Aromatase expression in *Xenopus* oocytes: a three cell-type model for the ovarian estradiol synthesis

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Abstract

In contrast to the classical model describing the synthesis of androgens and estrogens as restricted to somatic cells, a previous study demonstrated that *Xenopus laevis* oocytes participate in androgen synthesis. The objective of our study was to determine whether *Xenopus* oocytes are also involved in estrogen synthesis. More precisely, we analyzed aromatase expression by *in situ* hybridization and RT-QPCR and measured aromatase activity. Aromatase, the enzyme responsible for estrogen synthesis, appears to be expressed and active not only in the follicular cells but also in the vitellogenic oocytes. During late oogenesis, aromatase oocyte expression and activity decreased concomitantly with the trend observed in surrounding follicular layers. In order to investigate the role of estradiol-17 β (E₂), we studied its effect on oocyte meiotic resumption. It appears that, as in *Rana pipiens*, E₂ inhibited the follicle-enclosed maturation of *Xenopus* oocytes, likely through inhibition of LH-induced maturation-inducing steroid synthesis. In addition, E₂ exerted a slight enhancing action on denuded oocyte maturation whose biological significance remains unclear. Together, our results demonstrate that *Xenopus* oocyte significantly participates in ovarian E₂ synthesis and this may be a common feature of vitellogenic vertebrates.

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Introduction

Estradiol-17 β (E₂) is known to have pleiotropic effects and important roles in female reproductive function in vertebrates, and characterizing the regulation and function of E₂ synthesis during ovarian follicular growth is a major challenge in reproductive biology. Throughout oogenesis, E₂ is synthesized by ovarian follicles and secreted into the blood. Growth and development of *Xenopus laevis* follicles are accompanied by shifts in steroidogenic abilities, with E₂ produced by medium-sized follicles and androgen and progesterone secreted by large, postvitellogenic follicles (Fortune 1983). In non-mammalian vertebrates, the best-known endocrine effect of E₂ is the stimulation of yolk synthesis through hepatic vitellogenins synthesis and secretion into blood circulation. Circulating vitellogenins are taken up by the oocyte leading to oocyte yolk accumulation and growth (Rasar & Hammes 2006). Yolk will provide nutritional reserves necessary for embryo development. More recently, E₂ was also shown to enhance progesterone-induced ovulation, possibly through a stimulatory effect on gonadotropin synthesis (Ogawa *et al.* 2011). In addition to E₂ endocrine effects, E₂ may have autocrine and/or paracrine effects, as E₂ inhibits follicle-enclosed oocyte maturation through inhibition of maturation-inducing

steroid (MIS) synthesizing enzymes in vitellogenic species such as *Rana pipiens* (Schuetz 1972, Spiegel *et al.* 1978, Lin & Schuetz 1983, 1985) or *Oncorhynchus mykiss* (Jalabert *et al.* 1984). This steroid may also activate transcription of particular genes within the oocyte that would play a role in oocyte development, such as IGFBP3, the expression of which has been shown to be positively regulated by E₂ in trout oocytes (Kamangar *et al.* 2006).

E₂ is synthesized by the cytochrome P450 aromatase enzyme that converts androstenedione and testosterone into estrone (E1) and E₂ respectively. In *Xenopus*, two variants are expressed in the gonads and a third variant is expressed in the brain (Iwabuchi *et al.* 2007). As for mammals (Gore-Langton & Armstrong 1988) and fish (Nagahama *et al.* 1995), a two cell-type model has been proposed to describe amphibian E₂ synthesis by the whole follicle (Kwon & Ahn 1994, Ahn *et al.* 1999). According to this classical model, theca cells provide androgens to granulosa cells that, in turn, aromatize androgens into estrogens. Nevertheless, in *Xenopus laevis*, some steroidogenic activity had been previously reported in oocytes (Yang *et al.* 2003). Besides oocyte ability to metabolize exogenous steroid precursors (Reynhout & Smith 1973, Sanyal *et al.* 1973, Fouchet *et al.* 1975, Thibier-Fouchet *et al.* 1976), 17-hydroxylase/17,20-lyase (Cyp17) appeared to be expressed only by

the oocyte in *Xenopus* ovary (Yang *et al.* 2003). Cyp17 is a key enzyme mediating androgen production, suggesting that *Xenopus* oocytes participate in synthesis of estrogen precursors. Moreover, *Xenopus* oocytes were shown to be required for E₂ production by growing stage IV follicles in response to gonadotropin (Sretarugsa & Wallace 1997). Thus, the objective of this study was to determine oocyte contribution to E₂ synthesis in *Xenopus laevis* by characterizing the expression and activity of the estrogenic enzyme, the aromatase. Moreover, contradictory results have been reported concerning E₂ effects on progesterone-induced oocyte maturation in *Xenopus laevis*. Some authors have shown less potentiating effects after E₂ pretreatment on *in vitro* denuded oocyte maturation (Hanocq-Quertier & Baltus 1981, Pickford & Morris 1999), while antagonistic effects have also been reported (Baulieu *et al.* 1978). Therefore, we finally investigated the effect of E₂ on meiotic resumption of either follicle-enclosed or denuded oocytes.

Materials and Methods

Animals and tissue collections

Investigation and animal care were conducted in compliance with French and European regulations on the care and use of laboratory animals. *Xenopus laevis* ovarian pieces were surgically removed from anesthetized adult females, purchased from NASCO (Fort Atkinson, WI, USA). Stage IV (st IV, ~800 µm diameter (Dumont 1972)), and stage VI (st VI PI, >1200 µm diameter, in prophase I of meiosis) ovarian follicles were manually isolated from ovarian pieces of each female in modified OR2 buffer (83 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.4). Corresponding oocytes and follicular cells were manually isolated from ovarian pieces or from individual follicles after incubation in calcium-free OR2 buffer supplemented with collagenase (type 1A, Sigma-Aldrich; final concentration = 275 IU/ml). Ovarian tissues were frozen in liquid nitrogen and stored at -80 °C until use. For *in situ* hybridization, ovarian tissues were fixed in Dietrich's fixative (10% formaldehyde 40%, 29% ethanol 95%, and 2% glacial acetic acid) overnight at 4 °C, rinsed in diethylpyrocarbonate (DEPC) water five times for 10 min, and then stored at 4 °C in 50% ethanol until paraffin embedding.

In vitro oocyte maturation

Follicle-enclosed and denuded oocytes were incubated for 15 h at 20 °C in modified OR2 buffer supplemented with 40 IU/ml human chorionic gonadotropin (hCG; Organon, Puteaux, France; LaMarca *et al.* 1985) or

with 0.1–1 µM progesterone (Baulieu *et al.* 1978). E₂ (1 µM final concentration; Steraloids, Newport, RI, USA) was added simultaneously. Approximately 30 pg E₂ diluted in PBS with 1 mg/ml BSA and 10% ethanol were injected directly into stage VI PI oocytes. Oocytes injected with equivalent volume of steroid vehicle (~15 nl PBS containing 10% ethanol and BSA 1 mg/ml) were used as control. After incubation, germinal vesicle breakdown (GVBD), which reflects meiosis resumption, was assessed by direct observation under a stereomicroscope of the appearance of a white spot on the animal pole of the follicle-enclosed or denuded oocytes.

In situ hybridization

In situ hybridization was performed as described previously (Mourot *et al.* 2006, Vizziano *et al.* 2007). In brief, ovarian tissue was processed in a citadel 1000 tissue processor (Shandon, Pittsburgh, PA, USA). Dehydrated tissues were embedded in paraffin in plastic molds using a HistoEmbedder (TBS88; Medite, Burgdorf, Germany). *In situ* hybridization was performed using the *In situ* Pro, Intavis AG robotic station on 10 µm sections. *P450-arom* sense and antisense probes were synthesized from *Xenopus* cDNA clone (BC079750.1) using Riboprobe Combination System (Promega). PCR products were generated using M13 reverse and forward primers (CCCAGTCACGACGTT-GTAAACGA/AGCGGATAACAATTTCACACAGGA) and were incubated (300 ng) for 2 h at 37 °C with sense (SP6) or antisense (T7) primers in transcription buffer as described previously (Gohin *et al.* 2011). Probe concentration and quality were assessed using nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Hybridization specificity was controlled by comparing signals obtained with the antisense and the sense probes on adjacent sections. Mounted sections were photographed with an ECLIPSE 90i microscope and NIS Advanced Research Software (Nikon Instruments, Amstelveen, The Netherlands).

P450-arom mRNA synthesis and injection

The cDNA corresponding to *Xenopus laevis* p450-arom open reading frame (ORF; GenBank accession number: NM_001085653) was subcloned into the pCS2+ expression vector (Rupp *et al.* 1994, Turner & Weintraub 1994). Capped *p450-arom* RNA was prepared after NotI linearization using mMessage mMachine Kit (Ambion) and purified according to the manufacturer's instructions. *In vitro* transcribed capped *p450-arom* RNA was injected into stage VI prophase I denuded oocytes (~15 nl of a 1 µg/µl solution). Microinjected oocytes were then incubated at room temperature (20 °C) for 12–24 h in modified OR2 buffer before any treatment or freezing.

RNA purification, RT, and real-time quantitative PCR

Total RNA was extracted from *Xenopus* follicles or denuded oocytes using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. Total RNA was repurified using a Nucleospin RNA II kit (Macherey Nagel, Germany) and 2 µg RNA were reverse transcribed using 200 units Moloney murine leukemia virus reverse transcriptase (Promega) and 1 µg random hexamers (Promega) in a master mix containing 2 mM dNTPs, 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, pH 8.3, and supplemented with 25 units of RNase inhibitor (RNasin, Promega) as described previously (Bobe *et al.* 2006). Control reactions were run without enzyme and used as negative controls in the real-time PCR study.

QPCR was carried out using a real-time PCR Step One Plus system (Applied Biosystems, Foster City, CA, USA). RT products were diluted to 1/25. Triplicates were run for each RT product. Real-time PCR was performed using a kit provided with a SYBR Green fluorophore (Fast SYBR Green Master Mix, Applied Biosystems). Primer concentrations were 200 nM for 18S and 600 nM for *p450-arom*, *star*, E₂ receptor 1 (*esr1a* and *esr1b*). A pool of reverse-transcribed RNA was serially diluted and used to calculate a standard curve for each gene. 18S was used as an internal standard to normalize QPCR signal. Amplification cycle and fusion curve protocol followed the same steps as described previously (Gohin *et al.* 2010). The primers used are presented in Table 1.

Aromatase activity assay

A tritiated water release assay was used to measure aromatase activity using androstenedione as a precursor. One hundred stage IV and 50 stage VI PI follicles, denuded oocytes, and corresponding follicular layers were homogenized in 1.5 ml phosphate buffer (20 mM Na₂HPO₄, 0.2 M NaCl, 0.15 M KCl, 0.25 M saccharose, 5 mM dithiothreitol, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (Sigma-Aldrich), pH 7.55) using an ultra-turrax. Extracts were

centrifuged at 12 800 *g* for 15 min at 4 °C and the assay was performed using the supernatants.

The tritiated water release assay for aromatase activity determination was adapted from previous studies (Gore-Langton & Dorrington 1981, Monod *et al.* 1993) using 450 µl *Xenopus* extracts in duplicates adjusted to 500 µl after adding the cofactor NADPH (1 mM, Sigma-Aldrich) and 1β ³H-androstenedione (s.a.=0.87 TBq/mmol, PerkinElmer, Boston, MA, USA; 19 kBq to a final concentration of 50 nM). The reaction was performed at 16 °C under agitation for 20 h and terminated by adding 50 µl 3 M trichloroacetic acid. After adding 450 µl water, samples were centrifuged (3500 *g*, 15 min, 4 °C). Supernatants (700 µl) were transferred to 5 ml glass tubes containing a charcoal pellet (50 mg/tube). Tubes were shaken for 7 h at 4 °C and then centrifuged (3500 *g*, 15 min, 4 °C). Duplicates of 400 µl supernatants were mixed with 4 ml liquid scintillation fluid (Pico-Fluor 40, PerkinElmer) for radioactivity counting for 10 min.

Steroid extraction

One hundred stage IV and 50 stage VI PI denuded oocytes were homogenized in 1 ml phosphate buffer. A tracer quantity of tritiated E₂ (2,4,6,7-³H-E₂ Amersham, GE Healthcare Europe GmbH, Saclay, France; s.a.=3.26 TBq/mmol; 4000 d.p.m. in 100 µl buffer) was added to estimate steroid recovery. Homogenates were placed in 15 ml glass tubes, supplemented with 5 ml of 100% ethanol, and vortexed. After centrifugation at 3500 *g* for 15 min at 4 °C, supernatants were collected and placed into 10 ml glass tubes. Ethanol was evaporated at 50 °C under air and the aqueous phases were extracted three times with 5 ml dichloromethane. After evaporation of the organic phase under air, dry residues were stored overnight at -20 °C. E₂ was further purified by a solvent partition into aqueous NaOH (Chatterton *et al.* 2004). Dry residues were dissolved in 1 ml xylene and E₂ was extracted twice with 1.5 ml NaOH 1 N. The alkaline solutions were neutralized with 0.8 ml HCl 4 M and E₂ was re-extracted twice with 5 ml dichloromethane. The final organic phases were evaporated and extracts were dissolved in 500 µl

Table 1 QPCR primer 5'-sequences-3' and corresponding GenBank accession number

Gene	Accession number	Forward primer	Reverse primer
<i>Cyp19</i>	NM_001085653	CTTCGGGGAGCAGTTTGTTA	CCTGTCATGCACAACCATCT
<i>Star</i>	AF220437	ACGGAGAAGCTGGTGACAAC	CTCTGCTGCTTTCTCGTGTG
<i>Esr1a</i>	NM_001089617.1	TAGCTGGAACAGTGGACAGC	TCCAGTGGTTTGTGGGTAG
<i>Esr1b</i>	NM_001089615	TGGGGTAAACCCCTTATGTG	GTCCCTCTCAATTCCGATGC
<i>18S</i>	X02995.1	CGGAGGTTCTGAAGACGATCA	TTCACGCCCTCTTGAACCTCT

phosphate RIA buffer (NaH_2PO_4 10 mM, Na_2HPO_4 10 mM, and NaCl 150 mM, pH 7.25). One hundred microliters were used for radioactivity counting in 4 ml Picofluor (PerkinElmer) for 10 min per sample to determine extraction yield and 100 μl duplicates were used for E_2 RIA.

E_2 RIA

E_2 was measured by RIA as described previously (Fostier *et al.* 1982) using an anti- E_2 rabbit antibody, 30 000 d.p.m./tube of 2,4,6,7 ^3H - E_2 (s.a. = 3.26 TBq/mmol, PerkinElmer) as tracer and 5–3000 pg/tube unlabeled E_2 (Steraloids) as a reference.

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. Statistical analyses were performed using Statistica 7.0 Software (Statsoft, Tulsa, OK, USA). Differences among groups were analyzed using Mann–Whitney U test or χ^2 test for non-parametric samples. Differences between groups were considered significant when $P < 0.05$.

Results

Aromatase is expressed in late vitellogenic oocytes and transcript levels dramatically decrease during late oogenesis in follicle-enclosed and denuded oocytes

Aromatase transcript was detected in follicular cells of *Xenopus* vitellogenic follicles and appeared not homogeneously distributed (Fig. 1A and C). Under our experimental conditions, it was difficult to distinguish granulosa from theca cells. Aromatase transcript was also detected in the cytoplasm of previtellogenic stage I (100 μm diameter; Fig. 1E) to vitellogenic stage III and IV oocytes (600 μm diameter; Fig. 1A). Consistently with *in situ* hybridization results, aromatase was detected by RT-QPCR in *Xenopus* vitellogenic stage IV (st IV) oocytes (Fig. 2, left panel). During late vitellogenesis, the amount of aromatase transcript represented 25% of the signal in whole follicles. During oocyte maturation, aromatase expression dramatically decreased in both whole follicles and oocytes (Fig. 2, left panel: st VI MII).

In order to explore a possible contamination of oocyte extracts by follicle cell material, *star* expression was monitored under the same conditions in whole follicles and oocytes (Fig. 2, middle panel), as *star* has been described as expressed exclusively by the follicular cells in another non-mammalian vertebrate, the rainbow trout (Kusakabe *et al.* 2002, Gohin *et al.* 2011). Although *star* expression reached a very high level in *Xenopus*, no oocyte expression was detected significantly above

Figure 1 Localization of aromatase transcripts in *Xenopus laevis* ovaries. *Xenopus p450-arom* antisense (A, C, and E) and sense (B, D, and F) probes were hybridized on adjacent ovarian sections. *Xenopus p450-arom* transcripts were present in stage III oocytes (st III, >500 μm diameter) and in the corresponding follicular cells (st III foll) but not in the stage V oocytes (st V oo >1000 μm diameter). Gv, germinal vesicle; bv, blood vessel; me, melanosomes; fc, follicular cells.

background level (amplification from RT products without reverse transcriptase). As a control, *18S* expression appeared stable among tissues (Fig. 2, right panel).

Aromatase is active in oocytes and aromatase activity in follicle-enclosed and denuded oocytes dramatically decreases during late oogenesis

To determine whether the corresponding protein was expressed in oocytes and because none of the custom-made or commercially available antibodies that we tested cross-reacted with *Xenopus* aromatase, an enzyme activity assay was performed. From vitellogenic stage to postvitellogenic stage VI PI, a 70-fold decrease in aromatase activity occurred in the whole follicles (Fig. 3A). High levels of aromatase activity were also detected in *Xenopus* isolated follicular layers (follicular cells only). A significant activity was also detected in *Xenopus* late vitellogenic oocytes. In agreement with RT-QPCR data, *Xenopus* aromatase activity in the oocyte represented nearly 25% of the activity in the whole

Figure 2 QPCR analysis of aromatase and *star* mRNA expression in whole *Xenopus* follicles and oocytes during late oogenesis. The mRNA levels of *p450-arom* (left panel), *star* (middle panel), and *18S* (right panel) were monitored in whole follicles and denuded oocytes sampled during vitellogenic stage IV (st IV) and matured stage VI (st VI MII). For each sample, three separate RT reactions were carried out using three separate RNA samples originating from three different animals. Analysis of mRNA levels was standardized to levels of *18S* rRNA. Mean $\pm$ S.E.M. are shown ($n=3$). Different letters indicate significant differences (non-parametric Mann–Whitney U test at $P<0.05$). #Not significantly different from background levels. For each gene, the mRNA abundance was arbitrarily set to 100 for stage IV follicles.

follicle. Finally, a low but significant level of aromatase activity (1.14 ± 0.24 vs 0.53 ± 0.12 fmol/20 h in control) was measured in postvitellogenic, stage VI, follicles but no activity was detected in the corresponding denuded oocytes (0.98 ± 0.2 fmol/20 h; Fig. 3A). In order to demonstrate the specificity of the aromatase assay used in this study, we further showed that the activity measured by this assay was significantly increased in extracts of stage VI – prophase I arrested – oocytes previously (12–24 h) injected with 15 ng *in vitro* synthesized and capped mRNA encoding *Xl p450-arom* ORF whereas no significant activity was detected in non-injected or water-injected oocytes (Fig. 3B).

E₂ inhibits follicle-enclosed oocyte gonadotropin-induced maturation but facilitates progesterone-induced maturation of denuded oocytes

In order to further understand E₂ function in the oocyte, we measured E₂ concentrations in *Xenopus* oocytes. E₂ concentration in denuded oocytes (100 stage IV versus 50 stage VI PI oocytes from one female in duplicates) slightly decreased during late oogenesis from 7 to 5 pg per oocyte. Thus, in stage IV (800 μ m diameter and 0.3 μ l volume) and stage VI (1250 μ m diameter and 1 μ l volume) oocytes, E₂ concentration may be estimated to be 100 and 20 nM respectively. E₂ added simultaneously in culture medium dramatically inhibited *Xenopus* follicle-enclosed oocyte maturation induced by 40 units/ml hCG: only 8% of follicle-enclosed oocytes resumed meiosis after incubation with hCG supplemented with 1 μ M E₂ while 65% matured in absence of E₂ (Fig. 4A). To address the direct effect of E₂ on the oocyte, we then checked its action on denuded oocytes (Fig. 4B and C). Female maturational competence was evaluated by the maturing response to 1 μ M progesterone after a 12 h treatment. Females in

whom more than 50% of oocytes resumed meiosis were classified as highly responsive to progesterone stimulation, while females in whom <50% of oocytes resumed meiosis were classified as poorly responsive. First, in neither group did 1 μ M E₂ inhibit resumption of meiosis of denuded oocytes contrarily to the results obtained with whole follicles. In contrast, it rather potentiated oocyte maturation induced by a sub-saturating dose of progesterone (0.1 μ M; Fig. 4B and C). At high progesterone concentration (1 μ M = saturating

Figure 3 Aromatase activity in *Xenopus* whole follicles and denuded oocytes during late oogenesis. (A) Aromatase activity was measured by tritiated water release assay from whole follicles (foll) and denuded oocytes (oo) sampled at stage IV (st IV) and stage VI prior to meiosis resumption (st VI PI), and follicular layers (f.l.) from stage IV follicles. Activity is expressed as femtomoles of estrone (E1) synthesized over 20 h from three females in duplicates. (B) Aromatase activity in non-injected stage VI oocytes (not inj.), after injection of water (H₂O), or after 12–24 h following the injection of 15 ng *p450-arom* mRNA (12 h *p450-arom* and 24 h *p450-arom*). Activity was measured in 50 prophase I stage VI oocytes from three females in duplicates. The controls (ctrl) correspond to phosphate buffer processed under the same conditions. Mean $\pm$ S.E.M. are shown ($n=3$). Different letters indicate significant differences (non-parametric Mann–Whitney U test at $P<0.05$).

Figure 4 Effect of estradiol-17 β (E₂) on resumption of meiosis of follicle-enclosed or denuded *Xenopus* oocytes induced by gonadotropin and progesterone respectively. Follicle-enclosed or denuded oocytes were incubated for 15 h in modified OR2 buffer supplemented with 40 IU/ml of human chorionic gonadotropin (hCG) (A) or with 0.1–1 μ M progesterone (Pg; B and C). 1 μ M E₂ was added simultaneously. Females with more than 50% of maturing oocytes were classified as highly responsive to progesterone stimulation (B), while females with <50% of maturing oocytes were classified as poorly responsive (C). After incubation, germinal vesicle breakdown (GVBD) was assessed by direct observation under a stereomicroscope of the appearance of a white spot on the animal pole. Mean $\pm$ S.E.M. are shown. Different letters indicate significant differences (non-parametric χ^2 test at $P < 0.05$).

dose), E₂ also exerted a slight but significant potentiating effect only in poorly responsive females (Fig. 4C). Finally, E₂ alone did not stimulate oocyte maturation in any of the two groups (data not shown).

In order to confirm this slight but reproducible effect of E₂ on oocyte maturation, 30 pg E₂ (which represent

around four times the physiological concentration in stage IV according to the results reported here and in previous studies (Fortune 1983)) were directly injected in 50 stage VI oocytes of three females. Oocytes were then stimulated with progesterone within 30 min following microinjection. No significant effect was detected on the final GVBD rates after 12 h incubation, but a potentiation was reproducibly observed during the time course of progesterone-induced oocyte maturation (Fig. 5) as sometimes evidenced in our E₂ balneation experiments, especially with oocytes from low responsive females (data not shown).

ESR1a and b are expressed by the *Xenopus* oocyte

In order to address whether E₂ may exert its effect through ligand activation of the major estrogen receptor (ER α ; ESR1; two described isoforms in *Xenopus*: ESR1a and b), we analyzed their mRNA expression by RT-QPCR in follicle-enclosed and denuded oocytes (Fig. 6). Both receptors were expressed in oocytes and their mRNA level, which represented 50% of the signal measured in whole follicles, remained stable at least throughout late oogenesis.

Discussion

Our results showed that in addition to the follicular cells, aromatase, the enzyme responsible of E₂ synthesis is also expressed and active in *Xenopus* vitellogenic oocytes. Moreover, vitellogenic and postvitellogenic oocytes do express ER α mRNA, suggesting that estrogens may participate in oocyte late development.

Ovarian follicle E₂ synthesis, a three cell-type model

In this study, aromatase expression and activity were detected in the follicular cells of *Xenopus* follicular cells as evidenced by *in situ* hybridization. Previous studies have already shown that aromatase is expressed by the follicular cells in the amphibian *Rana rugosa* during gonad development (Kato *et al.* 2004). However, our observations also demonstrate that follicular somatic cells are not the unique site of aromatase expression in *Xenopus*. Aromatase appears indeed expressed and active in vitellogenic oocytes. This observation leads to reconsider the two-cell cooperation model that has been proposed for amphibians (Ahn *et al.* 1999). Moreover, a significant level of aromatase activity was detected in *Xenopus* oocyte that corresponds to 25% of whole follicle aromatase activity. This steroidogenic activity is consistent with the mRNA expression that also corresponds to 25% of whole follicle expression. As a follicular control, we observed that *star* was exclusively

Figure 5 Effect of estradiol (E_2) microinjection on progesterone-induced resumption of meiosis of *Xenopus* denuded oocytes. Fifty stage VI prophase I oocytes were microinjected with 30 pg E_2 or with vehicle buffer (veh.) and then incubated at room temperature in modified OR2 buffer completed with 1 μ M progesterone (Pg + E_2 or Pg + veh. respectively), whereas 50 oocytes were only incubated with or without 1 μ M progesterone (Pg (n.i.) or cont. respectively). Germinal vesicle breakdown (GVBD) was assessed by direct observation under a stereomicroscope of the appearance of a white spot on the oocyte animal pole. Results are representative of three independent experiments.

expressed by the follicular cells. Star is involved in cholesterol shuttling to the inner mitochondrial membrane and is thus necessary for androgen and estrogen synthesis. As Cyp17 (Yang *et al.* 2003) and p450-arom are expressed by the oocyte, it suggests that a constant interaction exists between the somatic and the germinal follicular compartments for ovarian steroidogenesis to occur. Therefore, the contribution of the oocyte compartment to the overall follicular aromatase activity appears more important than initially believed. So far, aromatase expression in the oocyte of non-mammalian vertebrates was never clearly reported or discussed, even though a thorough analysis of existing literature revealed a positive protein signal in the oocyte of medaka (Park *et al.* 2008) and *Rana rugosa* (Kato *et al.* 2004), and we recently evidenced such an oocyte expression of *cyp19a1a* in rainbow trout (Gohin *et al.* 2011). In order to further investigate whether oocyte contribution to E_2 synthesis is specific to a few vitellogenic species or, in contrast, a mechanism shared by evolutionary distant vertebrates, it would be interesting to perform such studies in other non-mammalian species.

The expression in the *Xenopus* oocyte of both Cyp17 (Yang *et al.* 2003) – the enzyme able to metabolize progestins into androgens – and p450-arom – the enzyme able to aromatize androgens – is consistent with an autonomous production of E_2 by the oocyte. It is also very likely that steroid exchanges exist between oocyte and surrounding somatic follicular compartments, leading to difficulties to access the specific role of E_2

synthesized by one compartment in comparison to the other. Moreover, it is also possible that oocytes modulate E_2 production by the follicular somatic cells and reciprocally, as previously reported (Sretarugsa & Wallace 1997). The regulation of aromatase expression and activity in the germinal compartment of the ovarian follicle during final oocyte maturation would be in favor of an active participation of E_2 from oocyte origin in the intra-follicular dialog. Therefore, our findings and a previous report (Yang *et al.* 2003) clearly indicate that a third participant – the oocyte – has to be added to the two cell-type model of E_2 synthesis in *Xenopus*. Nevertheless, determining the physiological role of E_2 synthesized by the oocyte in comparison to the role of E_2 of somatic origin is difficult to perform for technical reasons as local inhibition of oocyte aromatase within each compartment of the whole follicle cannot be performed by microinjection or use of aromatase inhibitors.

Modulation of *Xenopus* oocyte maturation by E_2

Oocyte aromatase expression and activity decreased during late oogenesis concomitantly with a decrease in aromatase expression and activity in the whole follicle

Figure 6 QPCR analysis of estradiol (E_2) receptor 1 (*esr1a* and *b*) mRNA expression in whole follicles and oocytes during late oogenesis. The mRNA levels of E_2 receptor 1 (*esr1a* and *b*) were monitored in whole follicles and denuded oocytes sampled during vitellogenic stage IV (st IV) and matured stage VI (st VI MII). For each sample, three separate RT reactions were carried out using three separate RNA samples originating from three different animals. Analysis of mRNA levels was standardized to levels of 18S rRNA. The mRNA abundance was arbitrarily set to 100 for stage IV follicle. Mean $\pm$ S.E.M. are shown ($n=3$). No significant difference could be detected (using non-parametric Mann–Whitney U test).

(Mulner *et al.* 1978, Fortune 1983, Gohin *et al.* 2010). Moreover, *Xenopus* intra-oocyte E_2 concentrations are in the range of E_2 content previously measured in the whole follicles (Fortune 1983) and in plasma varying from 1 to 28 ng/ml corresponding to a maximum of 100 nM (Pickford & Morris 1999, Hecker *et al.* 2004, Urbatzka *et al.* 2007). Moreover, E_2 appeared to be maternally inherited as E_2 is still detected in *Xenopus* unfertilized eggs (Kloas 2002). The physiological function of oocyte E_2 remains unclear, particularly during oocyte maturation. As reported in *Rana pipiens* (Schuetz 1972, Spiegel *et al.* 1978, Lin & Schuetz 1983, 1985), E_2 appeared to inhibit *Xenopus* follicle-enclosed oocyte maturation likely through an inhibition of gonadotropin/cAMP-induced progesterin synthesis by follicular layers (Lin *et al.* 1988). However, a direct inhibitory effect of E_2 on the oocyte could not be ruled out, as demonstrated in previous studies in *Xenopus* (Baulieu *et al.* 1978) and in *Rana pipiens* (Lin & Schuetz 1983). Moreover, several studies reported a slight significant effect of high E_2 dose pretreatment on *Xenopus* denuded oocyte maturation (Hanocq-Quertier & Baltus 1981, Pickford & Morris 1999). In our study, E_2 , added simultaneously and at nearly a tenfold oocyte physiological concentration, did not inhibit steroid-triggered maturation in denuded oocytes, suggesting that E_2 inhibitory effects are occurring in follicular cells. We even surprisingly observed that E_2 exerted a slight enhancing action on progesterone-induced meiotic resumption, especially when used in combination with a sub-saturating dose of the MIS. The potentiating effect of E_2 evidenced previously and in this study may be due to E_2 antioxidant effect.

The effect previously observed after E_2 pretreatment may also suggest that its potentiating effect results from a classical genomic mechanism of gene expression activation by ligand-dependent activation of the classical – intracellular – Esr1 and/or Esr2. This is strengthened by the fact that *esr1* (*a* and *b*) mRNAs are also detected in *Xenopus* oocytes and their content remains stable throughout late vitellogenesis and maturation. Nevertheless, E_2 may also act through a non-genomic mechanism after activation by ERs of signaling pathways (via c-Src or MAPK pathways) that would subsequently interact with the maturation-promoting factor induction. Indeed, it has been shown in other cell types that ER α (Esr1) directly interacts with and activates Src (Fox *et al.* 2009), the latter being able to accelerate progesterone-induced oocyte maturation (Tokmakov *et al.* 2005). Moreover, MAPK activation has been shown to participate in maturation promoting factor (MPF) activation and thus to stimulate *Xenopus* oocyte maturation (Palmer & Nebreda 2000). Finally, a modulator of non-genomic action of the ER, MNAR, is also expressed in *Xenopus*

oocytes (Haas *et al.* 2005). All these results suggest that E_2 genomic and non-genomic effects likely play important and unsuspected roles at the oocyte level during late oogenesis that will need to be further characterized.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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