# Expression and localization of the novel and highly conserved gametocyte-specific factor 1 during oogenesis and spermatogenesis

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**Objective:** To determine the onset of gametocyte-specific factor 1 (*Gtsf1*) expression in embryogenesis and its relation to *Nobox*; and to determine its localization during gonadal development and gametocyte maturation.
**Design:** Developmental animal study.
**Setting:** University reproductive biology laboratory.
**Animal(s):** Mice ranging in age from embryonic day 12.5 to 8 weeks.
**Intervention(s):** Polymerase chain reaction and quantitative polymerase chain reaction were performed to determine the onset of and relative messenger RNA expression. Western blot was performed to confirm protein expression and antibody specificity. In situ hybridization and immunohistochemistry were used determine localization of expression.
**Main Outcome Measure(s):** *Gtsf1* messenger RNA expression levels during embryogenesis through adulthood in wild-type mice and in newborn *Nobox* knockout mice; GTSF1 expression and localization in postnatal mice.
**Result(s):** *Gtsf1* functions downstream of *Nobox* and is highly expressed in embryonic male and female gonads, localizing to germ cells throughout development. GTSF1 expression is confined to the cytoplasm in all stages of postnatal oocyte maturation and to prespermatogonia during early postnatal testicular development.
**Conclusion(s):** The expression pattern of *Gtsf1* and its high conservation suggests that it may play an important role in germ cell development. Further characterization of *Gtsf1* may elucidate mechanisms involved in premature ovarian failure. (Fertil Steril® 2009;91:2020–4. ©2009 by American Society for Reproductive Medicine.)

**Key Words:** *Nobox*, premature ovarian failure, folliculogenesis, germ cell, gametes

In mammals, oocyte maturation is a complex process involving the interaction of many genes during embryogenesis (1). In mice, 45 primordial germ cells (PGCs) appear at embryonic day 7 after conception (E7.5) and give rise to the germ cell lineage (2, 3). From E9.5 to E11.5, PGCs migrate from the hindgut epithelium to the urogenital ridge to form germ cell clusters called *cysts* (4–8). Primordial germ cells within the cysts then undergo mitosis with cytokinesis to form oocyte clusters (1). At E13.5, the oocytes initiate meiosis and become arrested in the diplotene phase of meiosis I until ovulation (9). Shortly after birth these oocyte clusters separate and become associated with pregranulosa cells to form primordial follicles (1), with most germ cell cysts dissipating by postnatal day 7 (5). At postnatal day 3 some primordial follicles begin maturing into primary follicles (10). During this transition, numerous oocyte-specific genes critical to folliculogenesis, such as *Nobox*, *Figla*, *Gdf-9*, and *BMP-15* (10) are expressed.

*Nobox*, first isolated using in silico screening techniques to identify expressed sequence tags specific to the gonads (11), encodes a homeobox transcription factor critical for the transition of primordial to primary follicles (12, 13). Because *Nobox* deficiency in mice leads to a rapid postnatal loss of oocytes, it serves as a model for premature ovarian failure. Persistent expression of *Nobox* in the adult ovary throughout folliculogenesis suggests that it may also be responsible for maintaining the expression of genes in growing oocytes (10). Gametocyte-specific factor 1 (*Gtsf1*) (MGI:1921424) was identified on an Affymetrix 430 2.0 microarray chip as having a 13-fold reduction in messenger RNA (mRNA) expression in *Nobox*-deficient as compared with wild-type mice (14). It is highly conserved among 27 species, including humans, with evolutionary conservation extending to Euteleostomi (bony vertebrates). We aim to further characterize *Gtsf1* to determine its involvement in folliculogenesis and spermatogenesis; here we describe its expression during embryogenesis and early postnatal development in the ovary and testis.

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## MATERIALS AND METHODS

### Experimental Animals

Animals used in this research were maintained in accordance with the Institutional Animal Care and Use Committee at Baylor College of Medicine. Postnatal days were determined by counting the number of days after birth (postnatal day 0.5). All tissues used in reverse transcriptase polymerase chain reaction (RT-PCR) or immunochemistry were derived from C57BL/6/129S5 SvEvBrd hybrid background.

### Determination of Relative mRNA Expression Levels of *Gtsf1* Using Real-Time Quantitative PCR

Complementary DNA was derived from ovarian mRNA isolated from newborn ovaries using the RNeasy Mini Kit (Qiagen, Germantown, MD). Real-time quantitative PCR was performed using SYBR Green with oligonucleotide primers corresponding to *Gtsf1* (forward primer: 5′ tgc cct cct tgt gat gaa gac 3′; reverse primer: 5′ gaa ctc gca tgc ctg aag c 3′) and *Gapdh* (forward primer: 5′ caa tgt gtc cgt cgt gga tct 3′; reverse primer: 5′ gcc tgc ttc acc acc ttc tt 3′) as a control. Real-time quantitative PCR was performed three times, as previously described (10), to verify results. Student's *t* test was used to calculate level of significance of the change in mRNA levels.

### Determination of Tissue Specificity and Onset of *Gtsf1* mRNA Expression with RT-PCR

Complementary DNA was derived from mRNA isolated from multiple adult tissues as well as the ovaries and testis of both embryonic and adult mice using the RNeasy Mini Kit (Qiagen). Equal amounts of mRNA were reverse transcribed using Jumpstart Taq polymerase (Sigma, St. Louis, MO). Reverse transcriptase PCR was performed using *Gtsf1* primers (5′-GCC AAG CTT CTG TGC ATT TCC ATT GTT TTT CCA T-3′ and 5′-CCG GAT CCG ATG GAA GAC ACT TAC ATC GAC TCC CT-3′) to amplify a 501–base pair product. Reverse transcriptase PCR was performed for 29 cycles at 94°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing), and 72°C for 30 seconds (extension). Amplification was performed using *actin*-specific primers as a positive control for equivalent mRNA levels.

### Confirmation of GTSF1 Expression in Gonadal Tissue

Tissue from the ovaries, testis, and liver of postnatal day 1–3 mice were homogenized in Buffer C (20 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 25% glycerol, 0.42 $\mu$ NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L ethylenediaminetetraacetic acid). One hundred micrograms of protein extract from each specific tissue was loaded onto a 4%–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) and subjected to electrophoresis, and then the protein was transferred onto a nitrocellulose membrane (Whatman, Dassel, Germany). Nitrocellulose membranes were blocked in a 5% dry nonfat milk, 0.1% Tween in Tris-buffered saline solution (50 mmol/L Tris base, pH 7.5, 150 mmol/L NaCl, and 0.1% [wt/vol] Tween 20) for 1 hour at room temperature. The membranes were incubated at 4°C overnight with affinity-purified anti-GTSF1 antibodies at 1:100 dilution. The membranes were then incubated with a rabbit anti-guinea pig peroxidase-conjugated secondary antibody at 1:10,000 for 1 hour at room temperature. The enzyme products were visualized using enhanced chemiluminescence (ECL Western Blotting Analysis System; GE Healthcare, Buckinghamshire, United Kingdom).

### In Situ Hybridization of *Gtsf1*

In situ hybridization was performed as previously described (15). In summary, ovaries were initially fixed in 4% paraformaldehyde and then embedded in paraffin. Five-micrometer sections were hybridized to 35S-labeled sense and antisense riboprobes as previously described (11). The signal was detected by using NTB-2 emulsion autoradiography (Eastman Kodak, Rochester, NY), and tissue was counterstained with hematoxylin.

### Localization of GTSF1 Expression with Immunohistochemistry

Antibodies against GTSF1 were created by expressing the full-length protein in the pET-23b vector and immunizing guinea pigs at Cocalico Biologicals (Lancaster, PA). The anti-GTSF1 antibodies were immunoaffinity purified over Affi-Gel 10 (Bio-Rad Laboratories, Hercules, CA) and used in immunohistochemistry as previously described (16).

## RESULTS

*Gtsf1* localizes to mouse chromosome 15, and its human orthologue localizes to chromosome 12q13.2. Murine GTSF1 (XP_900203) protein shares 100% and 92% homology with rat and human orthologues, respectively. According to publicly available databases (www.ensembl.org), *Gtsf1* is a highly conserved gene, showing 1-to-1 conservation with 27 species, including zebrafish and frogs (www.ensembl.org, http://www.ncbi.nlm.nih.gov/sites/entrez). It belongs to a functionally uncharacterized protein family (UPF0224) with an unknown functional domain (http://www.bmm.icnet.uk/servers/3djigsaw/). Use of publicly available prediction models for subcellular localization demonstrated different locations, including nuclear (http://psort.nibb.ac.jp/form2.html), cytoplasmic (http://www.cs.ualberta.ca/~bioinfo/PA/Sub/), and extracellular (http://www.bioinfo.tsinghua.edu.cn/SubLoc/eu_predict.htm) regions.

To further evaluate earlier Affymetrix findings, real-time quantitative PCR was performed on three independent samples to determine relative expression levels of *Gtsf1* mRNA in wild-type and *Nobox*-deficient newborn ovaries (Fig. 1). Relative expression levels of *Gtsf1* in wild-type ovaries and *Nobox*-deficient ovaries were 0.38 and 0.017, 0.71 and 0.055, 0.84 and 0.022, respectively, for the first, second, and third times that quantitative PCR was performed. The average relative expression of *Gtsf1* for all three experiments