT

A search for genes expressed more highly in mouse cumulus cells than mural granulosa cells by subtraction hybridization yielded Slc38a3. SLC38A3 is a sodium-coupled neutral amino acid transporter having substrate preference for L-glutamate, L-histidine, and L-alanine. Detectable levels of Slc38a3 mRNA were found by in-situ hybridization in granulosa cells of large preantral follicles, but levels were higher in all granulosa cells of small antral follicles; expression became limited to cumulus cells of large antral follicles. Expression of Slc38a3 mRNA in granulosa cells was promoted by fully-grown oocytes from antral follicles, but not by growing oocytes from preantral follicles. Fully-grown oocytes were dependent upon cumulus cells for uptake of L-alanine and L-histidine, but not L-leucine. Fully-grown, but not growing, oocytes secreted one or more paracrine factors that promoted cumulus cell uptake of all three amino acids, but of L-alanine and L-histidine to a much greater extent than L-leucine. Uptake of L-leucine appeared dependent primarily upon contact-mediated signals from fully-grown oocytes. Fully-grown oocytes also promoted elevated levels of Slc38a3 mRNA and L-alanine transport by preantral granulosa cells, but growing oocytes did not. Therefore fully-grown oocytes secrete one or more paracrine factors that promote cumulus cell uptake of amino acids that oocytes themselves transport poorly. These amino acids are likely transferred to oocytes via gap junctions. Thus, oocytes utilize paracrine signals to promote their own development via metabolic cooperativity with cumulus cells. The ability of oocytes to mediate this cooperativity is developmentally regulated and acquired only in later stages of oocyte development.
INTRODUCTION

Bidirectional communication between oocytes and companion somatic cells is essential for the development and function of both cell types and to coordinate the overall development of the ovarian follicle [1, 2]. That a metabolic cooperativity occurs between the oocyte and granulosa cells has been known since classic experiments conducted in the 1960s [3, 4]. Mouse oocytes only poorly take up and utilize glucose as an energy source. Although cumulus cell-enclosed oocytes resume meiosis when cultured in medium containing only glucose as an energy source, cumulus cell-denuded oocytes do not. Nevertheless, addition of pyruvate to the oocyte culture medium does provide support for the resumption of meiosis by denuded oocytes [3, 4]. The evidence indicates that cumulus cells take up and metabolize glucose to products that can be utilized by oocytes for the energy metabolism necessary to support meiotic maturation [5], an example of metabolic cooperativity. Recent studies show that fully-grown oocytes promote elevated steady state levels of transcripts encoding enzymes in the glycolytic pathway in cumulus cells [6]. Thus, oocytes promote a key metabolic function of cumulus cells that is necessary for oocyte meiotic maturation.

Another example of the metabolic support of oocytes by cumulus cells involves the uptake of some amino acids, such as L-alanine, which are poorly transported into mouse oocytes and require uptake first by cumulus cells and then transfer to the oocyte via gap junctions. When oocytes are cultured with radiolabeled L-alanine, the amount of radioactivity detected in the oocytes cultured enclosed by cumulus cells is greater than in oocytes cultured while denuded of cumulus cells [7]. Blocking the function of gap junctions in oocyte-cumulus cell complexes abrogates this difference [8]. We designate
this group of amino acids as “coupling” dependent, “coupling” referring to gap junctional coupling between the oocyte and companion cumulus cells. Other (coupling independent) amino acids, such as L-leucine, are not differentially incorporated into cumulus cell-enclosed oocytes compared with denuded oocytes [7]. Suppression subtraction hybridization was used to identify transcripts expressed at higher levels in cumulus cells than in mural granulosa cells (MGCs); cumulus cell cDNA was used as the tester and mural granulosa cell cDNAs as the driver. The rationale for this project was that cumulus cell-enriched transcripts may be regulated by the oocyte. The transcript encoded by the solute carrier family 38, member 3 (Slc38a3) gene (MGI:1923507) was identified as enriched in the cumulus cell cDNA library. In the first part of this report, we describe the localization of Slc38a3 mRNA in the mouse ovary, and studies conducted to determine whether the elevated steady state levels of this transcript in cumulus cells are maintained by oocytes. SLC38A3 is a sodium-coupled neutral amino acid transporter highly expressed in liver. Transporters in this family exhibit a preference for L-glutamate, L-histidine, and L-alanine. When Slc38a3 mRNA was injected into Xenopus oocytes, L-alanine transport into the frog oocytes was increased about 10-fold [9]. In the second part of this report, we determined whether oocytes affect the uptake of both coupling dependent and independent amino acids by cumulus cells.
MATERIALS AND METHODS

Animals

B6SJLF1 mice, bred and raised in the research colony of the investigators, were used for all experiments. Ovaries were obtained from 22-day-old mice either without gonadotropin priming or 44 h after priming by injection of 5 IU eCG to stimulate follicular development. The ovaries from 12-day-old mice were used for some experiments; no prior hormonal priming was used in this case. The mice were maintained according to the Guide for the Care and Use of Laboratory Animals (ILAR).

In situ hybridization

After sequencing, a 332 bp clone from a cDNA library enriched in cumulus cell transcripts was identified as encoding a transcript of Slc38a3 encompassing exons 9 and 10. The clone was linearized with MaeIII (Roche Applied Science, Indianapolis, IN) to yield a 186 bp antisense RNA labeled with $^{33}$P CTP for in situ hybridization, which was carried out as we described previously [10] using ovaries from 12-day-old or 22-day-old mice 44 h after eCG priming.

Culture of oocytes and granulosa cells

Cumulus cells and preantral granulosa cells were collected as previously reported [11]. Briefly, oocytes were surgically removed from granulosa cell-oocyte complexes either from antral follicles of 22-day-old primed mice or from preantral follicles of 12-day-old mice to collect either cumulus cells or preantral granulosa cells, respectively. These granulosa cell complexes are referred as ooyectomized complexes (OOXs) in this study. Fully-grown, meiotically competent, oocytes were isolated by gentle pipetting
of cumulus-oocyte complexes (COCs). Growing, meiotically incompetent oocytes were collected from the preantral follicles of 12-day-old mice by collagenase digestion as described previously [12, 13].

The culture medium used was bicarbonate-buffered MEMα (Life Technologies, Inc., Grand Island, NY) with Earles salts, supplemented with 75 mg/liter penicillin G, 50 mg/liter streptomycin sulfate, 0.23 mM pyruvate, and 3 mg/ml crystallized lyophilized BSA. To maintain oocytes competent to undergo germinal vesicle breakdown (GVB) at the GV stage, the phosphodiesterase inhibitor, milrinone (10 µM), was added to all culture medium regardless of whether oocytes were present, or whether the oocytes were incompetent to undergo spontaneous maturation. All medium components were purchased from Sigma Chemical Co. (St. Louis, MO). In some experiments, granulosa cells were co-cultured with fully-grown oocytes or growing oocytes at the concentration of either 2 oocytes/µl or 4 oocytes/µl of culture medium, respectively. These relative concentrations were based on comparable oocyte volume [14]. All cultures were performed in 48 well cell culture plates (#3548, Corning Incorporated, Corning, NY) in 100 - 150 µl volume of culture medium for 15 h, and were maintained at 37°C in a modular incubation chamber (Billups Rothenberg, Del Mar, CA) infused with 5% O₂, 5% CO₂, and 90% N₂.

**RNase protection assays**

Messenger RNA was isolated as described previously [15] from samples of granulosa cells obtained after the various culture experiments presented below. Protected RNA fragments were analyzed and quantified using a Fuji Phosphor Imaging
System (Fuji Medical Systems USA, Stamford, CT). Data are expressed as the relative steady state level of \textit{Slc38a3} mRNA normalized with mRNA encoded by the housekeeping gene \textit{Rpl19}. Results are presented as the mean +/- the standard error of the mean of at least 3 independent experiments.

\textbf{Uptake of radiolabeled amino acids}

To assess the uptake of amino acids, either intact oocyte-cumulus cell complexes, OOX cumulus cells, OOX cumulus cells co-cultured with oocytes, or cumulus cell denuded oocytes were collected after 15 h culture as described above. They were washed through 3 dishes of Whitten medium containing 3 mg/ml BSA and 10 µM milrinone and transferred to 150 µl of same medium in 48-well tissue culture plates. Importantly, Whitten medium does not contain free amino acids [16]. The cells were incubated for 3 h in 100 µCi/ml medium containing one of three \textsuperscript{3}H-labelled amino acids: L-alanine (66 Ci/mmol), L-histidine, (50 Ci/mmol), or L-leucine (300 mCi/mmol) purchased from Dupont NEN (Boston, MA). After incubation, the cells were washed again through three changes of Whitten medium with milrinone and BSA, but no labeled amino acids. Incorporation of amino acids into half of the intact oocyte-cumulus complexes was determined. Uptake of amino acids into all the other groups is expressed as a percentage of the counts per minute relative to the intact oocyte-cumulus cell complex. The cumulus cells were removed from the other half of the oocyte-cumulus cell complexes and incorporation of the amino acids into either the oocytes or cumulus cells was measured separately. When incorporation of amino acids into the OOX group that was co-cultured with oocytes was measured, the oocytes were removed before assessing uptake into the
OOX cumulus cells. Ten oocytes, complexes, or the cumulus cells from 10 complexes were assessed for incorporation of radio-labeled amino acids as described previously [17]. Briefly, the washed cells in 5.0 µl of medium from the final wash dish were transferred to scintillation vials. Two samples were assessed for each group in each experiment and the experiments were conducted three times independently. For each experiment, a duplicate set of medium blanks (5.0 µl of medium only from the final wash dish) was also prepared. One hundred µl of 0.1 N NaOH was added to each vial and samples were held at room temperature for 1 h to dissolve the cells. Then, 0.1 N HCL was added to neutralize the NaOH. The radioactivity in each sample was determined by scintillation spectroscopy. The counts per minute of the blank samples were subtracted from experimental samples.

Statistical analyses

All experiments were performed at least three times. The Student’s $t$ test was used for paired comparisons. A P value of < 0.05 was considered statistically significant.

RESULTS

Ovarian localization of Slc38a3 mRNA

The site(s) of Slc38a3 mRNA expression in the mouse ovary was assessed using in situ hybridization. No expression was detected in the preantral follicles of 12-day-old mice (not shown). Expression of Slc38a3 mRNA in 22-day-old eCG-primed mice was low or undetectable in primordial, primary, or early secondary follicles, or in oocytes at
any stage of follicular development. However, expression increased in the later stages of secondary (preantral) follicles and was highest in all the granulosa cells, both cumulus and mural, of early tertiary (antral) follicles (Fig. 1). With the progression of antral follicle development, expression diminished to undetectable levels in MGCs but remained elevated in cumulus cells (Fig. 1). This verified the outcome of subtraction hybridization indicating that the steady state levels of *Slc38a3* mRNA in cumulus cells was higher than in MGCs.

Effect of oocytes on steady state *Slc38a3* mRNA levels in cumulus cells

To determine whether oocytes of antral follicles affect the steady state level of *Slc38a3* mRNA expressed in cumulus cells, oocytes were microsurgically removed from COCs, (OOX), and intact COCs and OOX cumulus cells were cultured for 15 h before assessing relative levels of *Slc38a3* transcripts expressed in the cumulus cells using RNase protection assays. In addition, OOX cumulus cells were co-cultured with cumulus cell-free FGOs (2 oocytes/µl) isolated from large antral follicles. OOX resulted in a reduction (P<0.05) in the steady state *Slc38a3* mRNA to almost undetectable levels in cumulus cells, but co-culture of OOX cumulus cells with FGOs restored levels to about the same as seen in the cumulus cells of intact COCs (Fig. 2A). Thus, FGOs promote the expression of *Slc38a3* mRNA by cumulus cells.

To determine whether the ability of the oocyte to promote *Slc38a3* expression by cumulus cells is dependent on the stage of oocyte development, OOX cumulus cells were co-cultured with either FGOs (2/µl) or growing oocytes (GOs) (4/µl) isolated from the preantral follicles of 12-day-old mice and relative steady state levels of *Slc38a3*
transcripts present in cumulus cells were determined. The concentration of GO in the co-
cultures was 4/µl to compensate for an approximate 50% difference in the volume of
GOs versus FGOs [14]. FGOs promoted about a 5-fold elevation of \textit{Slc38a3} mRNA
levels in the cumulus cells, but GOs did not (Fig. 2B). Thus, the ability of oocytes to
promote elevated \textit{Slc38a3} steady state mRNA levels in cumulus cells is developmentally
regulated.

Little or no expression of \textit{Slc38a3} mRNA was detected in the preantral granulosa
cells (PAGCs) of 12-day-old mouse ovaries by in situ hybridization (not shown). This
raises the question of whether the PAGCs are able to respond to factors from oocytes and
elevate steady state levels of \textit{Slc38a3} transcripts. Therefore, OOX cumulus cells (from
antral follicles) or OOX PAGCs were cultured either alone or with FGOs for 15 h before
assay. Consistent with the results presented above, FGOs promoted a 7-fold elevation of
\textit{Slc38a3} mRNA levels by OOX cumulus cells. FGOs also stimulated an elevated level of
\textit{Slc38a3} mRNA expression in OOX PAGCs (Fig. 2C). Thus the expression of \textit{Slc38a3}
mRNA by granulosa cells during follicular development is probably up-regulated by
oocytes during their final growth phase, which coincides with their development in late
preantral and antral follicles.

\textit{Effect of oocytes on amino acid transport by granulosa cells}

When \textit{Slc38a3} mRNA was injected into \textit{Xenopus} oocytes, uptake of L-alanine
into the frog oocytes was increased 10-fold [9]. This transporter also exhibits a substrate
preference for L-histidine and L-glutamine [9]. Therefore, the effect of oocytes on the
incorporation of L-alanine and L-histidine into cumulus cells or PAGCs and oocytes was
determined. Since transporters in this family do not exhibit a preference for L-leucine [9, 18], the incorporation of this amino acid was also assessed for comparison.

Approximately 15-20% of the L-alanine, L-histidine, and L-leucine incorporated into the intact COC was incorporated into the oocyte, the remainder into the cumulus cells (Fig. 3). However, while about the same amount of L-leucine was incorporated into cumulus cell-denuded oocytes, significantly less L-alanine or L-histidine was incorporated into denuded oocytes compared to cumulus cell-enclosed oocytes (Fig. 3A). These data for L-alanine and L-leucine are consistent with those presented by Colonna and Mangia [7]; however, incorporation of L-histidine was not included in their study.

The incorporation of L-alanine and L-histidine, but not L-leucine, into the oocyte is dependent, either completely or partially, on the participation of cumulus cells, and is probably mediated by the gap junctions that metabolically couple oocytes and granulosa cells [8]. Incorporation of all three amino acids into OOX cumulus cells was much less than into the cumulus cells of intact complexes (Fig. 3B). Co-culture of OOX cumulus cells with denuded FGOs (2 oocytes/µl) dramatically stimulated incorporation of L-alanine and L-histidine by cumulus cells. For example, about 70% of the total incorporation of L-alanine into the intact COCs was incorporated into the cumulus cells (Fig. 3B). However, incorporation into OOX cumulus cells was only about 3% of the total L-alanine incorporation found in the intact COCs, but incubation of OOX cumulus cells with FGOs increased incorporation to 44% (Fig. 3B). Similar results were obtained when the incorporation of L-histidine was measured (Fig. 3B). FGOs also stimulated incorporation of L-leucine by OOX cumulus cells, but not to nearly the same extent as they did for L-alanine and L-histidine (Fig. 3B). Incubation of OOX cumulus cells with
FGOs increased L-leucine incorporation from 12% to 20% of the total incorporation into COCs (Fig. 3B). FGOs did not promote the incorporation of any amino acid into OOX cumulus cells to the same extent measured in the cumulus cells of intact complexes, even when the concentration of oocytes used in the co-culture was increased to 4/µl (not shown).

As shown above, expression of \textit{Slc38a3} mRNA in preantral follicles was either undetectable or only slightly above background. Nevertheless, growing oocytes (GOs) from preantral follicles might secrete factors that promote amino acid uptake but PAGCs could be unresponsive to them. Therefore, experiments were conducted to determine whether GOs secrete factors that can promote the incorporation of amino acids by OOX-cumulus cells (from antral follicles), which were shown to be responsive to the stimulatory factors from FGOs. The concentration of GO in the co-cultures was 4/µl to compensate for an approximate 50% difference in the volume of GOs versus FGOs [14]. As expected, FGOs increased the OOX cumulus cell incorporation of L-alanine by 11.5-fold and L-leucine by 2.3-fold. However, GOs did not promote the incorporation of either amino acid by OOX cumulus cells (Fig. 4A).

The relatively low level of expression of \textit{Slc38a3} mRNA by PAGCs could be due to either the failure of GOs to promote expression, as shown above, or to the inability of PAGCs to respond to the stimulus, or both. FGOs were not only able stimulate elevation in the steady state levels of \textit{Slc38a3} mRNA (Fig. 2), but they also promoted a 40-fold increase in the incorporation of L-alanine into OOX-PAGCs (Fig. 4B). Nevertheless, FGOs did not promote the uptake of L-leucine into OOX-PAGCs (Fig. 4B).
DISCUSSION

Suppression subtraction hybridization detected differential expression of Slc38a3 mRNA between cumulus cells and MGCs. This was confirmed by in situ hybridization; expression was low in the preantral follicles of 12-day-old mouse ovaries, increased in PAGCs of larger preantral follicles in 22-day-old mice primed with eCG, and was in both MGCs and cumulus cells in early antral follicles. With growth and development of antral follicles, expression of Slc38a3 mRNA became progressively localized only to the cumulus cells, with little or no expression detected in the MGCs of large antral follicles (Fig. 1C). FGOs from antral follicles, but not GOs from preantral follicles, promoted elevated steady state levels of Slc38a3 mRNA in cumulus cells and PAGCs. Therefore, the expression of Slc38a3 mRNA in granulosa cells is regulated by oocytes according to the stage of oocyte development.

SLC38A3 is a sodium-coupled neutral amino acid transporter exhibiting a preference for L-glutamate, L-histidine, and L-alanine, but not for L-leucine [9]. In the absence of FGOs, cumulus cell incorporation of L-alanine, L-histidine, and L-leucine was greatly reduced. FGOs co-cultured with OOX cumulus cells greatly stimulated the incorporation of L-alanine and L-histidine, but stimulated the uptake of L-leucine to a much lesser extent. FGOs were unable to restore the incorporation of any of the amino acids to levels measured in the intact COC even though the steady state levels of Slc38a3 mRNA were elevated to levels similar to that of the intact COC. Thus paracrine factors from FGOs stimulate cumulus cell incorporation of amino acids, particularly the amino acids categorized as coupling-dependent and are taken up poorly by the oocyte itself.

Maximal incorporation of amino acids by cumulus cells appears to require contact
with the oocyte. The most extreme example of this is the uptake of L-leucine by cumulus
cells. Expression of Amh mRNA by granulosa cells also appears largely dependent upon
contact or very close association with the oocyte, particularly in PAGCs, but also in
cumulus cells [19]. This apparent requirement for contact between oocytes and granulosa
cells for maximum expression of Amh mRNA or for amino acid uptake by the granulosa
cells may be dependent upon gap junction–mediated signaling. Such signals originating
in the granulosa cells may promote the secretion of paracrine factors by the oocyte that,
in turn, enhance granulosa cell function, in a regulatory loop.

Although it is tempting to assume that the uptake of L-alanine and L-histidine into
cumulus cells depends upon expression of Slc38a3 mRNA, there is no direct evidence
presented here that these are linked. Considering the many transporters involved in
amino acid uptake, it is likely that SLC38A3 is only one of several transporters involved
in the uptake of these amino acids by cumulus cells. In fact, studies in progress in our
laboratory suggest that cumulus cells also express the transporter SLC38A5, and this
transporter appears to have a similar substrate preference to SLC38A3 in rats [20].

In addition to Slc38a3, the expression of six genes (Aldoa, Eno1, Ldh1, Pfkp, Pkm2, and Tpi1), encoding enzymes participating in the glycolysis pathway, is greater in
cumulus cells than MGCs [6]. It is well documented from classical studies that mouse
oocytes, as well as preimplantation embryos, are deficient in their ability to metabolize
glucose, and that the products of glycolysis provided by the cumulus cells are essential
for oocyte development [3-5]. The steady state levels of all of these transcripts encoding
glycolytic enzymes are elevated by paracrine factors produced by FGOs, but not GOs.
Thus, before the LH-surge, oocytes probably promote the expression of genes, including
Slc38a3 and genes encoding glycolysis enzymes, in cumulus cells that, in turn, are beneficial for oocyte development. Although the role of such gene products in oocyte development is not always obvious, promoting expression of genes involved in amino acid transport, particularly the transport of coupling-dependent amino acids, seems evident. In fact, FGOs promote the incorporation of the coupling-dependent amino acids L-alanine and L-histidine to significantly greater extent than the coupling independent amino acid L-leucine. The increase in oocyte volume is the greatest in the later stages of preantral follicle and early antral follicle development [13], and the demand for amino acids to support oocyte growth must be greatest at this time. Since oocytes are deficient in their ability to transport some amino acids, such as L-alanine and L-histidine, they probably become increasingly dependent upon the cumulus cells to provide them. In effect, oocytes may use the cumulus cell membrane as specialized extension of the oolemma, and the coupling gap junctions as means of conveyance. The studies reported here show that oocytes enhance incorporation of crucial amino acids via signals using both paracrine factors and contact-mediated communication. At least part of the enhancement mechanism involves up regulation of cumulus cell transcripts, such as Slc38a3 and those from genes encoding glycolytic enzymes.

Oocytes regulate the rate of ovarian follicular development [21]. This is most dramatically illustrated by the fact that reaggregation of partly grown oocytes from preantral follicles with the somatic cells of newborn ovaries, which contain only primordial follicles, resulted in a doubling of the rate of follicular development [21]. It is probable that this regulation is mediated by paracrine factors from the oocyte, such as GDF9 and BMP15, and by signals transmitted by contact mediated mechanisms, such as
through gap junctions. Signals originating in the oocyte affect not only the rate of
follicular development, but also profoundly affect the differentiation of granulosa cells.
This is evidenced by the oocyte’s influence on different patterns of gene expression
among the granulosa cells at different times, and in the various populations of granulosa
cells. Fully-grown oocytes promote the untimely expression of *Slc38a3* mRNA and
greatly enhance the uptake of coupling dependent amino acids by preantral granulosa
cells. This is an example of how oocytes can drive the precocious development of
granulosa cells to assure the coordination of oocyte and granulosa cell development.
Similarly, the ability of oocytes to control energy metabolism by granulosa cells is
another mechanism that could regulate the rate of follicular development [6].

The developmental coordination between the oocyte and the somatic components
of the follicle assures that both are at appropriate stages of maturation. Thus, a
developmentally competent oocyte is available to be ovulated at the same time that the
follicle is competent to undertake the ovulatory process in response to LH. In fact, the
oocyte itself plays a key role in the ovulatory process since GDF9 produced by the oocyte
is required to promote the production of hyaluronic acid and other factors by cumulus
cells that are necessary for ovulation [1, 2]. We propose that oocytes promote the
expression of genes involved in cellular metabolism, such as *Slc38a3* and the genes
encoding glycolysis enzymes [6], to achieve two goals. First, some of these gene
products are not produced by the oocyte itself and promoting the expression of these
genes in cumulus cells enhances aspects of metabolic cooperation between the granulosa
cells and the oocyte that are essential for the development of the oocyte. Second, the
oocytes use their ability to regulate metabolic pathways in the granulosa cells to orchestrate the rate of follicular development.

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REFERENCES


**Figure Legends**

**FIG. 1.** Localization of *Sle38a3* transcripts. Light field (panel A) and dark field (panel B) demonstrating the localization of *Sle38a3* transcripts in follicles of the ovaries of 22-day-old mice 44 h after injection of 5 IU eCG. No *Sle38a3* mRNA was detected in oocytes. No expression was detected in small preantral follicles (arrow 1; panels A and B). Low levels of expression were seen in granulosa cells of large preantral follicles (arrow 2; panels A and B) and this increased dramatically in early antral follicles (arrow 3; panels A and B) wherein both cumulus and mural granulosa cells showed relatively high levels of expression. However, expression was limited to the cumulus cells of large antral follicles (arrow 4; panels A and B). Panel C is the interpretation of the localization of the transcripts and their relative levels: white cells = low to undetectable expression; light blue cells = detectable mRNA levels; dark blue cells = maximum level of expression (numbers correspond to numbered arrows in panels A and B). Scale bars indicate 100 µm.

**FIG. 2.** Effect of oocytes on relative steady state levels of *Sle38a3* mRNA expression.

**Panel A.** Effect of fully grown oocytes (FGO) on relative steady state levels of *Sle38a3* transcripts in cumulus cells. Intact cumulus-oocyte complexes (COC), OOX cumulus cells, and OOX cumulus cells + FGOs were cultured for 15 h. Cumulus cells were collected and assessed for relative levels of *Sc138a3* mRNA levels using RNase protection assays. Data were normalized to the levels of *Rpl19* mRNA, the values of the control (cumulus cells from the COCs) sample in each experiment were set at 1 and the values of the two experimental groups are expressed relative to those. The figure shows
the mean and SEM of three independent experiments. * indicates significant difference (P < 0.05) from COC control.

**Panel B.** Effect of growing oocytes (GO) on the relative steady state levels of *Slc38a3* mRNA in OOX cumulus cells. OOX cumulus cells (control) were co-cultured with either FGOs (2 oocytes/µl) or GOs (4 oocytes/µl) for 15 h and RNase protection data were normalized as in Panel A to the control, which in this case was OOX cumulus cells. The figure shows the mean and SEM of three independent experiments. * indicates significant difference (P < 0.05) from OOX control.

**Panel C.** Effect of FGOs on the relative steady state levels of *Slc38a3* mRNA in the granulosa cells of preantral follicles. OOX cumulus cells or OOX preantral granulosa cells (PAGC) were cultured with or without FGO (2 oocytes/µl) for 15 h and RNase protection data were normalized to the control as in Panel A, which in this case was OOX cumulus cells or OOX PAGC. The figure shows the mean and SEM of three independent experiments. A significant difference (P < 0.05) from the OOX cumulus cell control is indicated by (*), or from the PAGC control is indicated by (#).

**FIG. 3.** Uptake of L-alanine, L-histidine, and L-leucine by cumulus cells and oocytes, and the effect of FGO on uptake by OOX cumulus cells. Intact COCs, OOX cumulus cells, cumulus cell-denuded oocytes (DO), and OOX-cumulus cells were cultured for 15 h and then transferred to Whitten medium containing 3 mg/ml BSA and one of the three 3H-labelled amino acids (100 µCi/ml) for 3 h. The counts in each group of cumulus cells or oocytes are expressed as a percentage of the total counts in an intact COC. **Panel A** compares the uptake of amino acids into oocytes, cultured either as COCs or as DO. The
FIG. 4. Effect of growing oocytes (GO) on amino acid uptake by OOX cumulus cells (Panel A) or preantral granulosa cells (Panel B). **Panel A.** OOX cumulus cells were cultured alone (control) or with FGOs (2 oocytes/µl) or GOs (4 oocytes/µl) for 15 h and then transferred to Whitten medium to assess uptake of radiolabeled amino acids over a 3 h incubation. Results are presented as the fold increase of the experimental groups, OOX cumulus cells co-cultured with FGOs or GOs, over the uptake by OOX cumulus cells alone. The data are presented as the mean and standard error of the mean of three independent experiments. * indicates a significant difference (P < 0.05) from the OOX cumulus cell control.

**Panel B.** Effect of growing oocytes (GOs) on amino acid uptake by preantral granulosa cells (PAGCs). PAGCs were cultured alone (control) or with FGOs (2 oocytes/µl) or GOs (4 oocytes/µl) for 15 h and then transferred to Whitten medium to assess uptake of radiolabeled amino acids over a 3 h incubation. Results are presented as the fold increase of the experimental groups, PAGCs co-cultured with FGOs or GOs, over the PAGCs alone. The data are presented as the mean and standard error of the mean of three independent experiments. * indicates a significant difference (P < 0.05) from the OOX cumulus cell control.
independent experiments. * indicates a significant difference (P < 0.05) from the PAGC control.
Figure 2

2A

Relative mRNA Levels

COC  OOX  OOX+FGO

2B

Relative mRNA Levels

OOX  OOX+FGO  OOX+GO

2C

Relative mRNA Levels

OOX  OOX+FGO  PAGC  PAGC+FGO

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Figure 3

Uptake into oocytes

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Uptake into cumulus cells

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Uptake into cumulus cells

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<tr>
<td>Alanine</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
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</tr>
</tbody>
</table>

Uptake into preantral granulosa cells

<table>
<thead>
<tr>
<th></th>
<th>FGO</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>*</td>
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<tr>
<td>Leucine</td>
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</tr>
</tbody>
</table>

Figure 4