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Expression and Localization of RAP250 mRNA in Rat Ovary

Possible Implications in Follicular Development and Ovulation

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The expression levels of nuclear receptor coregulators in specific tissue compartments and cells are thought to influence the expression of hormone-responsive genes involved in metabolism, development, and reproduction. RAP250 is a novel nuclear receptor coactivator highly expressed in brain and reproductive organs. To investigate the possible involvement of RAP250 in tissue-specific regulation of ovarian function, untreated immature, pregnant mare's serum gonadotropin luteinizing hormone (PMSG-LH)–primed, cycling, and pregnant rat models were used to study the localization and expression of RAP250 mRNA in rat ovary by *in situ* hybridization (ISH) and reverse transcriptase polymerase chain reaction (RT-PCR). The results showed that RAP250 mRNA was primarily localized to granulosa cells of healthy follicles in immature, cycling, and pregnant rats and increased during PMSG-induced follicular development. In the preovulatory and ovulatory follicles from the LH-primed rats of 48-h post-PMSG administration, the signals for RAP250 mRNA increased further and remained high until early luteal formation. Only a subset of corpora lutea during diestrus 1, diestrus 2, and initiation of pregnancy was weakly positive, and atretic follicles were largely negative. The RT-PCR results confirmed the presence of RAP250 mRNA in the rat ovary and strengthen the data from ISH. These findings suggest that RAP250 may play potential roles in follicular development and ovulation.

Key Words: RAP250; mRNA; follicular development; ovulation.

Introduction

Nuclear receptor ligands, such as sex steroids (progestin, estrogen, and androgen), adrenal steroids, thyroid and retinoid hormones, as well as a variety of other metabolic ligands, regulate developmental and physiologic processes by activating intracellular members of the nuclear receptor superfamily *(1)*. The liganded nuclear receptors bind to their cognate hormone response elements, located in the promoter or enhancer regions of target genes, and transmit signals to the transcriptional machinery via direct protein-protein interactions to stimulate transcriptional activation of hormone-responsive genes *(2–4)*. In addition, another class of proteins, called coregulators, is recruited and serves as bridging molecules between the transcription initiation complex and nuclear receptors *(5,6)*. Recent data *(7)* have indicated that the coactivators, as well as the corepressors, exist in distinct steady-state precomplexes and may function as molecular gates to enable integration of diverse signal transduction pathways at nuclear receptor–regulated promoters. Coactivator genes are ubiquitously expressed in many organs, but there is a marked tissue-to-tissue variation in their mRNA levels, which hints that they may be involved in diverse developmental and physiologic processes *(8)*.

RAP250 is a putative nuclear receptor coactivator isolated from a mouse embryo cDNA library using the yeast two-hybrid system *(9)*. The determined target receptors with which RAP250 interacts include estrogen receptors (ERs), thyroid hormone receptors, and peroxisome proliferator-activated receptors. The interaction between RAP250 and nuclear receptors is ligand dependent or ligand enhanced, depending on the nuclear receptor, and involves only one short LXXLL motif called nuclear receptor box *(9)*. The gene is widely expressed with the highest mRNA level in the brain and reproductive organs, such as the ovary, testis, and prostate, which are important target organs of hormone action. The specifically high expression of RAP250 in these organs raises the question of whether the gene is involved in the neuroendocrine regulation on the reproductive system by conferring tissue specificity to related hormone action.

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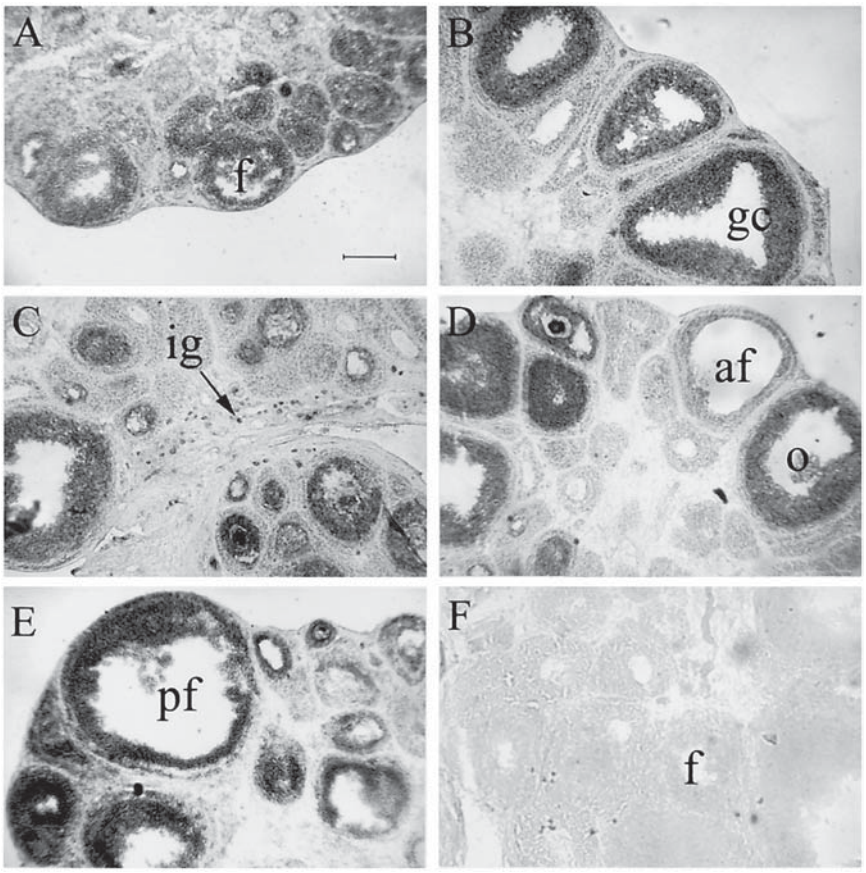


Fig. 1. *In situ* localization of RAP250 mRNA in rat ovary during gonadotropin-induced follicular development and ovulation. Cryostat sections were hybridized with digoxigenin (DIG)-UTP-labeled RNA probe, as described in Materials and Methods. (**A**) Ovaries from untreated immature rats; (**B–D**) ovaries from rats 48 h after PMSG treatment; (**E**) ovaries taken at 10 h after LH treatment; (**F**) representative negative control using RAP250 sense probe. f, follicle; gc, granulosa cell; ig, interstitial gland; o, oocyte; af, atretic follicle; pf, preovulatory follicle. Bar = 200 μm.

To investigate the potential role of RAP250 on the regulation of ovarian function, the present study was undertaken to localize RAP250 mRNA in rat ovaries during follicular development, ovulation, and early luteal formation.

Results

Differential Distribution of RAP250 mRNA in Rat Ovary

In situ hybridization (ISH) analysis was performed to determine which cell types were responsible for the expression of RAP250 mRNA in rat ovary. In ovaries from untreated immature rats (Fig. 1A), RAP250 mRNA was found to be predominantly present in granulosa cells and cumulus cells at different follicular development stages. Compared to the level of background, some follicles contained theca cells labeled weakly for RAP250 mRNA, but the signal was hardly discernible. Forty-eight hours post–pregnant mare's serum gonadotropin (PMSG) (15 IU), the follicles increased in size owing to the proliferation of granulosa and theca cells and an enlargement of the antrum (Fig. 1B). During the transition from the small antral to the large antral follicles, the intensity of the signal for RAP250 mRNA in granulosa cells increased to a high level, and the hybridization signals were also present in oocytes of the large antral follicles (Fig. 1D), as well as in the ovarian interstitial glands (Fig. 1C). By contrast, no RAP250 transcripts were detected in the follicles toward morphologic atresia (Fig. 1D). The stronger signal intensity was found in preovulatory follicles from luteinizing hormone (LH)–treated rats (Fig. 1E).

In the ovaries from cycling rats, strong signals for RAP250 mRNA were seen in the granulosa cells of different size follicles and remained at a stable level during all stages of the cycle. Weak but clearly evident signals were detected in a subset of the corpora lutea from the rats during diestrus 1 and diestrus 2 (Fig. 2C,D), whereas in the corpora lutea of proestrous and estrous ovary, the hybridization signal was almost indiscernible (Fig. 2A,B).

To investigate the expression pattern of RAP250 mRNA in the ovary during early pregnancy, the ovaries on d 1–7 of pregnancy were collected for ISH. RAP250 transcript was localized to the granulosa cells of healthy follicles and a subset of the corpora lutea on d 1 and 2 of pregnancy (Fig. 2E). From d 3 of pregnancy on, the intensity of hybridization signal in corpora lutea greatly diminished to an undetectable

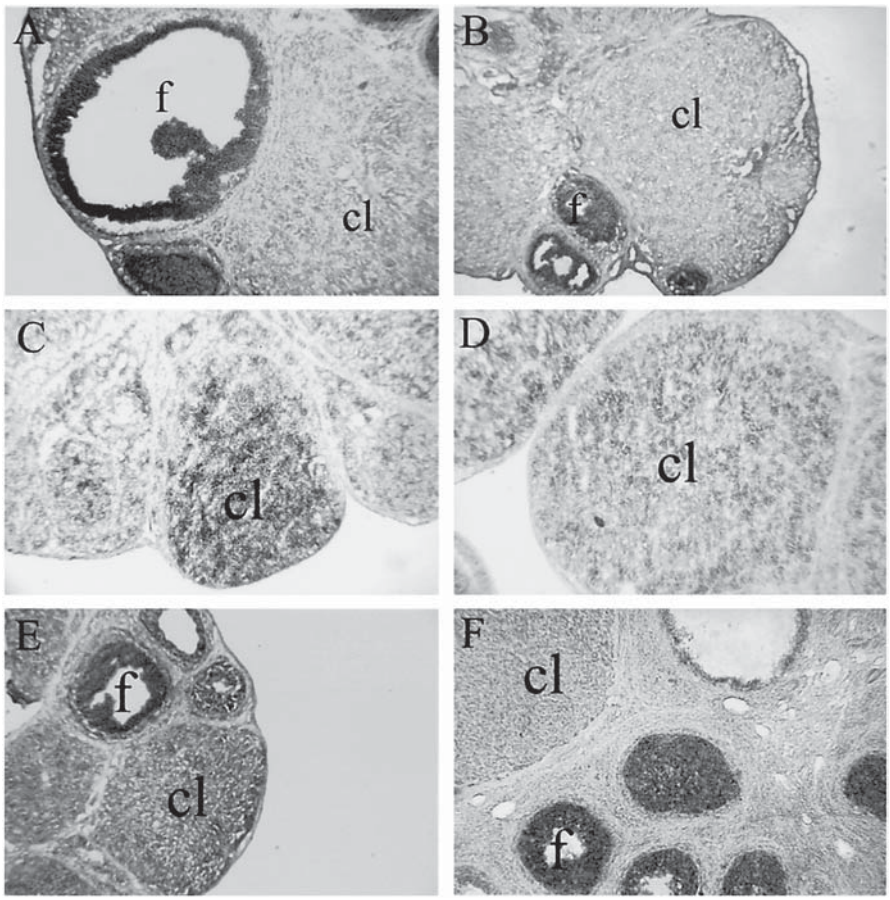


Fig. 2. Expression of RAP250 mRNA in ovary from cycling and pregnant rat. RAP250 is strongly expressed in granulosa cell of healthy follicles, weak but evident signals were present in a subset of the corpora lutea during diestrus 1, diestrus 2, and initiation of pregnancy. (**A**) Proestrus; (**B**) estrus; (**C**) diestrus 1; (**D**) diestrus 2; (**E**) d 2 of pregnancy; (**F**) d 4 of pregnancy. f, follicle; cl, corpus luteum. Bar = 200 μm.

level. By contrast, strong staining for RAP250 mRNA was still present in the granulosa cells of different size follicles. Figure 2F shows the localization for RAP250 mRNA in the ovary on d 4 of pregnancy, and the pattern of staining was similar in the ovary on d 3–7 of pregnancy (data not shown).

Reverse Transcriptase Polymerase Chain Reaction

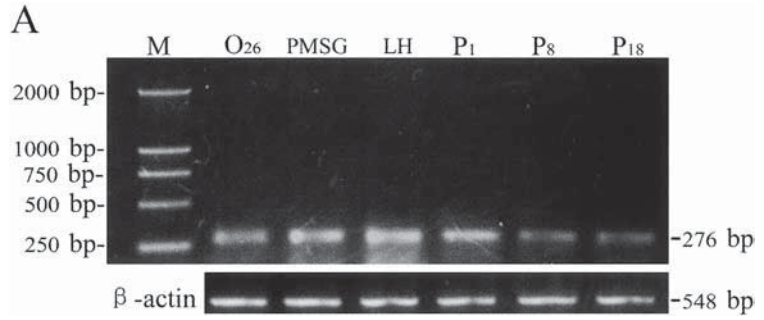
The results of reverse transcriptase polymerase chain reaction (RT-PCR) confirmed the presence of mRNA for RAP250 in the rat ovary. The cDNA of the predicted size was amplified (Fig. 3A). The product of the RT-PCR, corresponding to RAP250, was purified and sequenced and found to have 85% homology to the human analog (Fig. 3B).

Discussion

The current study reports, for the first time, the detailed expression and localization of mRNA for RAP250 in the rat ovary. Interestingly, the novel nuclear receptor coactivator is predominantly localized in the granulosa cells of healthy immature and mature follicles in prepubertal, naturally cycling, and pregnant rats, but the follicles destined to be atretic were devoid of RAP250 transcripts. In the corpora lutea, only relatively weak hybridization signals were detected in a subset of corpora lutea during diestrus 1, diestrus 2, and initiation of pregnancy.

Our observations raise the question of whether the high expression of RAP250 gene specifically in granulosa cells at various follicular development stages confers tissue specificity of related hormone action to folliculogenesis and ovulation. It has been reported that RAP250 has a large intrinsic glutamine-rich activation domain and can significantly enhance the transcriptional activity of several nuclear hormone receptors, among which ERs are the important target receptors with which RAP250 interacts *(9)*.

Estrogen originates from the ovary and also exerts local effects within this organ by activating the cognate ERs specifically expressed in ovarian tissue. As the ovarian follicles grow and differentiate, increasing amounts of estrogen are produced *(10)*, which, in turn, increase follicular expression of both follicle-stimulating hormone and LH receptors in granulosa cells *(11)*, control granulosa cell gap junction formation *(12)*, and inhibit granulosa cell apoptosis *(13)*, thereby promoting ovarian follicular growth. The interac-



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      gcagccacag ccgcagccac caccacctag tcagccacag  rat

3278  cagcagcagc agcagcaaca aatgatgatg atgctcatga  human
      tcgcagcagc ggcagcagca gatgatgatg atgctcatga  rat

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      tggttagttt gcaaggacct gcctctgtgc caccat      rat
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Fig. 3. RT-PCR analysis of RAP250 mRNA in rat ovary. (**A**) The expected band can be seen in ovaries from 26-d-old rats (D26) and becomes strong after PMSG and LH treatment. The band is also present in early developing (P_1) corpora luteum and decreases in mid (P_8) and late (P_{18}) corpora luteum of pregnancy. The results are representative of three independent experiments. (**B**) Alignment of rat RAP250 partial sequence and homolog region of human cDNA. The bases shown in black boxes differ from the corresponding human bases. The two RAP250 sequences share 85% identity.

tion of RAP250 with ERs is ligand enhanced *(9)*; therefore, increasing amounts of estrogen in growing follicles may intensify activation of hormone-responsive genes. There was evidence showing that ER-β is primarily localized in the granulosa cells of rat ovary, with only weak signals in corpora lutea (*14–16)*. It seems reasonable that interactions between RAP250 and ER-β mediate estrogenic actions in granulosa cells, and the increased expression of RAP250 observed in the ovarian grannulosa cells of gonadotropin-treated rat may facilitate the action of estrogen. Unlike the predominant granulosa cell expression of ER-β, ER-α is mainly present in theca cells, interstitial gland cells, and germinal epithelium cells *(17)*. The present survey demonstrated that RAP250 mRNA was not expressed in the ER-α-positive cell types except interstitial gland cells, implying that the novel coactivator is not the main coregulator of the ER-α subtype in rat ovary.

In summary, the present study demonstrates that RAP250 mRNA is expressed at different compartments and levels in rat ovary and may confer specific tissue to hormonal sensitivity, thus leading to a better understanding and further exploring of the molecular mechanisms of hormonal effects on ovarian function.

Materials and Methods

Animals and Tissue Recovery

All animal procedures were approved by the Committee on Animal Care and Use (Institute of Zoology, Chinese Academy of Sciences). Sprague-Dawley rats were kept under standardized environmental conditions (14 h of light:10 h of darkness) with free access to water and pelleted food. Immature female rats were injected with 15 IU of PMSG (Sigma, St. Louis, MO) on d 26 of age to initiate follicular development. At 48 h post-PMSG, ovaries were collected from a subset of these animals. The remaining animals received 20 IU of LH (Sigma) to stimulate ovulation. The estrous cycles of adult rats were determined by daily observation of vaginal smears for two consecutive cycles before the animals were killed. Time pregnant rats were obtained by normally caging male and female rats overnight. Midday of the day when the vaginal plug is present was considered pregnant day (P) 1. At each chosen time point, at least three animals were killed, and the ovaries were collected. Some corpora lutea at various stages of pregnancy were dissected from the ovaries under a stereoscopic microscope as previously described *(18)*.

Morphologic Characterization of Follicular Health

Follicles were identified as healthy on the following basis: no more than three pyknotic nuclei in membrana granulose layer, granulosa cells regularly apposed on an intact basement membrane, and no fibroblastic morphology in the granulose cell compartment. Follicles were classified as early atretic if they contained more than three pyknotic nuclei and an irregular basal lamina. In addition to these criteria, late atretic follicles also contained fibroblast cell in the granulose cell compartment, a small number of granulosa cells, and a larger follicular cavity.

Preparation of Probe

The full length of RAP250 cDNA in pSG5 expression vector was a generous gift from Dr. Eckardt Treuter (Department of Biosciences at Novum, Karolinska Institute, Huddinge, Sweden). A cDNA fragment (445 bp, 2829–3273 nucleotides) was obtained by PCR amplification from pSG5-RAP250 and subcloned into pGEM®-T easy vector (Promega, Madison, WI). The plasmid was linearized with either NcoI or NdeI, and DIG-UTP-labeled antisense or sense RNA probe was transcribed using SP6 or T7 RNA polymerase, respectively, according to the protocol by the supplier (Boehringer-Mannheim, Indianapolis, IN).

In Situ *Hybridization*

ISH was performed as detailed previously *(19)* with a slight modification. In brief, sections (10 μm) of rat ovary were collected onto poly-L-lysine-coated slides, fixed in 4% paraformaldehyde for 15 min, and then washed in phosphate-buffered saline containing 0.1% active diethylpyrocarbonate twice for 15 min each, followed by rinsing in 5× SSC (1× SSC is 0.15 *M* NaCl, 0.015 *M* sodium citrate) for 15 min. The slides were prehybridized with a hybridization mixture (50% deionized formamide, 5× SSC, and 120 μg/mL of salmon sperm DNA) without probes for 2 h at 55ºC. The DIG-labeled RNA probes, contained in hybridization mixture at a dilution of 400 ng/mL, were denatured and applied to hybridization reaction. The slides were sealed and incubated for 18 h at 55ºC. Unbound probes were removed by washing slides sequentially at room temperature for 30 min in 2× SSC, 65ºC for 1 h in 2× SSC, 65ºC for 1 h in 0.1× SSC. Bound probes were detected with anti-DIG antibody coupled to alkaline phosphatase according to the manufacturer's instructions (Roche, Hong Kong, China). Sense probe was used as a control for the hybridization specificity and background level.

Reverse Transcriptase Polymerase Chain Reaction

Total RNA was isolated from intact ovary or dissected corpus luteum using Trizol reagent (Gibco-BRL, Rockville, MD) according to the manufacturer's instructions. mRNA (2 μg) was reverse transcribed in a 20-μL volume by M-MLV RT (Gibco-BRL). The reverse-transcribed cDNA was amplified by PCR using specific primers of RAP250. The sense and antisense primers were 5'-AGCCACCACAGCAGCA GCCAC-3' and 5'-ATGGTGGCACAGAGGCAGGTCC-3', respectively, with an expected fragment of 276 bp. PCR reaction was carried out in 25-μL vol with 30 cycles of denaturing (95ºC, 45 s), annealing (63ºC, 45 s), and extension (72ºC, 45 s). The PCR product was electrophoresed on 1.2% agarose gels containing 0.5 μg/mL of ethidium bromide, and the size of the product was determined by comparison with a 2-kb DNA ladder. The amplified fragment in agarose gel was then excised and purified for sequencing.

Acknowledgments

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