

Characterization of a Novel Transcript of 14-3-3 Theta in Sertoli Cells

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ABSTRACT: The isoforms of the highly conserved and ubiquitously expressed 14-3-3 family of proteins function primarily as adapters that modulate interactions between components of various cellular signaling and cell cycle regulatory pathways. Low levels of 14-3-3 isoforms appear to be expressed in most tissues, but specific isoforms or combinations have been shown to be overexpressed in a cell-specific manner. In the present study we show that the theta isoform of 14-3-3 is expressed in Sertoli cells. Although previous reports have shown the presence of a 14-3-3 theta isoform in mouse testicular germ cells, this report demonstrates the presence of the 14-3-3 theta isoform in rat Sertoli cells. The 14-3-3 theta isoform isolated from rat Sertoli cells appears to have a truncated 3' UTR, which makes the transcript shorter by 244 bp, compared with its brain counterpart. Northern blot analysis suggests that the 14-3-3 theta isoform may also be present in other testicular cell types and

tissues. The truncation of the 3' UTR suggests a potential role in regulating cell-specific expression of 14-3-3 theta. The expression of 14-3-3 theta in Sertoli cells was confirmed by Northern blot, polymerase chain reaction, Western blot, and immunocytochemical analysis. The levels of 14-3-3 theta mRNA and protein in Sertoli cells remained unchanged in response to the gonadotropin, FSH. Consistent with the absence of the effect of FSH on the expression of 14-3-3 theta, an antisense oligonucleotide to 14-3-3 theta had no effect on FSH-induced activation of the transferrin promoter in Sertoli cells. The widespread expression of 14-3-3 theta in testis and the lack of effect of FSH on levels of its expression suggest that 14-3-3 theta influences Sertoli cell function in an FSH-independent manner.

Key words: Testis, seminiferous tubule, cell differentiation.

J Androl 2000;21:730-738

The 14-3-3 family of proteins were first identified in brain as regulators of hydroxylase activity, a rate-limiting step in the biosynthesis of neurotransmitters (Moore and Perez, 1967). Various isoforms named with Greek letters α through η (Aitken et al, 1992) and, more recently the θ isoform (Watanabe et al, 1994), have been identified in many different eukaryotic tissues. All the isoforms have multiple modular domains, including pseudosubstrate-like domain for protein kinase C (PKC) phosphorylation, a dimerization domain, a domain with annexin similarity, and multiple sites for *in vivo* phosphorylation. These domains are highly conserved across all the isoforms (reviewed in Aitken et al, 1995). Some isoforms have tissue-specific distribution, particularly γ in brain (Isobe et al, 1991), τ in T cells (Reuther et al, 1994), and σ in epithelial cells (Prasad et al, 1992). Observations suggest that a combination of various isoforms of 14-3-3 are expressed in all tissues. The strong conservation of 14-3-3 proteins across species suggests that these proteins

may play an important role in eukaryotic cells (Wang and Shakes, 1996).

14-3-3 proteins exist as both homodimers and heterodimers and most if not all the diverse functions that regulate many cellular processes are based on the ability of the dimer to physically interact with a number of cellular proteins (reviewed in Reuther and Pendergast, 1996). The formation of such multimeric protein complexes may mask or unmask potential phosphorylation sites or kinase domains. Some of the specific functions of the 14-3-3 family of proteins include regulation of kinases such as PKC (Toker et al, 1992), Raf serine kinase (Freed et al, 1994; Thorson et al, 1998), and the break point cluster region (Bcr) (Reuther et al, 1994). Other cellular processes influenced are Cdc25 phosphatase (Conklin et al, 1995), cell cycle control (Ford et al, 1994), apoptosis (Hsu et al, 1997), catecholamine biosynthesis (Furukawa, et al, 1993), exocytosis (Roth et al, 1993), adenosine diphosphate (ADP) ribosylation (Chen et al, 1994; Reuther et al, 1994), and chaperone function (Rosenfeld et al, 1991).

The theta isoform of 14-3-3 was recently shown to be specifically expressed in murine testis and brain (Rosenfeld et al, 1991; Watanabe et al, 1994; Pergo and Berruti, 1997). In the testis, 14-3-3 theta protein appears to be expressed in both meiotic and postmeiotic germ cells

Supported by grants from the National Institutes of Health.

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Received for publication January 10, 2000; accepted for publication March 7, 2000.

(Pergo and Berruti, 1997). Although not investigated, the authors predicted that 14-3-3 *theta* isoform may also be present in Sertoli cells. In adults, Sertoli cells are a post-mitotic and terminally differentiated somatic cell population that forms the seminiferous epithelium to provide both structural and functional support to developing germ cells (Griswold, 1998). Sertoli cell functions are generally regulated by the gonadotropin, follicle-stimulating hormone (FSH), which acts through the adenylate cyclase-cyclic adenosine monophosphate (cAMP)-protein kinase A pathway (Kangasniemi et al, 1990). Growth factors such as transforming growth factor beta (Le Magueresse-Battistoni et al, 1995), insulin-like growth factor (IGF-1; Schteingart et al, 1995), fibroblast growth factor (FGF; Smith et al, 1989), and epidermal growth factor (Oneda and Suarez-Quian, 1994) also regulate Sertoli cell functions. The action of these growth factors are mediated by PKC, Raf-1, MEK kinases, or a combination of these. The presence of diverse signaling pathways and the observation that Sertoli cells undergo minimal apoptosis suggests the 14-3-3 family of proteins may be present and regulate various Sertoli cell functions.

In the present study we report the identification, isolation, sequence, and function of a novel transcript of rat Sertoli cell 14-3-3 *theta*. This isoform was fortuitously isolated while we were screening a rat Sertoli cell complementary DNA library for the basic helix-loop-helix (bHLH) protein (rat E-box binding) REB alpha. Compared with the published nucleotide sequence of the rat brain 14-3-3 *theta* isoform (Rosenfeld et al, 1991), the rat Sertoli cell 14-3-3 *theta* is truncated at the 3' end. To our knowledge, the 3' truncation reported in this manuscript is the first observation suggesting that 14-3-3 *theta* may have alternate polyadenylation site(s).

Materials and Methods

Isolation of Sertoli Cells

Sertoli cells were isolated from testis of 20-day-old rats as described earlier (Whaley et al, 1995). The isolated Sertoli cells were then plated under serum-free conditions in 150 × 20 mm tissue-culture dishes (Nunc, Naperville, Ill) at a concentration of 10⁸ cells/plate. Cells were maintained in a 5% CO₂ atmosphere in Ham's F-12 medium (Gibco, Rockville, Md) with 0.01% bovine serum albumin (BSA) at 32°C. Sertoli cells were treated with either FSH (100 ng/mL; o-FSH-16, National Pituitary Agency, Bethesda, Md), or vehicle alone (Ham's F-12, control). The cells were cultured under serum-free conditions for a maximum of 5 days with a media change and treatment after 48 hours of culture. Cell number and viability did not change during the culture in the absence or presence of treatment.

RNA Preparation

Freshly isolated or cultured Sertoli cells were lysed directly using TRI Reagent (T9424, Sigma Chemical Company, St Louis,

Mo). The cell lysate was then passed several times through a pasture pipette to form homogenous lysate. The whole tissue (skeletal muscle, brain, and detunicated testis) in TRI Reagent (5% w/v; Sigma) was homogenized in a tissue homogenizer (Tissue Tearor, Biospec, Bartlesville, Okla). To avoid any possible contamination of extracellular material and high-molecular-weight DNA in the final RNA samples, the homogenate was centrifuged at 12000 × g for 10 minutes at 4°C. Total RNA was then isolated from the cell lysate and whole-tissue homogenate following the manufacturer's protocol for RNA isolation using TRI Reagent. The final RNA pellet was dissolved in distilled water at a concentration of 1 mg/mL.

Polymerase Chain Reaction

Total RNA (2 µg) was reverse transcribed in a final volume of 20 µL containing 20 U RNasin (Promega, Madison, Wis); 200 µM each of dATP, dCTP, dTTP, and dGTP; 1 µg oligo dT (Pharmacia, Peapack, NJ); 10 µM DTT; and 200 U of MMLV reverse transcriptase (BRL, Grand Island, NY) in the MMLV first strand synthesis buffer supplied by the manufacturer (BRL). The RNA and oligo dT primer in the buffer were first denatured for 5 minutes at 65°C, then cooled on ice before addition of nucleotides and enzyme. The reverse transcriptase (RT) reaction was carried out at 37°C for 1 hour. Polymerase chain reaction (PCR) was performed using the GeneAmp kit (PE Biosystems, Foster City, Calif) with 30 cycles as follows: 94°C, 1 minute (denaturation); 58°C, 2 minutes (primer annealing); and 72°C, 1 minute (primer extension). Each PCR reaction contained 250 pg reverse transcribed DNA; 1 µM of each 5' and 3' oligonucleotide primers; 2.5 U Taq polymerase (AmpliTaq, PE Biosystems); and 200 µM each of dATP, dCTP, dGTP, and dTTP. The 5' primer was derived from the amino acid sequence RR(K/M/I)(AN)(AN)(TN)(M/L/A)RER(RL) of the basic domain, whereas the 3' primer was derived from the degenerate amino acid sequence (L/V)(L/E)(IT)L(HQ)(QN)(AT)(VL)(QN)(YC)(VA) of the Helix II domain. The amino acids within the parentheses and between slashes show the degeneracy and were used to design degenerate primers. The sequences used for the primers were based on the comparison of at least 20 human, rat, and mouse bHLH proteins.

Cloning and Sequencing

The PCR fragments were run on 1.5% agarose gel, stained with ethidium bromide, and visualized. The bands were then dissected out and the DNA isolated from the gel using the Glass MAX DNA isolation system (BRL). The purified DNA fragments were subcloned into pBlueScript (Stratagene, La Jolla, Calif) plasmid. The cloned DNA fragments were sequenced using standard M13 forward and reverse primers in an automated fluorescence-based Sequencer (PE Biosystems). The sequences were then analyzed against the known sequences in GenBank using a FASTA search.

Sertoli Cell cDNA Library

The rat Sertoli cell cDNA library was constructed in HybriZapII by Stratagene from poly (A) + RNA isolated from purified Sertoli cells of 20-day-old rats. The HybriZap library is primarily designed for use in the yeast 2-hybrid screening, but also allows for nucleic acid screening. The standard procedures for nucleic

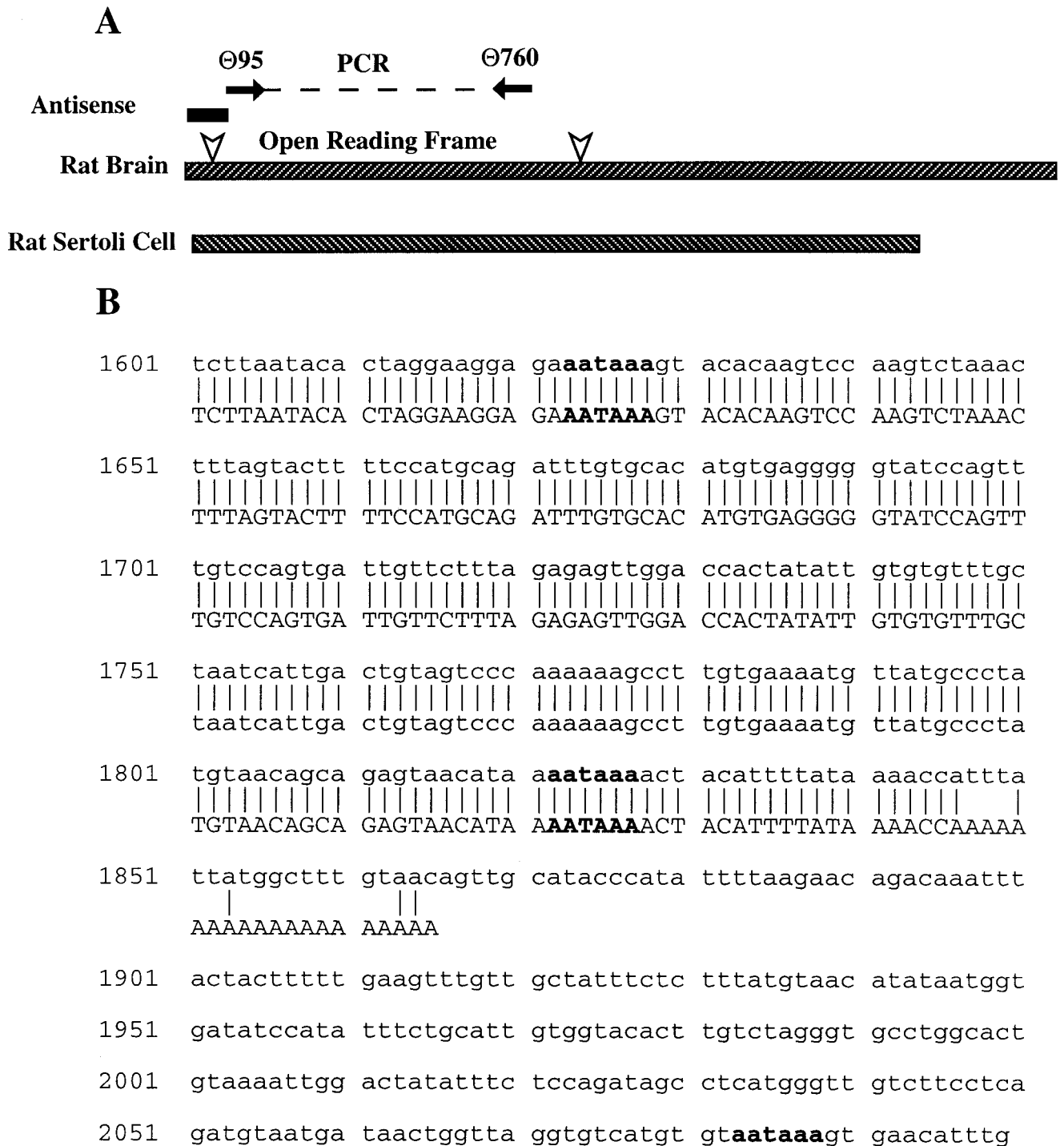


Figure 1. (A) Schematic of the rat brain and Sertoli cell 14-3-3 theta isoforms. The arrows indicate the location of the forward and reverse primers used for RT-PCR. The inverted arrowheads mark the beginning and end of the protein coding region. The solid bar represents the region targeted by the antisense oligonucleotide (antisense). (B) Comparison of the 3' end of the untranslated region of the rat brain (lower case letters) and Sertoli cell (upper case letters) 14-3-3 theta nucleotide sequence. The bold letters indicate the potential polyadenylation sites.

acid screening were followed as mentioned elsewhere (Sambrook et al, 1989). The REB probe was digested from the p-BlueScript plasmid using *Eco*R1, dephosphorylated, and end-labeled with γ P³²-ATP and T4 polynucleotide kinase. Individual

plaques (1.5×10^6) were screened with the 170-bp REB α PCR fragment. The positive clones were excised from the HybriZap library to obtain pAD plasmid-containing clones. The pAD plasmids were analyzed by restriction mapping and sequencing. The

sequences were subjected to a FASTA search against known sequences in GenBank.

The primer pair sequences used to investigate the presence of 14-3-3 *theta* in various tissues by RT-PCR were determined empirically and synthesized from commercial sources. Primer pairs were designed to amplify a 685-bp region. The possible contamination of RNA with DNA was distinguished by performing the RT reaction without MMLV reverse transcriptase. The absence of any product in the amplification reaction using such a reverse transcribed preparation indicated the absence of any contaminating DNA in our RNA samples. The sequence and position of the primers were as follows:

14-3-3 *theta* 5'- (95–120) ACC GAG CTG ATC CAG AAG GCC AAG CT

14-3-3 *theta* 3'- primer (780–766) TTC TCC TGC ACT GTC TGA TGT CCT

Each reverse transcription reaction was performed using 3 different samples. The PCR-based amplification reactions were carried out in duplicate on each reverse transcribed RNA sample. Simultaneous PCR reactions were also carried out using primers designed to rat cyclophilin to monitor the efficiency of the PCR. Cyclophilin was faithfully amplified in all the PCR reactions, indicating consistency in the quality of RT and PCR reactions. The data presented are therefore representative of 3 different RT-PCR reactions carried out in duplicate.

Northern Analysis

Total RNA from Sertoli cells, germ cells, and peritubular cells were isolated as described earlier using TRI-Reagent (Sigma). Approximately 10 µg of total RNA was fractionated on a 1% formaldehyde-agarose gel. Following fractionation, the RNA was transferred onto Nylon membrane (Hybond N+, Amersham, Little Chalfont, United Kingdom) in 10× SSC buffer and UV-cross linked as described previously (Sambrook et al, 1989). The membranes were then prehybridized in Quick Hybridization buffer (Stratagene) for 30 minutes at 60°C. The hybridization was carried out at 60°C for 1 hour with ³²P-labeled 14-3-3 *theta* probe. The membrane was subsequently stripped and rehybridized with rat cyclophilin. All the probes were labeled using a prime-it II kit from Stratagene.

Transfections

The CAT reporter plasmid (pUC8-CAT) containing –581 bp (–581 bp mTf-CAT) of the proximal mouse transferrin promoter was generously provided by Dr G. Stanley McKnight (University of Washington, Seattle, WA). The mouse transferrin promoter used in the present study included the transcriptional initiation site of the transferrin gene, which is 54 bp upstream of the start site of translation (Idzerda et al, 1986). Sertoli cells cultured in 24-well cell culture plates for 48 hours were transfected with the reporter gene construct by the calcium phosphate method coupled with hyperosmotic shock (10% glycerol) as described previously (Whaley et al, 1995). In each experiment transfection efficiency was monitored by transfecting the Sertoli cells by the plasmid containing β-galactosidase gene driven by

a CMV promoter. Subsequent staining and counting the cells expressing β-galactosidase (blue color) resulted in 25% transfection efficiency. The antisense to 14-3-3 *theta* (Figure 1, 84–98 bp CCA TGG AGA AGA CCG) was synthesized using phosphorothioate modification. The antisense 14-3-3 *theta* (4 µM) was added to the Sertoli cells 4 hours following transfection. The cells were subsequently treated with FSH. The antisense was added to the culture wells every 12 hours for a total period of 72 hours, following which the cells were harvested for the CAT assay as described previously (Whaley et al, 1995). The average conversion of CAT substrate for treated cells ranged between 20% and 30%. This assay was found to be linear with the protein concentration used.

Sequence Motif Analysis

GCG DNA analysis software was used to generate the restriction map and translation to the corresponding protein sequence. FASTA and tBlastX (NCBI BLAST) against GenBank, Entrez, and SRS links were used to generate a homology table of 14-3-3 *theta*.

Immunocytochemistry Procedure

Sections (6 mm) of paraffin-embedded testes from 20-day-old rats fixed in buffered formaldehyde (4% in 0.1 M PBS) were incubated either with rabbit anti-rat 14-3-3 *theta* (Santa Cruz Laboratories, Santa Cruz, Calif; 1:200) or normal rabbit serum (negative control) for 2 hours at room temperature. Before application of the primary antibody, a microwave antigen retrieval technique was used as described previously (Cattoretti et al, 1992). The sections were then rinsed in Tris-buffered saline and incubated with goat anti-rabbit biotinylated antibody (Vectastain Elite kit, Vector Laboratories, Burlingame, Calif; dilution 1:100). After rinsing, the sections were incubated in ABC complex (Vector Laboratories, dilution 1:100) and the reaction was visualized with DAB (Sigma).

Western Blot Analysis

A Western blot analysis was performed to demonstrate that 14-3-3 *theta* protein was expressed in Sertoli cells. Sertoli cells were cultured in 6-well plates and were left either untreated (control) or treated with FSH, dibutyl cAMP, or 10% fetal bovine serum. Following the 48-hour treatment period, the cells were rinsed twice in PBS and solubilized in 200 µL of cell lysis buffer (PBS, 0.2% Triton X-100, 5 mM DTT, and protease inhibitor cocktail tablet, complete mini; Boehringer, Indianapolis, Ind) for 45 minutes on ice. The lysate was cleared by centrifugation and protein concentration measured by Bio-Rad protein assay kit (Bio-Rad, Hercules, Calif). Two micrograms of protein was electrophoresed and protein transferred to the nitrocellulose membrane. The membrane was saturated overnight in PBST (PBS with 0.1% Tween 20) containing 5% milk and subsequently incubated with the 14-3-3 *theta* antibody (1:3000, Santa Cruz) for 2 hours. The presence of the specific protein-antibody complex was detected using goat anti-rabbit immunoglobulin (Ig)G conjugated to alkaline phosphatase (1:3000) and chemiluminescent substrate (Immun-Star, Bio-Rad).

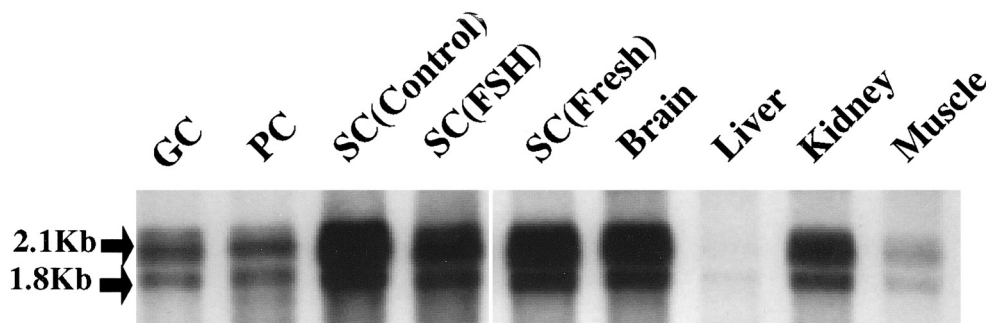


Figure 2. Northern blot analysis of 14-3-3 theta in brain, kidney, lung, and testicular cells. Freshly isolated (Fresh) or cultured and treated with either vehicle alone (Control) or follicle stimulating hormone (FSH). Two transcripts of approximately 2.1 and 1.8 kb were detected when 685-bp PCR product was used as the probe. The data are representative of 3 different Northern blots performed on separate RNA samples collected at different times. GC indicates germ cells; PC, peritubular cells; SC, Sertoli cells.

Results

In order to identify transcription factors of the basic helix-loop-helix family in Sertoli cells, an RT-PCR of the RNA obtained from cultured Sertoli cells was performed using degenerate oligonucleotides designed toward the highly conserved basic and helix II domain of bHLH proteins. The ~170 bp PCR fragment spanning the basic helix loop helix domain was cloned into the pBlueScript plasmid. Ten different clones were isolated, sequenced, and analyzed. All the clones matched 100% to the ubiquitously expressed bHLH transcription factors, REB or E47. Based on the observation that bHLH domain is highly conserved, a rat Sertoli cell cDNA library was screened with the 170-bp bHLH domain PCR product of REB. The screening of the library resulted in the identification of the full-length clone of the REB alpha cDNA, a spliced variant of the REB gene and an additional clone that was similar to the rat brain 14-3-3 theta sequence (Acc No D17614). 14-3-3 Theta has no recognizable basic helix loop helix domain (based on motif search) and showed no homology to the REB nucleotide sequence. Therefore, 14-3-3 theta was identified because of apparent nonspecific interactions. The nucleotide sequence of 14-3-3 theta revealed an insert of 1865 bp, which is 234 bp shorter than the reported rat brain 14-3-3 theta sequence. The 5' coding region and the corresponding protein sequence were similar to the rat brain 14-3-3 theta nucleotide and protein sequence (Figure 1A). The only difference is observed at the 3' end of the rat Sertoli cell 14-3-3 theta nucleotide sequence. Comparison of brain and Sertoli cell 3' regions of the 14-3-3 theta sequence suggested that the Sertoli cell 14-3-3 theta sequence is truncated at the 3' end (Figure 1B). This truncation is based on the observation that the new Sertoli cell 14-3-3 theta isoform has a poly A tail at the 3' end, which is about 250 base pairs upstream of the 3' end of the reported brain 14-3-3 theta sequence. Therefore, the 3' truncation accounts for the smaller transcript of the rat Sertoli cell 14-3-3 theta by

234 bp compared with its counterpart in the brain. The truncated form of the 14-3-3 theta transcript isolated from Sertoli cells is therefore the first example of a subisoform of the member of 14-3-3 family. This subisoform with an alternative polyadenylation site is henceforth referred to as 14-3-3 theta-1.

Northern blot analysis suggested that the 2.1-kb transcript of 14-3-3 theta was highly expressed in enriched populations of Sertoli cells (Figure 2). Two transcripts at 1.8 kb and 2.1 kb were observed when 685-bp 14-3-3 theta probe was used to detect message levels. The presence of 2 transcripts seen in the Northern blot shown in Figure 2 is consistent with data reported elsewhere (Pergo and Berruti, 1997). The 14-3-3 theta message was low in freshly isolated germ cells and peritubular cells, but was high in freshly isolated, cultured, or FSH-treated Sertoli cells. Northern analysis indicated that FSH, the known regulator of Sertoli cell differentiated functions, does not influence 14-3-3 theta expression levels. Northern blot data were normalized with the expression of a constitutively expressed gene cyclophilin (data not shown). Low levels of 14-3-3 theta expression were also observed in liver and muscle. Northern blot data support the previous observations that 14-3-3 theta is highly expressed in brain and the expression levels were comparable to those seen in Sertoli cells. High and comparable levels of 14-3-3 theta expression were also observed in kidney, whereas low levels were present in muscle. The 14-3-3 theta probe used in the present study will likely not distinguish between the full-length and 3'-truncated isoforms of 14-3-3 theta. It is possible that the smaller transcript seen in the Northern blot represents the truncated isoform, which may be present in other tissues as well.

To complement the Northern blot data, the expression of 14-3-3 theta was also analyzed by RT-PCR using primers specific to 14-3-3 theta (Figure 3). As shown in Figure 3 and consistent with the Northern blot data shown in Figure 2, 14-3-3 theta expression was observed in all the

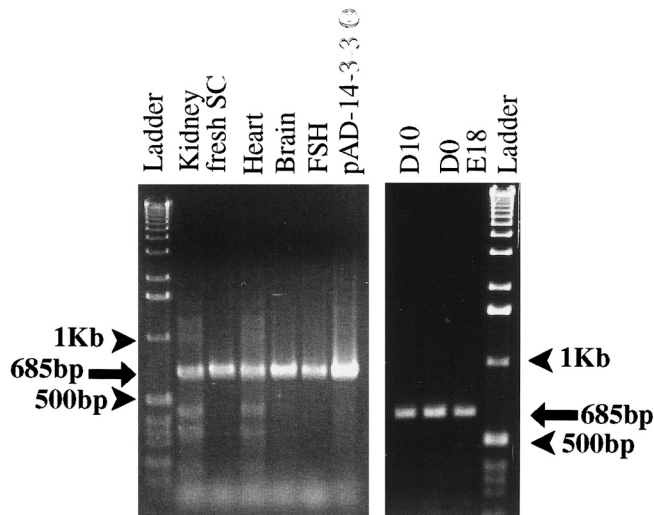


Figure 3. RT-PCR using primers specific for 14-3-3 theta on RNA obtained from kidney, freshly isolated Sertoli cells (Fresh SC), heart, brain, FSH-treated cultured Sertoli cells (FSH), pAD-GAL4 plasmid containing full length 14-3-3 theta sequence isolated from Sertoli cells (pAD 14-3-3-0) and whole testis from various stages of development (E18, embryonic Day 18; D0 and D10, postnatal Day 0 and Day 10, respectively). The data are representative of 3 separate PCRs carried out on at least 3 different RT-mRNA samples.

tissues analyzed. Two different approaches were used to confirm the identity of the 685-bp 14-3-3 PCR band. First, the PCR product was digested with the restriction enzyme, *StyI*, which resulted in 2 fragments of 427 and 257 bp. Second, the PCR product was subcloned and sequenced. Sequence analysis confirmed that the 685-bp PCR product is the expected 14-3-3 theta fragment. Although RT-PCR is not quantitative, it is concluded that low levels of 14-3-3 theta message are present in all the tissues examined. Attempts to show that the full-length 14-3-3 theta transcript is not expressed in Sertoli cells by amplifying the 3' region found in the wild type 14-3-3 theta sequence were not successful because of the failure to obtain good primer pairs in this region.

Data obtained from Northern blot and RT-PCR suggest that 14-3-3 theta is expressed in testis and in Sertoli cells. The expression of 14-3-3 theta protein in Sertoli cells was confirmed by Western blot analysis using an antibody specific to the 14-3-3 theta isoform (Figure 4). The absence of any effect of FSH, cAMP, or serum on levels of 14-3-3 theta protein expression correlates well with Northern blot data. Immunocytochemical studies were then performed to show the localization of protein. Immunolocalization of 14-3-3 theta by 14-3-3 antibody suggested that the protein is expressed in Sertoli cells (Figure 5). Consistent with previous observations (Pergo and Berruti, 1997), the expression was also observed in germ cells, peritubular cells, and interstitial cells.

An antisense approach was used to investigate the functional significance of 14-3-3 theta in Sertoli cells.

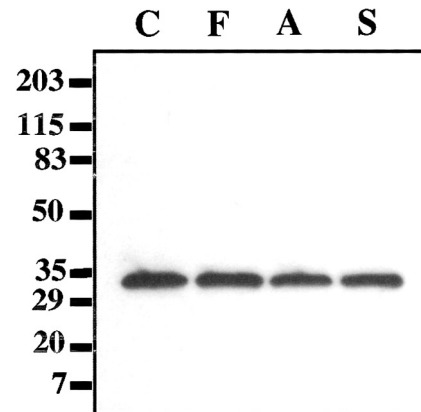


Figure 4. Western blot analysis for 14-3-3 theta protein expression in cultured, untreated Sertoli cells (control) or treated with FSH (F), dibutryl cAMP (A), and 10% fetal bovine serum (S). The experiment is representative of at least 3 Western blots performed on protein samples obtained at various time points.

Cultured rat Sertoli cells transfected with a CAT reporter plasmid driven by the proximal 581 bp of the mouse transferrin promoter (mTf-CAT) were treated with an antisense to 14-3-3 theta. The 14-bp antisense oligonucleotide spanned 84 to 98 bp (GenBank D17614, Figure 1). As controls, the 14-3-3 theta sense oligonucleotide, a non-specific, computer-generated, scrambled 14-3-3 theta antisense oligonucleotide and a *cfos* antisense oligonucleotide were used. Consistent with our previous observation, the *cfos* antisense oligonucleotide significantly inhibited (50%) the FSH-induced activation of the mTf-CAT reporter activity (data not shown). It is interesting that the 14-3-3 theta antisense oligonucleotide had no effect on basal or FSH-induced activation of the mTf-CAT reporter construct (Figure 6). Observations suggest that the signaling pathway in Sertoli cells elicited in response to FSH appears to be independent of 14-3-3 theta (Figure 6). In addition, 14-3-3 may regulate transferrin gene expression without an increase in the levels of mRNA and protein. This mechanism would not be affected by the presence or absence of the antisense oligonucleotides.

The Sertoli cell 14-3-3 theta isoform was identified while screening for the bHLH protein, REB. Although no sequence homology was observed between these 2 proteins, we tested the possibility that 14-3-3 theta and bHLH proteins may interact at the protein level. GST pull-down assays were performed to test this hypothesis. Recombinant GST-E12, GST-E47, GST-MyoD, and GST-Id, while still bound to glutathione-sepharose 4B matrix, were incubated with 35S-Met labeled *in vitro* translated 14-3-3 theta. Following incubation, the matrix was washed multiple times to remove any unbound *in vitro* translated 14-3-3. After the washes, the matrix was resuspended in reducing buffer and loaded onto SDS-PAGE minigels and visualized by autoradiography. The absence of any band

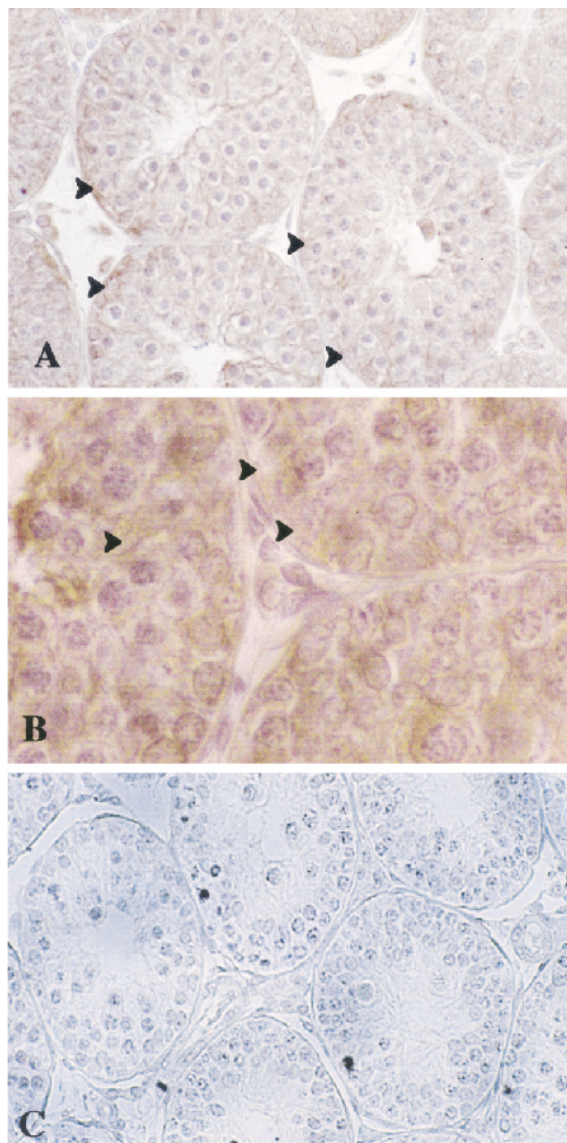


Figure 5. (A) Immunocytochemical analysis for the presence of localization of 14-3-3 theta in the testis; 14-3-3 theta is primarily localized in the cytoplasm of Sertoli cells (arrowheads). Low levels of 14-3-3 theta expression are also seen in germ cells and the interstitium. Magnification 400 \times . (B) Higher magnification (1000 \times) showing the presence of 14-3-3 theta in Sertoli cell cytoplasm. (C) Control using nonimmune serum. The data are representative of immunocytochemistry performed in triplicate on 3 different testis samples from 20-day-old rats.

shift or alteration of the positive controls (ie, GST-E47 and in vitro translated ^{35}S labeled MyoD) suggests that the 14-3-3 theta protein does not interact with any of the bHLH proteins tested in the in vitro binding assay (data not shown).

Discussion

A novel truncated subisoform of the 14-3-3 theta transcript from rat Sertoli cells has been cloned and se-

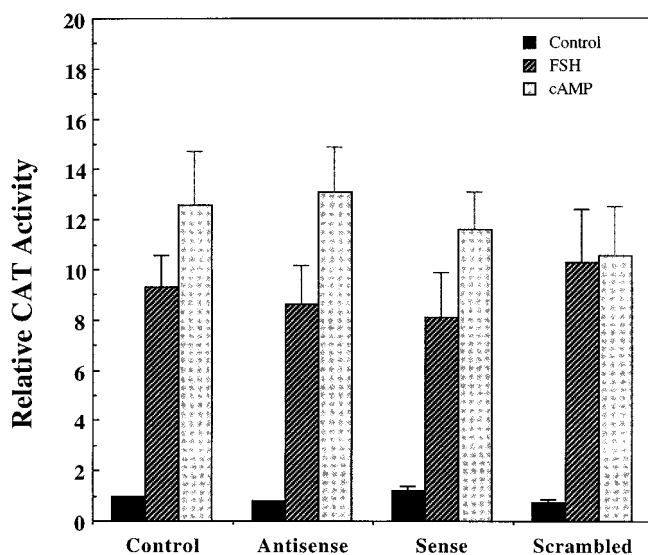


Figure 6. Regulation of the -581 bp transferrin promoter, chloramphenicol acetyltransferase (mTf-CAT) construct in Sertoli cells by an antisense (Antisense) or sense (Sense) oligonucleotide to 14-3-3 theta. As an additional control, a computer-generated scrambled oligonucleotide (Scrambled) from the antisense oligonucleotide sequence was also used. The cells were cultured in the absence (control) or presence of FSH and dibutyl-cAMP. The data are expressed as relative CAT expression with respect to the untreated control (control) set to 1 and presented as mean \pm SEM from 3 different experiments performed in duplicate. ANOVA analysis of the data indicates that the presence or absence of any of the oligonucleotide had no significant effect on either FSH- or cAMP-stimulated promoter activation.

quenced. The open reading frame of the Sertoli cell 14-3-3 theta isoform is identical to the published sequence of 14-3-3 theta isolated from rat brain. However, a significant difference was observed between the 3' UTR of Sertoli and brain 14-3-3 theta isoforms. The Sertoli cell 14-3-3 theta isoform had a truncated 3' UTR. This truncation resulted in the 3' UTR being 234 bp shorter than its counterpart in the brain. The truncation was apparent because a poly A tail was present at the 3' end of the Sertoli cell 14-3-3 theta isoform, whereas no such sequences were observed in the brain 14-3-3 theta isoform. The truncation at the 3' UTR suggests that the expression of 14-3-3 theta protein may be regulated by post-transcriptional events. Although both the transcripts are present in brain and testis at high levels, as observed by Northern blot analysis, the post-transcriptional events regulating protein expression in the different cell types may be different and under the control of distinct stimuli.

The expression of the 2 14-3-3 theta transcripts were observed in all the tissues examined, but was significantly higher in brain and Sertoli cells, followed by intermediate levels in germ cells and peritubular cells. The expression of 14-3-3 theta protein in rat germ cells observed by immunocytochemistry in the present study is consistent with the observation that mouse germ cells also express 14-3-3 theta (Pergo and Berruti, 1997). In addition to the pres-

ence of 14-3-3 theta in testicular cells and brain, Northern blot data also show high levels of expression in kidney and low levels of expression in muscle and liver. By using specific primers, 14-3-3 theta expression was observed in all the tissues examined by RT-PCR. This is not surprising given the increased sensitivity of RT-PCR over Northern blot analysis. Observations suggest that other tissues apart from brain and testis also express 14-3-3 theta. This supports the previous hypothesis that most tissues may express multiple isoforms of 14-3-3 protein, but higher levels of expression of specific isoforms of 14-3-3 may be restricted to individual cell type(s).

The endocrine hormone, FSH, is known to regulate the majority of Sertoli cell functions (Means et al, 1980). FSH primarily acts on Sertoli cells through the adenylate cyclase system by increasing the amount of cAMP that activates the protein kinase A pathway (Suire et al, 1995). Another signaling system that is active in Sertoli cells is the PKC pathway (Eikvar et al, 1993; Meroni et al, 1997). Growth factors are known to stimulate Sertoli cell function through the Ca⁺⁺ dependent/independent PKC pathway. It is interesting that 14-3-3 isoforms can modulate PKC activity (Toker et al, 1992), but their role in regulating protein kinase A activity is not known. An antisense oligonucleotide to 14-3-3 theta was found to not influence the activation of the basal- or FSH-stimulated mouse transferrin promoter. The activation of mouse transferrin promoter by FSH is primarily mediated by the cAMP-dependent protein kinase A pathway (Suire et al, 1995; Chaudhary and Skinner, 1999), which may not be the target for 14-3-3 regulation. Growth factors such as EGF, bFGF, IGF-1, and PDGF, which act primarily through PKC, Raf-1 kinase, and MEK kinases, may be more direct targets for regulation by 14-3-3 theta in Sertoli cells. Surprisingly, the tau isoform of 14-3-3 was recently shown to associate with the FSH receptor in human ovarian granulosa cells (Cohon and Dias, 1999), although the functional significance of such an association remains unknown. Experiments are currently in progress to determine if 14-3-3 theta is involved in the regulation of PKC, or Raf-1 kinase activity, or both by transiently overexpressing 14-3-3 theta protein in Sertoli cells.

The role of 14-3-3 in the regulation of kinase activity by physically associating with kinases is one of the many functions of the 14-3-3 family of proteins (Toker et al, 1992; Freed et al, 1994; Reuther et al, 1994). The other functions, which are mainly interactions with other proteins and appear to be regulated by phosphorylation, may include the role of 14-3-3 proteins as chaperones (Alam et al, 1994), molecular bridges (Jones et al, 1995), and effectors (Reuther and Pendergast, 1996). One such interaction that may be particularly important for Sertoli cell survival is the ability of 14-3-3 to prevent apoptosis by interacting with BAD, a member of Bcl-2 family (Hsu,

1997). The eta isoform of 14-3-3 has been shown to associate with homeodomain transcription factor TLX-2 and regulate cytoplasmic to nuclear localization (Tang et al, 1998), which suggests the possibility that 14-3-3 theta may associate with Pem, a homeodomain transcription factor expressed in Sertoli cells (Sutton et al, 1998). The functional importance of 14-3-3 theta-1 in Sertoli cells remains to be elucidated.

In conclusion, we have demonstrated that 14-3-3 theta is highly expressed in rat Sertoli cells. This extends the previous suggestion that 14-3-3 is expressed in spermatogenic cells (Pergo and Berruti, 1997). The presence of short 3' UTR in the 14-3-3 theta transcript is particularly interesting because no other isoform of the 14-3-3 family has been previously shown to have a truncated 3' UTR. An interesting speculation is that this isoform with a potential alternative polyadenylation site has altered activity (eg, constitutively active) then the other theta isoforms. Both Sertoli cells in the testis and neurons in the brain are postmitotic terminally differentiated cell populations. The high level of 14-3-3 theta in brain and testis may have a functional role in this process.

Acknowledgments

We thank Rachel Mosher and Gene Herrington for technical assistance and Susan Cobb for manuscript preparation assistance.

References

- Aitken A, Collinge DB, van Heusden BPH, Isobe T, Roseboom PH, Rosenfeld G, Soll J. 14-3-3 proteins: a highly conserved widespread family of eukaryotic proteins. *Trends Biochem Sci.* 1992;17:498–501.
- Aitken A, Jones D, Soneji Y, Howell S. 14-3-3 proteins: biological function and domain structure. *Biochem Soc Trans.* 1995;23:605–611.
- Alam R, Hachiya N, Sakaguchi M, Kawabata S, Iwanaga S, Kitajima M, Mihara K, Omura T. cDNA cloning and characterization of mitochondrial import stimulation factor (MSF) purified from rat liver cytosol. *J Biochem.* 1994;116:416–425.
- Cattoretti G, Becker MH, Key G, Duchrow M, Schluter C, Galle J, Gerdes J. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *J Pathol.* 1992;16:357–363.
- Chaudhary J, Skinner MK. E-box and cyclic adenosine monophosphate response elements are both required for follicle-stimulating hormone-induced transferrin promoter activation in Sertoli cells. *Endocrinology.* 1999;140:1262–1271.
- Chen Z, Fu H, Liu D, Chang PF, Narasimhan M, Ferl R, Hasegawa PM, Bressan RA. A NaCl-regulated plant gene encoding a brain protein homology that activates ADP-ribosyltransferase and inhibits protein kinase C. *Plant J.* 1994;6:729–740.
- Cohon B, Dias J. Identification of follicle-stimulating hormone receptor interacting proteins by yeast two-hybrid analysis. *Biol Reprod.* 1999; 60(suppl 1):595.
- Conklin DS, Galaktionov K, Beach D. 14-3-3 protein associate with cdc25 phosphatases. *Proc Natl Acad Sci USA.* 1995;92:7892–7896.
- Eikvar L, Tasken KA, Eskild W, Hansson V. Protein kinase C activation

- and positive and negative agonist regulation of 3', 5'-cyclic adenosine monophosphate levels in cultured rat Sertoli cells. *Acta Endocrinol (Copenh)*. 1993;128:568-572.
- Ford JC, al-Khodairy F, Fotou E, Sheldrick KS, Griffiths DJ, Carr AM. 14-3-3 protein homologs required for the DNA damage checkpoint in fission yeast. *Science*. 1994;265:533-535.
- Freed E, Symons M, Macdonald SG, McCormick F, Ruggieri R. Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. *Science*. 1994;265:1713-1716.
- Furukawa Y, Ikuta N, Omata S, Yamauchi T, Isobe T, Ichimura T. Demonstration of the phosphorylation-dependent interaction of tryptophan hydroxylase with the 14-3-3 proteins. *Biochim Biophys Acta*. 1993;194:144-149.
- Griswold MD. The central role of Sertoli cells in spermatogenesis. *Semin Cell Dev Biol*. 1998;9:411-416.
- Hsu SY, Kaipia A, Zhu L, Hsueh AJW. Interference of BAD (Bcl-xL/Bcl-2-associated death promoter)-induced apoptosis in mammalian cells by 14-3-3 isoforms and P11. *Mol Endocrinol*. 1997;11:1858-1867.
- Idzerda RL, Huebers H, Finch CA, McKnight GS. Rat transferrin gene expression: tissue-specific regulation by iron deficiency. *Proc Natl Acad Sci USA*. 1986;83:3723-3727.
- Isobe T, Ichimura T, Sunaya T, Okuyama T, Takahashi N, Kuwano R, Rosenfeld G, Takahashi Y. Distinct forms of the protein kinase dependent activator of tyrosine and tryptophan hydroxylases. *J Mol Biol*. 1991;217:125-132.
- Jones DH, Ley S, Aitken A. Isoforms of 14-3-3 protein can form homo and heterodimers in vivo and in vitro: implications for function as adapter proteins. *FEBS Lett*. 1995;368:55-58.
- Kangasniemi M, Kaipia A, Mali P, Toppari J, Huhtaniemi I, Parvinen M. Modulation of basal and FSH-dependent cyclic AMP production in rat seminiferous tubules staged by an improved transillumination technique. *Anat Rec*. 1990;227:62-76.
- Le Magueresse-Battistoni B, Morera AM, Benahmed M. In vitro regulation of rat Sertoli cell inhibin messenger RNA levels by transforming growth factor-beta 1 and tumour necrosis factor alpha. *J Endocrinol*. 1995;146:501-508.
- Means AR, Dedman JR, Tash DJ, Tindall DJ, van Sickle M, Welsh MJ. Regulation of the testis Sertoli cell by follicle stimulating hormone. *Ann Rev Physiol*. 1980;43:59-70.
- Meroni S, Canepa D, Pellizzari E, Scheitingaart H, Cigorraga S. Regulation of gamma-glutamyl transpeptidase activity by Ca(2+)- and protein kinase C dependent pathway in Sertoli cells. *Int J Androl*. 1997;20:189-194.
- Moore BW, Perez VJ. Specific acidic proteins of the nervous system. In: Carlson FD, ed. *Physiological and Biochemical Aspects of Nervous Integration*. Englewood Cliffs, NJ: Prentice-Hall; 1967:343-359.
- Oneda M, Suarez-Quian CA. Modulation of transferrin secretion by epidermal growth factor in immature rat Sertoli cells in vitro. *J Reprod Fertil*. 1994;100:541-550.
- Pergo L, Berruti G. Molecular cloning and tissue specific expression of the mouse homologue of the rat brain 14-3-3-0 protein: characterization of its cellular developmental pattern of expression in the male germ line. *Mol Reprod Dev*. 1997;47:370-379.
- Prasad GL, Valverius EM, McDuffie E, Cooper ML. Complementary DNA cloning of a novel epithelial cell marker protein, HME1, that may be down regulated in neoplastic mammary cells. *Cell Growth Differ*. 1992;3:507-513.
- Reuther GW, Fu H, Cripe LD, Collier RJ, Pendergast AM. Association of the protein kinase c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. *Science*. 1994;266:129-133.
- Reuther GW, Pendergast AM. The roles of 14-3-3 proteins in signal transduction. In: Litwak G, ed. *Vitamins and Hormones*. San Diego, Calif: Academic Press; 1996:149-175.
- Roth D, Morgan A, Burgoyne RD. Identification of a key domain in annexin and 14-3-3 proteins that stimulate calcium-dependent exocytosis in permeabilized adrenal chromaffin cells. *FEBS Lett*. 1993;320:207-210.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
- Schteingart HF, Meroni SB, Pellizzari E, Perez AL, Cigorraga SB. Regulation of Sertoli cell aromatase activity by cell density and prolonged stimulation with FSH, EGF, insulin and IGF-1 at different moments of pubertal development. *J Steroid Biochem Mol Biol*. 1995;52:375-381.
- Smith EP, Hall SH, Monaco L, French FS, Wilson EM, Conti M. Regulation of cfos messenger ribonucleic acid by fibroblast growth factor in cultured Sertoli cells. *Ann NY Acad Sci*. 1989;564:132-139.
- Suire S, Fontaine I, Guillou F. Follicle stimulating hormone (FSH) stimulates transferrin gene transcription in rat Sertoli cells: cis and trans-acting elements involved in FSH action via cyclic 3', 5'-monophosphate on the transferrin gene. *Mol Endocrinol*. 1995;9:756-766.
- Sutton KA, Maiti S, Tribble WA, Lindsey JS, Meistrich ML, Bucana CD, Sanberm BM, Joseph DR, Griswold MD, Cornwall GA, Wilkinson MF. Androgen regulation of the PEM homeodomain gene in mice and rat Sertoli and epididymal cells. *J Androl*. 1998;19:21-30.
- Tang SJ, Suen TC, McInnes RR, Buchwald M. Association of the TLX-2 homodomain and 14-3-3 eta signaling proteins. *J Biol Chem*. 1998;273:25356-25363.
- Thorson JA, Yu LW, Hsu AL, Shih NY, Graves PR, Tanner JW, Allen PM, Piwnicka-Worms H, Shaw AS. 14-3-3 proteins are required for maintenance of Raf-1 phosphorylation and kinase activity. *Mol Cell Biol*. 1998;18:5229-5238.
- Toker A, Sellers LA, Amess B, Patel Y, Harris A, Aitken A. Multiple isoforms of a protein kinase C inhibitor (KCIP/14-3-3) from sheep brain: amino acid sequence of phosphorylated forms. *Eur J Biochem*. 1992;206:453-461.
- Wang W, Shakes DC. Molecular evolution of the 14-3-3 protein family. *J Mol Evol*. 1996;43:384-398.
- Watanabe M, Isobe T, Ichimura T, Kuwano R, Takahashi Y, Kondo H, Inoue Y. Molecular cloning of rat cDNAs for the ζ and θ subtypes of 14-3-3 protein and differential distributions of their mRNAs in the brain. *Brain Res Mol Brain Res*. 1994;25:113-121.
- Whaley PD, Chaudhary J, Cupp A, Skinner MK. Role of specific response elements of the cfos promoter and involvement of intermediate transcription factor(s) in the induction of Sertoli cell differentiation (transferrin promoter activation) by the testicular paracrine factor PModS. *Endocrinology*. 1995;136:3046-3053.