Dev. Reprod. Vol 7, No 1, 2003(Suppl). pp 21-23

Localization of Weel and Other Cell Cycle Machinery in the Mouse Primordial and Growing Follicles

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ABSTRACT
Mechanisms regulate the arrest and growth of the resting primordial follicles are very poorly understood. To elucidate genes involved in the early folliculogenesis, we conducted suppression subtractive hybridization using mRNA from day1 and day5 ovaries and selected weel for further analysis, since it was most frequent gene in the day1-subtracted cDNA library (1). Expression of weel and correlated components of the cell cycle machinery, such as cdc2, cyclin B1, cdc25C, and phosphorylated cdc2 was evaluated by immunohistochemistry. In primordial follicles, expression of weel, cdc2, and cyclin B1 was cytoplasmic in oocytes, but phosphorylated cdc2 was weakly expressed in oocytes. While cdc25C expression was in ovarian somatic and in some theca cells. None of components was expressed in the pre-granulosa cells of the primordial follicles, while weel weakly, and cdc2 and cyclin B1 was strongly expressed in the granulosa cells of the growing follicles. Results from the present study suggest that 1) the meiotic arrest of the oocytes may not due to lack of cell cycle machinery, and 2) the weel may arrest meiosis by sequestering cdc2 and cyclin B1 in the cytoplasm by protein-protein interactions and/or by inhibitory phosphorylation.

INTRODUCTION

Mammalian folliculogenesis is a complex process regulated by various extraovarian and intraovarian factors (2). Compare to many studies on the growth and maturation of antral follicles, relatively small number of studies has been conducted on the early folliculogenesis. When ovarian follicles are formed, they enter the resting, primordial stage that persists for a period of time that varies from follicle to follicle. Mechanisms of the arrest and the initiation of follicular growth of these primordial follicles, however, are completely unknown (3).

To date, a major research has been the analysis of mostly known genes, but in the previous study, we used a suppression subtractive hybridization (SSH), a genome-wide method, to identify differentially expressed genes as well as to discover novel genes that may play important roles in primordial follicle arrest and/or activation to growth into primary follicle (1). We used day1 and day5 whole mouse ovaries for SSH rather than isolating each stage of follicles, since the day1 ovary consists mainly of primordial follicles while day5 ovary consists of primordial and primary follicles, without further developed follicles. We focused to weel and its related components to study further, since weel was the most frequent clone found in the day1-subtracted cDNA library after SSH (1). While all other previously identified protein kinases are positive regulators of cell proliferation, weel is an inhibitor of the cyclin-dependent kinase cdc2 (4, 5). The weel tyrosine

kinase carries out the inhibitory phosphorylation of the cdc2 kinase on tyrosine 15 and hence negatively regulates entry into mitosis (6, 7). We hypothesized that the weel, the well-known mitotic inhibitor, in the early stage follicles may act as a tonic inhibitor to ensure the meiotic cell cycle arrest of the primary oocytes as well as the follicular growth at the primordial stage. The concept of tonic inhibition of growth initiation in vivo originated from the observation that the spontaneous activation of follicular growth of most primordial follicles in serum-free medium occurred when cultured the neonatal, fetal ovaries, or ovarian cortex in vitro (3).

MATERIALS AND METHODS

Suppression Subtractive Hybridization (SSH)

Total RNAs were isolated from ovaries using Trizol (Gibco BRL, Grand Island, NY) and mRNAs were subsequently purified by the Oligotex mRNA purification system (QUIAGEN, Santa Clara, CA), and treated DNaseI to remove genomic DNAs followed by acid phenol extraction and ethanol precipitation. Both forward and reverse SSH was conducted to obtain the day1- and day5-subtracted cDNA libraries, respectively (1). After the PCR subtraction, the amplified products were cloned and transformed into E. coli strain of competent JM109 (Promega). The individual cDNA inserts were isolated and subjected to sequencing. DNA sequencing was performed using an automated ABI 310 DNA Analyzer (Perkin-Elmer APLLIED Biosys., Foaster City, CA). Identity of each sequenced data was confirmed in a sequence homology analysis using the BLAST and RIKEN databases.

Immunohistochemistry

The ovaries were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, sectioned at 5μm thickness, and mounted on positively charged slides (ProbeOn Plus, Fisher Scientific, Pittsburgh, PA). Tissue sections were deparaffinized and preincubated with 10% swine non-immune serum for overnight at RT and followed by incubation with primary antibodies. After incubation for 3 hrs at RT, the sections were rinsed in TBS and subsequently treated for 20min at RT with biotinylated swine secondary antibody (DAKO, Carpinteria, CA). Finally, biotinylated alkaline phosphatase complex (Dako) were treated for 20min at RT, and alkaline phosphatase activity was developed with Fuchsin (DAKO). Hematoxylin was used for counterstaining. The primary antibody used were listed as followed: weel affinity purified rabbit polyclonal antibody(C-20, 1:100; Santa Cruz Biotechnology, sc-325), p-Cdc2(Tyr15)-R affinity purified rabbit polyclonal antibody(1:100; Santa Cruz Biotechnology, sc-7989-R), Cdc2 p34(17) mouse monoclonal IgG2a antibody(1:100; Santa Cruz Biotechnology, sc-54), mouse monoclonal anti-human cyclin B1 antibody(clone CB169, 1:200; Upstate Biotechnology, Lake Placid, NY; This antibody was raised against a bacterially produced GST-fusion protein(IgG2b) corresponding to residues 130-433 of human cyclin B1), Cdc25C affinity purified rabbit polyclonal antibody(C-20, 1:100; Santa Cruz Biotechnology, SC-327).

RESULTS AND DISCUSSION

By immunohistochemisty, strong expression of the weel protein was observed in the cytoplasm of all oocytes in the arrested primordial as well as in the growing follicles. While relatively weaker expression compared to that of oocytes was observed in the surrounding granulosa cells of the growing follicles.

We observed concurrent cytoplasmic expression of cdc2 and cyclin B1, to the weel expression in all oocytes, suggesting oocytes from the primordial follicles have the cell cycle machinery. However, expression of cdc25C was observed neither in the oocytes nor in the granulosa cells, but in some theca cells of the growing follicles and in some ovarian somatic cells. We expected to

observe concurrent cytoplasmic expression of the phosphorylated cdc2 in the oocytes, since the weel exerts inhibitory phosphorylation of the cdc2 in the mitosis (6, 7).

Phosphorylated cdc2 expression was weak in the primordial follicular oocytes, but stronger in oocytes, granulosa cells, and theca cells of the growing follicles. Progression through cell cycle transition is due to the sequential appearance of cyclins and activation of their cdk partner (8). Several modes of regulating the presence of the cyclins and cdks are used, such as transcription, translation, turnover, phosphorylation/dephosphorylation, inhibitors, and subcellular localization (8). It has been suggested that the nuclear localization of some of the cyclin/cdk complexes may be important for mitosis or meiosis I progression (9). We concluded that the high cytoplasmic expression of weel in the primary oocytes may act as a tonic inhibitor of the meiotic cell cycle, and it may work through phosphorylation and/or sequestering of the cdc2/cyclin B1 in the cytoplasm.

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