Follicles in pregnant rat ovary are incapable of steroidogenesis

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Abstract Antral follicles are present in the ovaries throughout gestation. These follicles are "physically immature" and cannot ovulate under the induction of LH/hCG. The purpose of the present study is to examine whether follicles in pregnant rat ovaries are capable of steroidogenesis. StAR is believed to be the key regulator of steroid hormone biosynthesis. Antisense StAR probe and anti-StAR rabbit serum have been used to detect the StAR expression in ovarian follicles at various stages of pregnant rats. The results indicate that theca-interstitial cells and the membrane granulosa cells at the stages from the estrous or pre-estrous in the normal cycling rat ovary express StAR mRNA and its protein, whereas neither granulosa cells nor the theca cells in pregnant ovary throughout gestation express StAR. These results indicate that the pregnant follicles throughout gestation are incapable of steroidogenesis.

Keywords: follicles in pregnant ovary, steroidogenic acute regulatory protein, luteinizing hormone receptor, *in situ* hybridization.

THE steroidogenic acute regulatory protein (StAR) is believed to be the key regulator of steroid hormone

biosynthesis[1]. The synthesis of steroid hormone requires intramitochondrial translocation of cholesterol to the cholesterol sidechain cleavage complex which is located on the matrix side of the inner mitochondrial membrane, and this is the rate-limiting step of the steroidogenesis. It depends on *de novo* synthesis of StAR protein[2]. The precursor form of StAR is imported into the mitochondria by the leading of its signal peptide and then the signal peptide is cleaved and processed to its mature forms. In this process the inner and outer membranes of mitochondria adhere together and cholesterol enters the mitochondria from the adhesion site. Now it is proved that StAR is expressed in all the tissues capable of steroidogenesis and only the tissues synthesizing steroid hormone express StAR. Temporally only when steroid hormone is synthesized do these tissues express StAR[3,4]. The synthesizing ability in human whose StAR gene has mutated is severely damaged, and this leads to the so-called lipoid CHA. The StAR knock-out mouse is incapable of steroidogenesis[6]. So the expression of StAR is necessary for the cell to synthesize steroid hormone.

Antral follicles are present throughout gestation. These follicles are "physically immature" and cannot ovulate under the induction of LH or hCG. Therefore, whether the follicles in pregnant rats ovaries are capable of steroidogenesis is an interesting question to be investigated. Using antisense StAR probe and anti-StAR rabbit serum, we have detected the StAR expression in ovarian follicles of pregnant rats at various stages.

1 Materials and methods

(ⅰ) Animals. Sprague-Dawley rats were obtained from the Institute of Zoology, the Chinese Academy of Sciences. One male and three female sexually mature rats were put in a cage in the evening. When sperms were found in the vaginal smear the next morning, that day was considered as the first day of pregnancy. On days 2, 4, 9, 13, 18 and 22, the animals were decapitated and the ovaries were removed and fixed in Bouin's fluid. The normal estrous or pre-estrous rats were also decapitated and the ovaries were removed and also fixed in Bouin's fluid. The tissues were embedded with paraffin and sectioned about 4—6 μm, adhered to the gelatin-treated slides.

(ⅱ) *In situ* hybridization. StAR and LH receptor probes were labeled with digoxigenin *in vitro*. The deparaffinized sections were treated with 5—10 μg/mL proteinase K for 10 min at 37℃ and washed in PBS for 2 min. The sections were then fixed in 4% paraformaldehyde for 5 min and washed in PBS for 5 min. After being dehydrated through a graded ethanol series and followed by air dry, the sections were prehybridized with 50% formamide and double-strength SSC buffer (200 ng/mL DIG-labeled probe, 10 mmol/L Tris-CL, pH 7.5, double-strength SSC, 50% deionized formamide, single-strength, Denhardt's, 5% dextran sulfate, 2.5 mmol/L dithiothreitol, 0.5% SDS, and 400 μg/mL yeast tRNA) at 45—50℃. After hybridization, the sections were thoroughly washed in double-strength, single-strength, and 0.1-strength SSC, each twice, for 15 min each time at 40℃. The sections were then rinsed in DIG buffer Ⅰ (0.1 mol/L maleic acid, 150 mmol/L NaCl, pH 7.5) for 5 min, blocked with 1% blocking reagent in DIG buffer Ⅰ for 1 h, incubated with alkaline phosphatase-conjugated anti-DIG IgG diluted 1∶500 in DIG buffer Ⅰ containing 1% blocking reagent for 30 min, and washed in DIG buffer Ⅰ 3 times for 10 min each, and then washed in DIG buffer Ⅲ (0.1 mol/L NaCl, 50 mmol/L $MgCl_2$, 0.1 mol/L Tris-CL, pH 9.5) for 5 min. Then the sections were incubated in alkaline phosphatase substrate (5-bromo-4-chloro-3-indoly) phosphate and nitro blue tetrazolium for 3—6 h. The sections were mounted. For control hybridization, the sections were hybridized with StAR sense RNA probe. The control was not specifically dyed, and then dyed slightly with hematin.

(ⅲ) Immunohistochemistry. Deparaffinized sections were incubated with 0.3% H_2O_2 in methanol for 30 min, and then washed in PBS for 3 times, 5 min each. The sections were antigen recovered by a microwave oven for 5 min (1.8 mmol/L citric acid, 8.2 mmol/L sodium citric, pH 6.0), cooled at room temperature for 10 min. The sections were incubated with normal goat serum (NGS) for 20 min, and then incubated with anti-StAR rabbit serum for 30 min, and washed with PBS for 3 times, 10 min each. Then the sections were incubated with biotinylated second antibody for 30 min, washed in PBS for 3 times, 5 min each. After incubation with avidin biotin-perexidase complex in PBS for 1 h, and washed in PBS 3 times (5 min each), the sections were incubated in diaminobenzidine tetrahydrochloride with

0.01% H_2O_2 for 2—7 min, washed in tap water. The sections were dehydrated and mounted. The control was incubated with NGS instead of the primary antibody. The control was not dyed specifically, and then dyed slightly with hematin.

2 Results and discussion

The theca cells and membrane granulosa cells adjacent to the basal membrane expressed StAR mRNA and antigen in the pro-estrous and estrous rat ovaries (fig. 1). We observed that medium and large antral follicles with a diameter of about 500—700 μm were present in the ovaries throughout gestation, but neither the granulosa cells nor theca cells in these follicles could express StAR mRNA or antigen, indicating that they are incapable of steroidogenesis. Only corpus luteum expressed StAR. However, both the granulosa cells and theca cells of pregnant follicles expressed LHR mRNA (figure 2).

Cholesterol enter the follicles from the capillary vessels web and the granulosa cells can synthesize pregnenolone. Due to the invasion of the cholesterol from the outside to the inside there was a concentration ladder of cholesterol from the outside to the inside in the granulosa cell layer. The expression of StAR in the membrane granulosa cells was dramatically stronger than that of the granulosa cells in the deeper

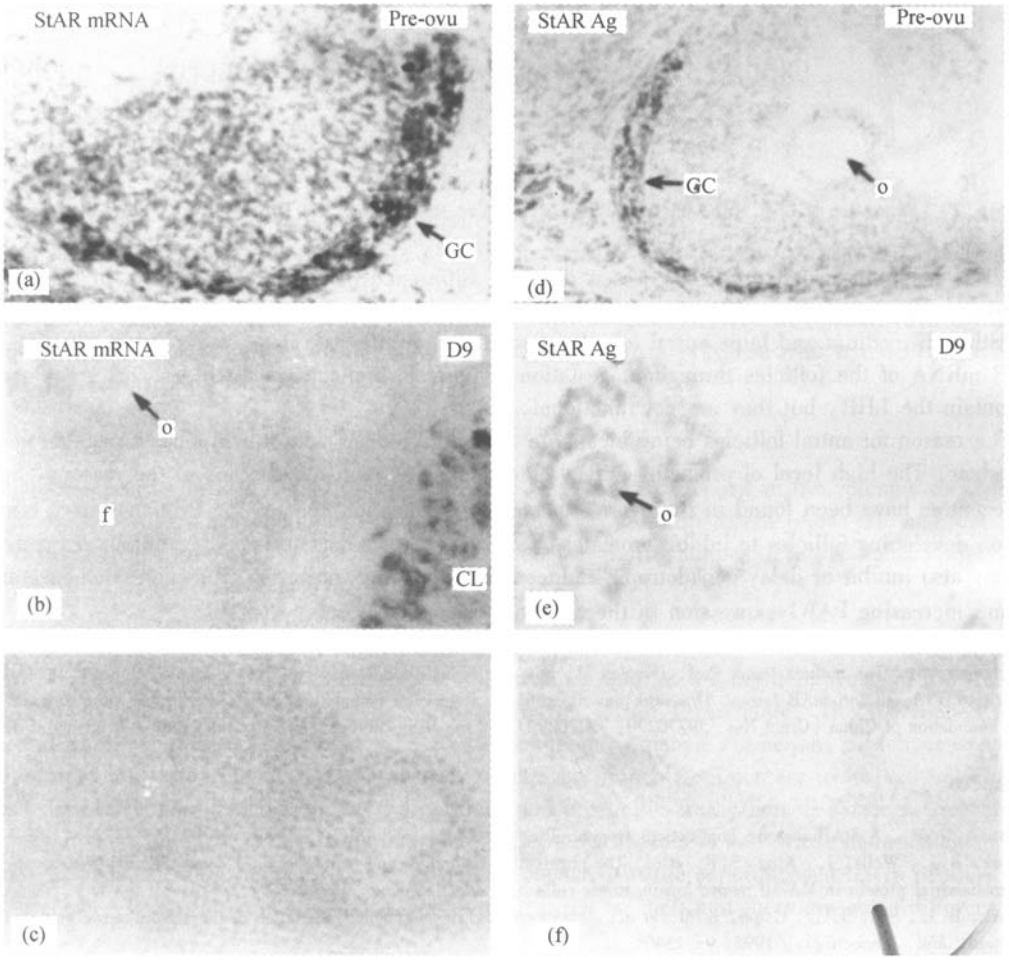

Fig. 1. Expression of StAR mRNA and antigen (Ag) in antral follicles (Pre-ovu) of pre-estrous or estrous rat ovaries and in follicles of the ninth day pregnant rat ovaries. (a), (b), (c), *in situ* hybridization results; (c) the negative control; (d), (e), (f), immunohistochemistry results; (f) negative control. CL, corpus luteum; f, follicle; o, oocyte; GC, membrane granulosa cell, × 200.

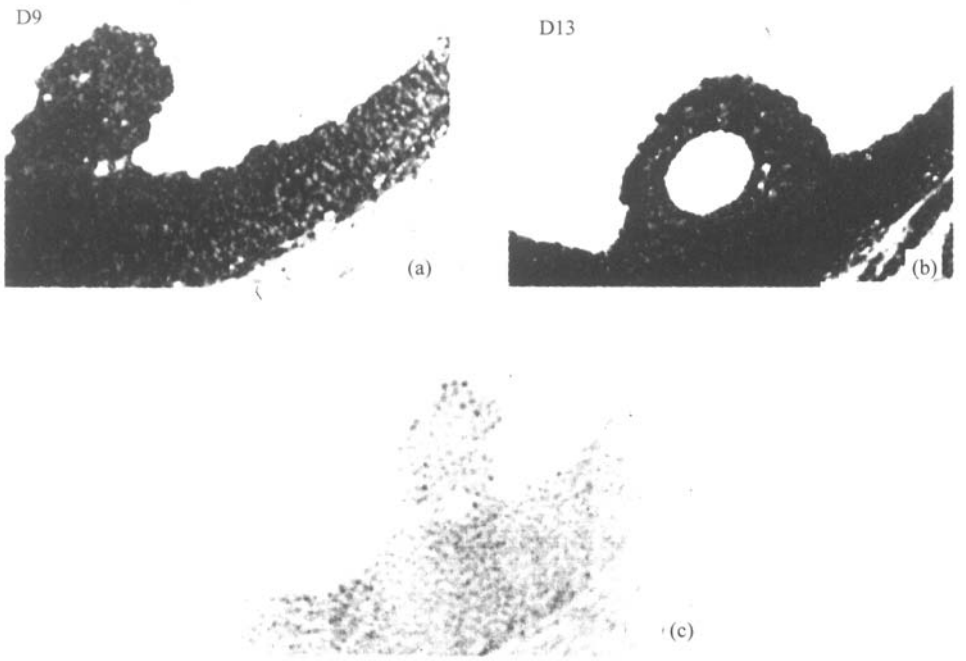

Fig. 2. Expression of LHR mRNA in the follicles of pregnant rats D9 (a) and D13 (b), (c) negative control, × 200.

layer. This result indicated that there was a dramatic difference between the two cells in the ability of synthesizing pregnenolone, and the membrane granulosa cells did the main job.

Although medium and large antral follicles throughout gestation do not express StAR. the expression of LHR mRNA of the follicles throughout gestation is normal, indicating that theca and granulosa cells may contain the LHR, but they are not functional.

The reason for antral follicles being incapable of steroidogenesis and of ovulation throughout gestation is not clear. The high level of prolactin (PRL) throughout gestation may be one of the reasons. Specific PRL receptors have been found in the ovaries of several mammalian species[7]. PRL may have effect directly on developing follicles to inhibit aromatase activity and steroidogenesis[8—11], follicular maturation. PRL may also inhibit or delay gonadotropin-induced ovulation, by inhibiting tPA expression in granulosa cells and increasing PAI-1 expression in theca cells[12].

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References

1 Stocco, D.M., A StAR search: implications in controlling steroidogenesis, *Biol. Reprod.*, 1997, 56: 328.
2 Clark, B.J., Wells, J., King, S.R. *et al.* The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells, *J. Biol. Chem.*, 1994, 269: 28314.
3 Clark, B.J., Soo, S.C., Caron, K.M. *et al.*, Hormonal and developmental regulation of the steroidogenic acute regulatory protein, *Mol. Endocrinol.*, 1995, 9: 1346.
4 Choi, Y.S., Stocco, D.M., Freeman, D.M., Diethylumbelliferyl phosphate inhibits steroidogenesis by interfering with a long-lived factor acting between protein kinase A activation and induction of the steroidogenic acute regulatory(StAR) protein, *Eur. J. Biochem.*, 1995, 234: 680.
5 Lin, D., Sugawara, T., Strauss III. J.F. *et al.*, Role of steroidogenic acute regulatory protein in adrenal and gonadol steroidogenesis, *Science*, 1995, 267: 1828.
6 Caron, K.M., Soo, S.C., Wetsel, W.B. *et al.*, Targeted disruption of the mouse gene encoding steroidogenic acute regula-

tory protein provides insights into congenital lipoid adrenal hyperplasia, *Proc. Natl. Acad. Sci. USA*, 1997, 92: 11540.
7 Saito, T., Saxena, B.B., Specific receptors for prolactin in the ovary, *Acta Endocrinol. Copenh.*, 1975, 80: 126.
8 Tsai-Morris, C.H., Ghosh, M., Hirshfield, A.N., Inhibition of ovarian aromatase by prolactin *in vitro*, *Biol. Reprod.*, 1983, 29: 342.
9 Hu, Z.Y., Liu, Y.X., Effect of prolactin on gonadotropin-induced ovarian estrogen and progesterone production in mouse, *Acta Physiologica Sinica*, 1995, 47: 96.
10 McNatty, K.P., Sawers, R.S., McNeilly, A.S., A possible role of prolactin in control of steroid secretion by the human Graafian follicle, *Nature*, 1974, 250: 653.
11 McNeilly, A.S., Prolactin and the control of Gonadotropin secretion, *J. Endocrinol.*, 1987, 115: 1.
12 Liu, Y.X., Peng, X.R., Chen, Y.J. *et al.*, Prolactin delays hCG-induced ovulation and down-regulates plasminogen activator system in rat ovary, *Human Reprod.*, 1997, 12: 2748.

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