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Changes in ovarian protein expression during primordial follicle formation in the hamster

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ABSTRACT

Although many proteins have been shown to affect the transition of primordial follicles to the primary stage, factors regulating the formation of primordial follicles remains sketchy at best. Differentiation of somatic cells into early granulosa cells during ovarian morphogenesis is the hallmark of primordial follicle formation; hence, critical changes are expected in protein expression. We wanted to identify proteins, the expression of which would correlate with the formation of primordial follicles as a first step to determine their biological function in folliculogenesis. Proteins were extracted from embryonic (E15) and 8-day-old (P8) hamster ovaries and fractionated by two-dimensional gel electrophoresis. Gels were stained with Proteosilver, and images of protein profiles corresponding to E15 and P8 ovaries were overlayed to identify protein spots showing altered expression. Some of the protein spots were extracted from SyproRuby-stained preparative gels, digested with trypsin, and analyzed by mass spectrometry. Both E15 and P8 ovaries had high molecular weight proteins at acidic, basic, and neutral ranges; however, we focused on small molecular weight proteins at 4–7 pH range. Many of those spots might represent post-translational modification. Mass spectrometric analysis revealed the identity of these proteins. The formation of primordial follicles on P8 correlated with many differentially and newly expressed proteins. Whereas Ebp1 expression was downregulated in ovarian somatic cells, Sfrs3 expression was specifically upregulated in newly formed granulosa cells of primordial follicles on P8. The results show for the first time that the morphogenesis of primordial follicles in the hamster coincides with altered and novel expression of proteins involved in cell proliferation, transcriptional regulation, and metabolism. Therefore, formation of primordial follicles is an active process requiring differentiation of somatic cells into early granulosa cells and their interaction with the oocytes.

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1. Introduction

The formation of primordial follicles requires the interaction of the oocytes with surrounding somatic cells, which differentiate into early granulosa cells (Byskov, 1986; Pepling, 2006). This event constitutes the critical first step in folliculogenesis and affects fertility (Skinner, 2005; van den Hurk and Zhao, 2005). However, the mechanism controlling this process remains obscure. Evidence indicates that certain hormones and growth factors may facilitate primordial follicle formation. FSH (Roy and Albee, 2000) (FSH), growth differentiation factor-9 (GDF9) (Dong et al., 1996; Wang and Roy, 2006), bone morphogenetic protein (BMP15) (Hashimoto et al., 2005) have been found to affect follicle formation, including primordial follicles. Estrogen plays a critical role in primordial follicle formation (Wang and Roy, 2007) though the mechanism(s) of its action remains undefined. A physiological concentration of estradiol-17b (E2) facilitates whereas higher doses compromise the formation and development of primordial follicles (Wang and Roy, 2007). Moreover, blocking the action of endogenous E2 causes a decline in follicle formation (Wang and Roy, 2007). Using cDNA array of fetal human ovaries, Fowler et al. (2009) have documented changes in gene expression during early folliculogenesis. However, changes in the gene expression during the precise period of primordial follicle formation remain undetermined. Further, whether all transcripts undergo translation during primordial folliculogenesis remains unknown, and there are inconsistencies in upregulated or downregulated transcriptome in rat versus human ovaries

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during the formation of primordial follicles (Fowler et al., 2009; Kezele et al., 2004). Because proteins carry biological functions, we focus on the expression of proteins during the critical period of primordial follicle formation to understand the mechanism regulates this process. The objective of the present study was to use proteomics approach to identify proteins, the expression of which would correlate with the formation of primordial follicles. We used hamsters because primordial follicles formed on 8th day of postnatal life, thus providing the opportunity to obtain ovaries completely devoid of primordial follicles and ovaries with the first cohort of primordial follicles (Lyall et al., 1989; Roy and Albee, 2000; Wang and Roy, 2007). We presumed that these two widely separated time points in ovary morphogenesis would allow us to identify novel proteins expressed in ovarian cells when the oocytes and pregranulosa cells assembled to form the primordial follicles.

2. Materials and methods

2.1. Chemicals and animals

The rabbit polyclonal antibody to Ebp1 was purchased from Lifespan Biosciences (Seattle, WA), isoelectrophoresis gel strips and chemical were from GE Healthcare (Piscataway, NJ), PCR chemicals were from Roche Molecular Biochemicals (Indianapolis, IN), Pharmacia Biotech Boehringer (Piscataway, NJ), and Invitrogen. Quantitative PCR primers and probes were synthesized in the Eppley DNA synthesis Core Facility (University of Nebraska Medical Center, Omaha, NE). Second antibodies for Western blotting chemiluminescence were from Jackson Immunoresearch, Inc. (West Grove, PA); ECL advance Western blotting detection kit was from GE Healthcare (Buckinghamshire, UK); Optitran nitrocellulose transfer membrane was from Schleicher & Schuell Bioscience (Dassel, Germany); the RNeasy mini kit was from Qiagen Inc. (Valencia, CA). All other molecular-grade chemicals were purchased from Sigma Chemical Company, Fisher Scientific Corporation (Pittsburgh, PA), or United States Biochemical (Cleveland, OH).

Golden hamsters (90–100 g) were purchased from Charles River Laboratories (Charles River, MA) and maintained in a climate controlled room with 14 h light and 10 h dark with free access to food and water. The use of hamsters for this study was according to the Institutional Animal Care and Use Committee and the US Department of Agriculture guidelines, and was approved by the Institutional Animal Care and Use Committee. Females with at least three consecutive estrous cycles were mated with males in the evening of proestrus, and the presence of sperm in the vagina the next morning was considered the first day of pregnancy. Hamster gestation lasts for 16 days, and pups are born on the 16th day of gestation, which was considered the first day of postnatal life (P1).

2.2. Preparation of ovarian samples for two-dimensional (2D) gel electrophoresis

Ovaries were collected from 15-day-old (E15) fetuses when ovaries contained undifferentiated somatic cells and oocytes in the oocyte clusters, and from 8-day (P8) old hamsters when ovaries contained the first cohort of primordial follicles, but no other stage of follicles (Roy and Albee, 2000). Ovaries were either homogenized by Omni 2000 homogenizer or sonified in 50 mM Hepes, pH 7.4, containing 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 1% Triton X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate, 0.1 ml/ml of a protease inhibitor cocktail and 200 mM Na-orthovanadate on ice. The homogenate was centrifuged and the supernatant was used for protein estimation by BCA method (Pierce, Rockford, IL).

2.3. Two-dimensional gel electrophoresis

Protein samples were processed and cleaned using 2D electrophoresis kit (GE Healthcare) according to the manufacturer instructions. Proteins were subjected to isoelectric focusing (IEF) using immobiline dry strip gels with a non-linear immobilized pH gradient (NL-IPG) of pH 3–10 or pH 4–7 (GE Healthcare). Gels were loaded with 60 μg protein for analytical gels or 900 μg protein for preparative gels by rehydrating the gels for 20 h in IPG buffer containing 100 μl Destreak, 10 mM DTT, and sample protein. Focusing was done in the IPGphor 3 (GE Healthcare) isoelectric focusing apparatus as instructed by the manufacturer. Immediately following the IEF, focused strips were equilibrated for 15 min in reducing SDS-equilibration buffer (6 M urea, 75 mM Tris–HCl, pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue, and 100 μg dithiotheritol (DTT) per 10 ml), and 15 min in alkylating SDS-equilibration buffer (6 M urea, 75 mM Tris–HCl, pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue, and 250 μg iodoacetamide (IAA) per 10 ml).

Second dimension gel electrophoresis was performed in a 20 cm 12% polyacrylamide gel. Equilibrated IEF strips were placed on top of the resolving gels and proteins were fractionated at 25 mA per gel. Analytical gels were stained with Proteosilver stain (Sigma Chemical Company, St. Louis, MO), whereas preparative gels were stained with SyproRuby (Sigma) as per manufacturer's instructions.

2.4. In gel digest

Proteins of interest were excised from the gel using sterile glass capillaries, excess liquid was removed, and gel pieces were destained using 100 μl of 50% acetonitrile (ACN) and 25 mM ammonium bicarbonate (ABC). Gel pieces were shaken at room temperature for 10 min, and the liquid removed. The destaining steps were repeated again, then 100 μl ACN was added. Samples were incubated for 3–5 min at room temperature, all excess liquid was removed, and samples were dried in a biosafety cabinet for 10 min. One hundred microliters fresh reducing agent (1.5 mg DTT per ml 25 mM ABC) was added and samples were incubated for 30 min at 37 °C. The supernatant was removed, and 100 μl fresh alkylating agent (10 mg IAA per ml 25 mM ABC) was added and samples were incubated for 30 min at room temperature in the dark. After removing the liquid, samples were washed with 100 μl of 50 mM ABC and then with 100 μl of 50% ACN in 25 mM ABC. The samples were washed with 100 μl of 100% ACN and dried for 5–10 min in a biosafety cabinet. Proteins were digested with 15 μl of trypsin (25 ng/μl) at room temperature for 15 min. Excess liquid was removed, 25 mM ABC was added, and samples were kept overnight at 37 °C in an air incubator. Peptides were extracted with 1 μl of 10% formic acid with shaking at room temperature for 10 min. The liquid was transferred to a fresh 1.5 ml tube and placed on ice (extract #1). The gel pieces were reextracted with 60% ACN/0.1% formic acid with shaking for 10 min at room temperature, the supernatant was added to extract #1 and samples were concentrated using a speedvac to 10 μl. Samples were brought up to 10 μl using 0.1% formic acid if necessary.

2.5. LC/MS/MS analysis

Extracted peptides were analyzed via LC/MS/MS on an Agilent ion trap (model 6340) Mass spectrometer with an HPLC-chip interface at the laboratory of Dr. Nichole Reisdorph at the NJH Mass Spectrometry Facility (University of Colorado Medical Center, Denver, CO). All HPLC components were Agilent 1100. Buffer A of the nanopump was comprised of 0.1% formic acid in HPLC grade water, and buffer B was 90% acetonitrile, 10% HPLC water, and 0.1% formic