Expression patterns of the DAZ-associated protein DAZAP1 in rat and human ovaries

Hsien-An Pan, M.D.,[a,b] *Yue-Shan Lin, M.D.,*[c,d] *Ko-Hung Lee, M.D.,*[a] *Jin-Ru Huang, M.Sc.,*[e] *Ying-Hui Lin, M.D.,*[a] *and Pao-Lin Kuo, M.D.*[a]

[a] Department of Obstetrics and Gynecology, [b] Institute of Clinical Medicine; [c] Department of Obstetrics and Gynecology, Chi-Mei Hospital; [d] Center of General Education, National Chung Cheng University, Kaohsiung, Taiwan; and [e] Institute of Molecular Medicine, College of Medicine, National Cheng Kung University, Kaohsiung, Taiwan

Objective: To evaluate the expression of DAZAP1 (deleted in azoospermia–associated protein 1) in rat and human ovaries.
Design: Experimental study.
Setting: University hospital.
Patient(s): Twelve corpus luteum (CL) specimens were collected during operation, either by laparoscopic surgery for CL rupture or by laparotomy for benign gynecologic conditions.
Intervention(s): Surgical excision of 12 human CL.
Main Outcome Measure(s): Proteins analyzed by immunohistochemical staining, Western blotting, and co-immunoprecipitation experiments.
Result(s): DAZAP1 is expressed in rat and human luteal cells. Expression of DAZAP1 decreases with advancing stages of CL. Co-immunoprecipitation experiments show in vivo interaction of DAZ-like (DAZL) protein with DAZAP1 in the ovarian tissues.
Conclusion(s): The expression patterns of DAZAP1 and DAZL are identical within rat and human ovaries. In mammalian species, DAZAP1 may be involved in diverse reproductive functions, ranging from cell cycle regulation and maturation of oocytes to differentiation of luteal cells. (Fertil Steril® 2005;84(Suppl 2):1089–94. ©2005 by American Society for Reproductive Medicine.)

Key Words: Corpus luteum, DAZAP1, DAZL

Proteins that bind RNA play important roles in the posttranscriptional regulation of gene expression. They also participate in the processing, transport, localization, and translational control of messenger RNA (1, 2). Ribonucleic acids that localize to the vegetal cortex of *Xenopus* oocytes are involved in early embryonic patterning and cell fate specification. Vg1 is a maternal mRNA that becomes localized to the vegetal cortex of the mature oocyte and is required for generating dorsal mesoderm at the blastula stage of *Xenopus* embryogenesis (3, 4). Proline-rich RNA-binding protein (Prrp) binds to Vg1 mRNA and VegT mRNA, which are essential for the proper localization of Vg1 mRNA to the vegetal cortex of the *Xenopus* oocytes (5). The ortholog of Prrp in the mammalian species is DAZAP1 (deleted in azoospermia–associated protein 1). DAZAP1 shares 89% similarity and 81% identity in amino acid sequences with *Xenopus* Prrp (6). DAZAP1 also is an RNA-binding protein, identified through its interaction with putative male infertility factors, DAZ and DAZ-like (DAZL), in a yeast two-hybrid system (7). DAZAP1 contains two RNA-binding domains in the N-terminal portion and a proline-rich domain in the C-terminal portion and is expressed most abundantly in the testis (8).

The *DAZ* gene family consists of three members in the human being: *DAZ* on Yq11.2, *DAZL* on 3p25, and *BOULE* on 2q33. The *DAZ* gene is present only on the Y chromosomes of great apes and Old World monkeys (9, 10). The autosomal *DAZL*gene is present in all vertebrates studied (10–13), and *BOULE* orthologs have been isolated from *Drosophila* and *Caenorhabditis elegans* in addition to mice and humans (14–16). Both *DAZ* and *DAZL* are thought to have evolved from the same ancestral *BOULE* gene, and they share common characteristics (16). They both encode RNA-binding proteins that are expressed mainly in germ cells. The *DAZ* gene encode several isoforms with a polymorphic *DAZ* repeat region containing 8–24 copies of a 24–amino acid *DAZ* repeat, whereas the DAZL protein contains a single *DAZ* repeat unit (9–11, 17). Deletion of the *DAZ* repeat region dramatically reduced the binding of DAZ/DAZL to DAZAP1 (7). *DAZL*is unique in terms of its tissue distribution patterns among *DAZ* gene family members, considering its expression not only in germ cells but also in somatic cells. In the male reproductive system, the DAZL protein is expressed in multiple stages of germ cell development, including gonocyte, spermatogonia, spermatocyte, spermatid, and spermatozoa (18–20). In the female reproductive system, DAZL is expressed in the fetal oogonium, adult oocytes,

Received November 2, 2004; revised and accepted March 13, 2005.
Supported by research grants from the National Science Council, Taiwan (NSC 92-2314-B-006-122 and NSC 92-2314-B-006-133).
Reprint requests: Pao-Lin Kuo, M.D., Department of Obstetrics and Gynecology, National Cheng-Kung University Medical Center, 138 Sheng-Li Road, Tainan 70428 Taiwan (FAX: 886-6-2766185; E-mail: paolink@mail.ncku.edu.tw).

granulosa cells, and theca interna (21–23). In a report elsewhere, we showed evidence that DAZL protein is expressed in granulosa-lutein cells from different phases of the corpus luteum (CL) (24).

Like DAZ/DAZL, DAZAP1 also binds to RNA homopolymers, with a preference for polyuridylic acid and polyguanylic acid (7). In vitro, DAZAP1 binds to both the Y chromosome–encoded DAZ and an autosome-encoded DAZL protein. However, the in vivo interaction of *DAZ* gene family members with DAZAP1 has not been demonstrated. Most proteins function through their interaction with other proteins. In this study, we showed expression patterns of DAZAP1 in rat and human ovaries. We also demonstrated the in vivo interaction of DAZL with DAZAP1.

MATERIALS AND METHODS

Collection of Human Ovarian Tissues

This study was approved by the institutional review board of National Cheng Kung University Hospital. We collected 12 specimens of CL: 4 in the early luteal phase, 4 in the mid-luteal phase, and 4 in the late luteal phase. The specimens were taken during operation, either by laparoscopic surgery for CL rupture or by laparotomy for benign gynecologic conditions. Each patient had regular menstrual periods, and the dating had been confirmed by the histology of the endometrium. The luteal phase was subdivided into an early luteal phase (days 15–19 of the cycle, or young CL, 1–5 days of age), mid-luteal phase (days 20–24 of the cycle, or mature CL, 6–10 days of age), and late luteal phase (days 25–27, or old CL, 11–13 days of age). Cycle day 14 was considered to be CL day 0. Human luteinized granulosa cells were obtained from four women undergoing IVF at our medical center. Granulosa cells were isolated, as described elsewhere (25), from aspirated follicular fluid after ovum retrieval. After centrifugation, the GLC layer on the top of a red blood cell pellet was resuspended in 1X phosphate buffer saline (PBS) containing 100μg/mL DNase I (Roche Molecular Biochemicals, Mannheim, Germany) and 2mg/mL type IV collagenase (Gibco, Grand Island, NY). The cell suspension was incubated at 37°C for 20 minutes and centrifuged at 300 X*g* for 10 minutes at room temperature. The pellet was resuspended in lysis buffer (120mM NaCl, 10mM Tris-HCl (pH 6.8), 1% Noidet P-40, 0.1% SDS and 1% deoxycholate) with protease inhibitor cocktail [2mM 4-(2-aminoethyl)benzenesulfony fluoride (AEBSF), 1mM EDTA, 130 μM bestatin, 14 μM E-64, 1mM leupetin and 0.3μM aprotinin] (Sigma, St. Louis, MO) for protein extraction.

Generation of Antibodies, Co-Immunoprecipitation, and Western Blotting

A goat anti-DAZAPl antibody was generated against the last 19 aa residues of the mouse DAZAP1 protein (26). A rabbit anti-DAZL antibody was generated against the 272nd to 290th amino acids of the DAZL protein (19, 24). Antibodies then were affinity purified on protein A columns (ImmunoPure-Plus Immobilized Protein A IgG Purification Kit; Pierce, Rockford, IL). Western blotting of rat and human testis and ovary extracts detected a band of ~50 kDa for DAZAP1 and a band of ~33 kDa for DAZL (19, 24, 26). For co-IP experiments, the tissues were homogenized and mixed with twice the volume of lysis buffer containing protease inhibitor (T-PER Tissue Protein Extraction Reagent; Pierce). After complete mixing, the samples were repetitively frozen–thawed in a liquid-nitrogen tank and a 37°C water bath several times to dissociate the chromosomal DNA and proteins. Approximately 500 μg of protein was added to 50 μL of protein A Sepharose (Amersham Bioscience, Uppsala, Sweden), gently mixed for at least 1 hour, kept at 4°C overnight, and processed by centrifuge at 12,000 $\times$ *g* for 20 seconds. Five microliters of polyclonal antibodies to DAZL or DAZAP1 and 50 μL of protein A Sepharose were added to the supernatant, gently mixed for 1 hour at room temperature, and kept at 4°C overnight. After centrifugation, the pellets were washed three times with lysis buffer and fractionated on a 12% sodium dodecyl sulfate–polyacrylamide gel, and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA) by using a BioRad transfer system at 110 V for 1 hour. The membranes were washed with Tris-buffered saline containing 0.5% of Tween 20 and then incubated in a blocking solution (5% milk powder in the wash buffer) for 1 hour. The membranes then were incubated in a 1:100 to 1:2,500 dilution of a primary antiserum overnight and washed three times with the wash buffer, followed by incubation with a 1:10,000 dilution of the secondary antibody (goat anti-rabbit immunoglobulin G, peroxidase conjugated; Pierce) in the wash buffer. The filters were then washed several times, and the peroxidase activities were visualized by using SuperSignal substrate, following the manufacturer's instructions (Pierce).

Immunohistochemical Detection of DAZAP1 and DAZL in Human CL and Dazap1 and Dazl in the Rat Ovary

Specimens were dehydrated, embedded in paraffin, and sectioned at 5 μm. Immunostaining of DAZAP1 or DAZL was carried out as described elsewhere (19, 24, 26). Briefly, sections were deparaffinized with 100% xylene and rehydrated with 100%, 95%, and 70% ethanol. The slides then were blocked with 3% hydrogen peroxide in absolute methanol for 5 minutes, washed with water for 5 minutes, and heated at 90°C for 5 minutes in preheated citrate acid buffer. After cooling, the slides were washed twice with Tris-buffered saline for 5 minutes each. The slides were incubated with primary antibody (1:1,000) for 60 minutes at room temperature. Binding of primary antibodies to tissue sections was detected by using a goat ABC staining system (Santa Cruz Biotechnology, Santa Cruz, CA). After the washing steps with Tris-buffered saline, sections were incubated with biotinated mouse anti-rabbit immunoglobulin G antibody (Dako, Carpinteria, CA) for 30 minutes at room temperature, washed with Tris-buffered saline, then incubated with avidin-biotinylated peroxidase complex for 30 minutes at room temperature, followed by