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# Role of the Basic Helix-Loop-Helix Protein ITF2 in the Hormonal Regulation of Sertoli Cell Differentiation

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**ABSTRACT** Sertoli cells are a post-mitotic terminally differentiated cell population that forms the seminiferous tubules in the adult testis and provides the microenvironment and structural support for developing germ cells. During pubertal development, Sertoli cells are responsive to follicle-stimulating hormone (FSH) to promote the expression of differentiated gene products. The basic helix-loop-helix (bHLH) and inhibitors of differentiation (Id) transcription factors are involved in the differentiation of a variety of cell lineages during development. Both bHLH and Id transcription factors have been identified in Sertoli cells. A yeast two-hybrid screen was conducted using a rat Sertoli cell cDNA library to identify bHLH dimerization partners for the Id1 transcription factor. The ubiquitous bHLH protein ITF2 (i.e., E2-2) was identified as one of the interacting partners. The current study investigates the expression and function of ITF2 in Sertoli cells. ITF2 was found to be ubiquitously expressed in all testicular cell types including germ cells, peritubular myoid cells, and Sertoli cells. Stimulation of cultured Sertoli cells with FSH or dibutryl cAMP resulted in a transient decrease in expression of ITF2 mRNA levels followed by a rise in expression with FSH treatment. ITF2 expression was at its highest in mid-pubertal 20-day-old rat Sertoli cells. ITF2 was found to directly bind to negative acting Id HLH proteins and positive acting bHLH proteins such as scleraxis. Transient overexpression of ITF2 protein in cultured Sertoli cells stimulated transferrin promoter activity, which is a marker of Sertoli cell differentiation. Co-transfections of ITF2 and Id proteins sequestered the inhibitory effects of the Id family of proteins. Observations suggest ITF2 can enhance FSH actions through suppressing the inhibitory actions of the Id family of proteins and increasing the actions of stimulatory bHLH proteins (i.e., scleraxis) in Sertoli cells. *Mol. Reprod. Dev. 73: 491–500, 2006.*


**Key Words:** sertoli cell; testis; ITF; E2-2; bHLH; inhibitor of differentiation (Id); cellular differentiation; follicle stimulating hormone (FSH)

## INTRODUCTION

During puberty, Sertoli cells undergo dramatic morphological and physiological changes as the cell becomes a post-mitotic and terminally differentiated somatic cell population forming the basal and apical surface of the seminiferous epithelium (Fawcett, 1975). Tight junctions between adjacent Sertoli cells form the blood–testis barrier and create a microenvironment within the seminiferous tubule (Waites and Gladwell, 1982). Due to the blood–testis barrier, the developing germ cells are dependent on Sertoli cells for nutrients (Griswold, 1988). Transport proteins, such as the iron binding protein transferrin, produced by Sertoli cells deliver nutrients to the developing germ cells, which are essential for spermatogenesis (Skinner and Griswold, 1980; Griswold, 1995).

The onset of embryonic testis determination occurs with the expression of SRY that facilitates the initiation of Sertoli cell differentiation (Swain et al., 1998). Along with SRY, a significant number of other diverse transcription factors regulate this developmental pathway. Transcription factors such as SOX9 (Kent et al., 1996), c-fos (Whaley et al., 1995), c-myc (Lim and Hwang, 1995), SF-1 (Morohashi and Omura, 1996), and CREB (Don and Stelzer, 2002) appear to regulate or maintain Sertoli cell differentiated functions during early development. At the onset of puberty, Sertoli cell functions are regulated by the gonadotropin follicle-stimulating hormone, FSH (Fakunding et al., 1976). FSH acts on Sertoli cells through the cAMP/protein kinase A pathway that activates transcription factors responsible for the expression of Sertoli cell specific genes (Fakunding et al., 1976; Skinner et al., 1989; Walker et al., 1995). The expression of specific genes such as transferrin (Skinner and Griswold, 1980),

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androgen-binding protein (Saxlund et al., 2004), inhibin (Moore et al., 1994), and the follicle-stimulating hormone receptor (Rannikko et al., 1996) increase and are actively expressed in mature Sertoli cells to maintain normal testicular function. The specific transcription factors that regulate and maintain Sertoli cell differentiated functions remain to be elucidated.

The basic-helix-loop-helix (bHLH) family of transcription factors have been shown to play a critical role in cell fate determination and differentiation in a variety of tissues (Jan and Jan, 1993; Weintraub, 1993; Olson and Klein, 1994). These proteins contain a common HLH structural motif that consists of two amphipathic helixes separated by a loop. The HLH domain mediates homo- and heterodimerization between other bHLH proteins (Murre et al., 1989a,b). The presence of an adjacent DNA-binding region rich in basic amino acids mediates binding of the dimer to a common DNA sequence (CANNTG) known as an E-box (Lassar et al., 1989).

bHLH proteins can be arranged into two distinct classes depending on their dimerization specificity and expression pattern. The class A bHLH proteins are ubiquitously expressed and consist of ITF2 (i.e., E2-2) (Henthorn et al., 1990; Corneliussen et al., 1991; Javaux et al., 1991), HEB (i.e., REBa) (Hu et al., 1992), and the differentially-spliced transcript of the *E2A* gene (El2 and E47) (Murre et al., 1989b). The second group is the tissue-restricted group also known as the class B bHLH proteins. Examples of these proteins include the myogenic bHLH factors MyoD and myogenin in muscle development (Hasty et al., 1993; Nabeshima et al., 1993; Weintraub, 1993; Olson and Klein, 1994), neuronal factors such as neurogenin and Mashl (Guillemot et al., 1993; Ma et al., 1996), and *TAL* gene involved in hematopoiesis (Begley et al., 1989). Typically, these class B bHLH proteins dimerize with the ubiquitously expressed class A bHLH proteins (Murre et al., 1989b). bHLH transcription factors form an interacting network that regulates transcription of a variety of genes.

The activities of the class A and B bHLH proteins are often negatively regulated by another class of HLH proteins that lack the basic domain and are known as inhibitors of differentiation (Id) (Benezra et al., 1990). Id proteins are considered to be positive regulators of cell proliferation and negative regulators of cell differentiation. The four known Id proteins, Id1–4, form inactive dimers with either the class A or B bHLH proteins by preventing the heterodimer from binding to E-box sites located in the promoters of a variety of genes (Einarson and Chao, 1995; Langlands et al., 1997; Rigolet et al., 1998). Thus, overexpression of Id genes can inhibit the differentiation of a variety of cells including B lymphocytes (Sun et al., 1991), mammary epithelial cells (Desprez et al., 1995), muscle cells (Jen et al., 1992), erythroid cells (Lister et al., 1995), and Sertoli cells (Chaudhary et al., 2001).

Recent studies have demonstrated the effects of Id1–4 on Sertoli cell differentiated function (Chaudhary et al., 2000). All of the Id proteins except Id4, increased in expression in the presence of serum (Chaudhary et al., 2001). Interestingly, Id1 was downregulated in the presence of FSH and cAMP while Id4 was upregulated suggesting that Id1 and Id4 may have opposing functions in regulating Sertoli cell differentiated functions (Chaudhary et al., 2001). It has also been shown that conserved E-box sequences are present in the promoters of Sertoli cell differentiated genes such as c-fos (Chaudhary and Skinner, 1999), transferrin (Chaudhary et al., 1997), SF-1 (Daggett et al., 2000), and the FSH receptor (Goetz et al., 1996). Overexpression of Id1 has been shown to downregulate the mouse transferrin promoter (Chaudhary et al., 1997, 2001). The stable integration and constitutive overexpression of Id1 or Id2 in pubertal or adult Sertoli cells makes the cells re-enter the cell cycle and proliferate with a corresponding reduction in differentiated functions (Chaudhary et al., 2005). Combined observations suggest the Id family of transcription factors regulate Sertoli cell differentiation during postnatal development.

In the attempt to isolate bHLH genes involved in regulating Sertoli cell differentiated functions, a yeast two-hybrid system (Fields and Song, 1989) was used to screen a 20-day-old rat Sertoli cell cDNA library whose products could dimerize with Id1. One of the clones that formed a functional dimer with Id1 was ITF2, a class A bHLH protein. ITF2, also known as E2-2, is ubiquitously expressed and has been shown to form heterodimers with tissue-specific class B bHLH proteins and promote the process of differentiation (Lassar et al., 1991; Skerjanc et al., 1996; Chen and Lim, 1997; Furumura et al., 2001; Parrinello et al., 2001). One of the interacting class B bHLH partners is MyoD. ITF2 has been shown to interact with MyoD and activate promoters containing E-box sequences suggesting that heterodimerization of ITF2 and MyoD act to promote differentiation in myogenesis (Lassar et al., 1991). ITF2 was shown to be upregulated during the switch to pheomelanogenesis in melanocytes and was able to modulate the transcription of melanogenic genes (Furumura et al., 2001). ITF2/E2-2 can influence neuronal cell differentiation (Persson et al., 2000; Jogi et al., 2002), placental cell differentiation (Scott et al., 2000), and mesoderm development (Miyagishi et al., 2000). ITF2 has also been shown to interact with Id1 (Parrinello et al., 2001), Id2 (Parrinello et al., 2001; Liu et al., 2004), and Id3 (Chen and Lim, 1997). When co-expressed with Id, ITF2 counteracted the inhibitory effects of the Id proteins suggesting that ITF2 may promote differentiation by sequestering the inhibitory effect of the Id proteins (Skerjanc et al., 1996; Chen and Lim, 1997). Overexpression studies using the muscle creatine kinase promoter revealed that ITF2 acts as an inhibitor of differentiation by sequestering promoter activity (Chen and Lim, 1997). Therefore, ITF2 can act as both an activator and inhibitor of cellular differentiation. The current study investigates the role of ITF2 in the hormonal regulation of Sertoli cell differentiated functions.

## MATERIALS AND METHODS

### Yeast Expression Vectors

The GAL4(DBD)-Id1 fusion vector was constructed by inserting the region encoding amino acids 50–84 of Id1 into the *Eco*RI site of the yeast expression vector pBDGal4 (Stratagene, La Jolla, CA). The construct generated is a chimeric cDNA containing the yeast Gal4 DNA binding domain in frame with Id1. The region of Id1 expressed contains the bHLH domain. The same was done for E47 (full length), Id2 (full length), Id3 (full length), ITF2 (full length), and scleraxis (full length). A Gal4(AD) fusion expression library from Sertoli cell cDNA was constructed in HybriZapII by Stratagene from poly (A) + RNA isolated from purified 20-day-old rat Sertoli cells (Muir et al., 2005).

### Yeast Two-Hybrid Procedure

The Gal4(DBD)-Id1 was transformed into the yeast strain AH109 (mat A) (Clontech, Palo Alto, CA) while the Gal4(AD)-Sertoli cell cDNA library was transformed into the yeast strain Y187 (mat a) (Clontech). Following Clontech's procedure for performing a mating two-hybrid system $6 \times 10^6$ colonies were screened (Muir et al., 2005). Colonies able to grow on SD-Trp-Leu-His plates were replica-plated onto SD-Trp-Leu-His-Ade plates and harvested after 5 days. Successful candidates were assayed for β-galactosidase activity. Plasmids from β-galactosidase positive colonies were isolated and transformed into DH5α cells (Invitrogen, Carlsbad, CA). DH5α cells containing Gal4(AD)-cDNA were selected with ampicillin. Plasmids were isolated and sequenced using Gal4(AD) primers that flanked the cDNA insert in conjunction with dideoxy sequencing using an automated fluoresence-based sequencer (WSU/UI Center for Reproductive Biology, Molecular Biology Core). The sequences were then analyzed against the known sequences in GenBank using a FASTA search.

### Yeast Two-Hybrid Interaction Screen

Yeast two-hybrid interaction screens were performed with the yeast strain AH 109 (Clontech) harboring *HIS3, ADE2,* and *β-gal* as reporter genes. Approximately 2 μg of either the pBDGal4 or pADGal4 constructs was introduced into 50 ml of yeast. Cells were transformed using the lithium acetate method and plated on appropriate drop out medium. To identify the expression of the β-gal reporter, a liquid culture assay using ONPG as a substrate was followed according to Clontech protocol (Muir et al., 2005).

### RNA Preparation and Polymerase Chain Reaction (PCR)

Cultured Sertoli cells were lysed using TRI reagent (Sigma, St. Louis, MO). The lysate was passed through a pasture pipette to form a homogenous lysate. The whole tissue samples were homogenized in a tissue homogenizer in the presence of TRI reagent. The homogenate was centrifuged at 12,000*g* for 10 min 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. Total RNA from Sertoli cells, germ cells, and peritubular cells were isolated as described earlier using TRI reagent.

Total RNA (2 μg) was treated with DNase I for 30 min at 37°C to eliminate contaminating DNA. Potential contamination of RNA with DNA was determined by performing PCR on samples without a reverse transcription (RT) reaction step. The absence of any product in the amplification reaction indicated the absence of DNA in the RNA sample. All RNA samples were tested for DNA contamination. RNA was reverse transcribed with oligo dT (Invitrogen) using MMLV reverse transcriptase (Invitrogen). Approximately 2 μl of the product was subjected to PCR amplification using the following ITF2 primers: ITF2 5′-ggg atg eta ggc aac tct cat ate c and 3′-ctg aga gaa ggc cag ttc cat ate c. Each RT reaction used a distinct RNA sample. For an internal control, two primers corresponding to the cyclophilin gene were utilized as a constitutively expressed gene and to monitor the efficiency of the PCR reaction. A minimum of three different experiments using different RNA samples was performed in replicate.

A two-step real-time PCR was carried out to analyze the expression pattern of ITF2. RT-PCR was used to generate cDNA from either hormone treated cultured Sertoli cells, freshly isolated Sertoli cells, or whole testis. Total RNA (2 μg) was reverse-transcribed into cDNA in a reaction primed by oligo dT using MMLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Reverse and forward oligonucleotide primers, specific to the chosen candidate genes, were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) as described by the manufacturer. The following primers were used: ITF2 5′-act gga cga cgc aat tea tgt and 3′-ttg tga gag ggt ccg atg ate; ribosomal RNA (S2) 5′-ctg etc ctg tgc cca aga ag and 3′-aag gtg gec ttg gca aag tt; transferrin 5′-aaa tgg tgc gca gtg tea ga and 3′-ctt gca gaa atg gec ttg atg. Real-time RT-PCR was performed in a 96-well plate using a 7000 ABI prism sequence detection system (Applied Biosystems). The previously synthesized cDNA was used as template. Samples from cultured Sertoli cells, freshly isolated Sertoli cells or testes were plated in triplicate PCR reactions. The PCR reaction contained 5–10 ng of cDNA, SYBR GREEN master mix (Applied Biosystems), and 100 nM of each reverse and forward primers of ITF2 in a final PCR reaction of 50 μl. Amplification parameters were: denaturation at 94°C for 10 min followed by 40 cycles of 94°C for 15 sec and 60°C for 60 sec. Samples were analyzed in triplicate and ribosomal RNA (S2) was used as an endogenous control and for calibration curves (Chaudhary et al., 2005). A minimum of three different experiments, each done in triplicate, were performed.

### Sertoli Cell Preparation and Culture

Sertoli cells were isolated from 10- and 20-day-old rats by a sequential enzymatic digestion procedure

previously described (Tung et al., 1984). Decapsulated testis fragments were digested first with trypsin (1.5 mg/ml; Life Technologies, Gathersburg, MD) to remove the interstitial cells and then with collagenase (1 mg/ml type I; Sigma Chemical Co.) and hyaluronidase (1 mg/ml; Sigma). Sertoli cells were plated under serum-free conditions in 24-well Falcon plates (Falcon Plastics, Oxnard, CA) at $1 \times 10^6$ cells/well. Cells were maintained in a 5% $CO_2$ atmosphere in Ham's F-12 medium (Life Technologies) with 0.01% BSA at 32°C. Sertoli cells were left untreated (control) or treated with either FSH (100 ng/ml; FSH-16, National Pituitary Agency), dibutryl cAMP (dbcAMP; 100 μM), or 10% bovine calf serum. The cells were cultured for up to 72 hr. Sertoli cells from 65-day-old rats were isolated by a similar method previously described (Anway et al., 2004). All animal procedures were approved by the Washington State University Animal Care and Use Committee.

### Transfection Procedure

Sertoli cells cultured for 48 hr were transfected with the mammalian reporter and expression vectors by the calcium phosphate method coupled with hyper osmotic shock (10% glycerol) (Kingston et al., 1993). The procedure used is as previously described with a transfection efficiency greater than 50% estimated (Chaudhary et al., 1999). The luciferase reporter construct contains 581 bp of the mouse transferrin (mTf) promoter that includes the transcriptional initiation site of the transferrin gene, which is 54 bp upstream of the start site of translation (Idzerda et al., 1989). Full length ITF2 was inserted into the *Fxo*RI site of pCMV-HA (Promega, Madison, WI). Full length Id1, Id2, and Id3 were subcloned into the *Eco*Rl site of the mammalian expression plasmid pCMV-myc (Promega). Approximately 1.5 μg of each vector was added to 150 μl of transfection buffer (250 mM NaCl, 50 mM HEPES, and 1.47 mM $Na_2HPO_4$, pH 7.05) and placed in each well of a 24-well plate containing $1 \times 10^6$ Sertoli cells. Incubation occurred for 4 hr and was followed by a 3-min 10% glycerol shock. Cells were washed twice with HBSS (Invitrogen) before adding fresh Ham's F-12. Cells were treated with serum, FSH, or dibutryl cAMP and incubated for 72 hr. Cells were lysed after 72 hr with 150 μl of lysis buffer (Promega). Luciferase activity was determined by measuring luminescence of 20 μl of the cell lysate plus 100 μl of luciferase assay reagent for 20 sec in a luminometer (LB96P; Wallac-Berthold, Bad Wildbad, Germany).

### Statistical Analysis

The data from quantitative real-time PCR and overexpression analysis were analyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The values were expressed as the mean ± SEM. Statistical analysis was performed using a paired comparison Student's *t*-test. Groups were considered significantly different for a two-tailed *P*-value if $P \leq 0.05$. Statistical analysis of multiple groups used an analysis of variance (ANOVA) with a Tukey's and Dunnett's post test (GraphPad Software, Inc.). A minimum of three different experiments were performed in replicate for all data sets.

## RESULTS

### Identification of Id1 Interacting Proteins

To identify Sertoli cell bHLH factors that form functional dimers with Id1, a yeast two-hybrid system was used to screen a 20-day-old rat Sertoli cell cDNA library. In this screen, the HLH domain of Id1 was fused to the DNA binding domain of yeast Gal4 while the cDNA library was fused to the activation domain of yeast Gal4. A screen of approximately $1.25 \times 10^6$ independent Sertoli cell library transformants yielded 95 clones. ITF2 was a clone isolated from the screen. The known bHLH proteins, E47 and REBa were also identified in Sertoli cells in the screen. The identification of ITF2 in the yeast-two-hybrid screen prompted the current study to investigate the role of ITF2 in Sertoli cells.

### ITF2 Expression

A reverse transcription polymerase chain reaction (RT-PCR) procedure was used to identify the ITF2 expression pattern in the mid-pubertal rat. Tissues isolated from 20-day-old postnatal rats (P20) showed that ITF2 is expressed in all the tissues examined including the heart, kidney, muscle, brain, testis, spleen, liver, ovary, prostate, and lung (Fig. 1). Cyclophilin was used as a constitutively expressed control

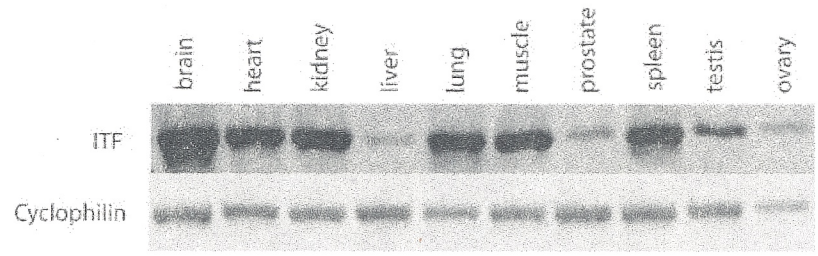


**Fig. 1.** ITF2 mRNA expression in a variety of tissues. Total RNA (2 μg) isolated from brain, heart, kidney, liver, lung, muscle, prostate, spleen, testis, and ovary was analyzed using RT-PCR. Cychlophilin gene was utilized as a constitutively expressed control gene and to monitor the efficiency of the PCR reaction. ITF2 PCR product 620 bp and cyclophilin PCR product 600 bp. The data is a representative of three separate experiments carried out on three different RNA samples.

gene. The RT-PCR procedure was used to determine what testicular cell types express ITF2. Cell types analyzed were the germ cells, peritubular cells, and Sertoli cells. ITF2 mRNA was detected in all the testis cell types analyzed (Fig. 2). ITF2 is ubiquitously expressed in the various tissues and cells of the testis.

Quantitative real-time PCR was used to analyze the expression levels of ITF2 in developing Sertoli cells. The RNA was isolated from pre-pubertal day 10 (P10), pubertal day 20 (P20), and adult day 65 (P65) rat Sertoli cells. Levels of ITF2 mRNA at P20 were eightfold greater than the mRNA levels of ITF2 at P10 (Fig. 3). ITF2 mRNA levels at P65 declined slightly from the level observed at P20. ITF2 mRNA levels at P65 are approximately sixfold greater than P10 mRNA levels. Transferrin is a Sertoli cell-differentiated gene product that increases in response to rising FSH levels. Therefore, transferrin is used as a positive marker of Sertoli cell differentiation. At P20 and P65, transferrin mRNA levels increase 100-fold and 500-fold, respectively as compared to the levels observed at P10 (data not shown). Observations indicate that ITF2 expression increases at a time when Sertoli cells begin pubertal differentiation and ITF2 expression is subsequently maintained at high levels in the adult.

### Hormonal Regulation of ITF2 Expression

Quantitative real-time PCR was used to analyze the hormonal regulation of ITF2 mRNA levels in Sertoli cells. Sertoli cells from 20-day-old rats were cultured and treated with either dibutryl cAMP or FSH for 0, 2, 4, 8, 24, and 72 hr. A decrease in ITF2 mRNA message was observed at 2 hr after FSH treatment and reached a minimum message level after 4 hr of treatment (Fig. 4A). In contrast, at 72 hr of FSH treatment the ITF2 expression increased. Although there was a consistent rise in ITF2 expression after 72 hr, it was not statistically significant ($P < 0.05$) above control levels. Treatment of the cells with dibutryl cAMP resulted in a similar transient reduction in ITF2 mRNA that was not noticeable until 8 hr after treatment with cAMP (Fig. 4B). Similar to FSH actions, after 72 hr of cAMP treatment, the ITF2 expression returned to control 0 hr levels.

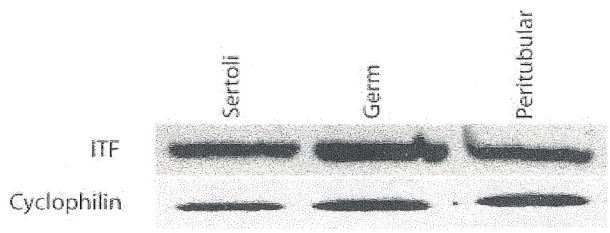


**Fig. 2.** ITF2 expression in different cell populations of the testis. RT-PCR analysis was used to detect the presence of ITF2 mRNA in germ cells, Sertoli cells, and peritubular cells. ITF2 mRNA message was detected when 2 μg of total RNA was analyzed. Cychlophilin gene was utilized as a constitutively expressed control gene and to monitor the efficiency of the PCR reaction. The data is representative of three separate experiments using three different RNA samples for each cell type analyzed.

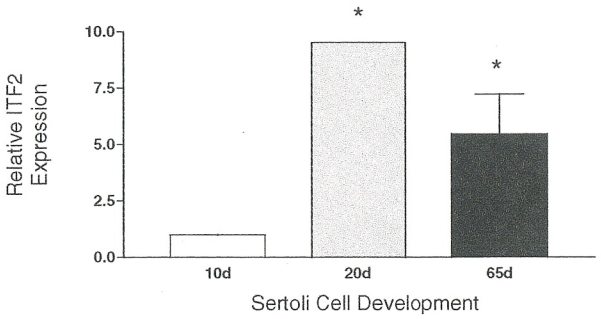


**Fig. 3.** ITF2 expression pattern in Sertoli cells during pubertal development. RNA was isolated from Sertoli cells of postnatal 10-, 20-, and 65-day-old rats. ITF2 and ribosomal RNA (S2) levels were analyzed using real-time PCR. All data was normalized to the P10 time point for each group. Data are mean ± SEM from three separate experiments with three replicates per experiment. Negligible error was found with 10- and 20-day-old development periods. Asterisk indicates a significant difference between the 10-day-old Sertoli cells and the other time points at $P < 0.01$ by Student's $t$-test.

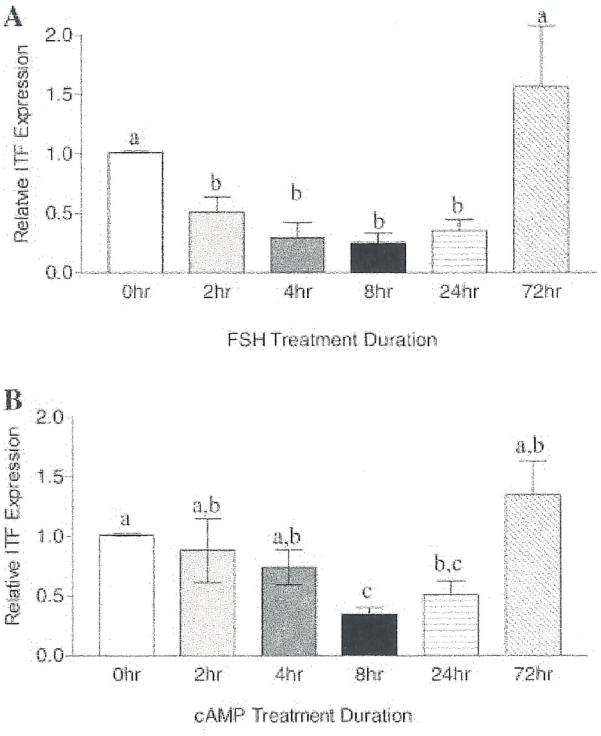


**Fig. 4.** ITF2 mRNA expression in response to dibutryl FSH (**A**) and cAMP (**B**). Analysis of ITF2 and S2 mRNA levels in Sertoli cells treated with FSH or dibutryl cAMP. Sertoli cells were cultured for 0, 2, 4, 8, 24, and 72 hr in the presence of FSH or cAMP. ITF2 and ribosomal RNA (S2) levels were analyzed using quantitative real-time PCR. All data were normalized to the 0 hr time point for each treatment group. Data are mean ± SEM from three separate experiments with three replicates per experiment. Different letter superscripts indicates a significant difference between treatment time points at $P < 0.05$ by ANOVA.

Therefore, in response to FSH and cAMP, ITF2 message levels transiently decrease suggesting that ITF2 expression is initially negatively regulated by FSH. However, long-term and likely indirect effects of FSH cause a rise in ITF2 expression to basal 0 hr levels.

### Functional Role of ITF2 in Sertoli Cells

The transferrin gene was used as a marker of Sertoli cell differentiation to study the functional significance of ITF2 on Sertoli cell differentiation. A chimeric reporter construct containing the luciferase gene driven by the proximal 600 bp-mouse transferrin promoter was transiently transfected into 20-day-old rat Sertoli cells. This reporter construct has been shown to be responsive to bHLH proteins (Chaudhary et al., 1997). An expression vector containing full length ITF2 was co-expressed with the transferrin-luciferase promoter construct. A basal expression vector was used as a control. After transfection, the Sertoli cells were left untreated or treated with cAMP. Compared to the control plasmid, the overexpression of ITF2 resulted in an increase of the transferrin promoter activity after cAMP stimulation (Fig. 5).

Id1, Id2, and Id3 were co-expressed with ITF2 to determine if ITF2 regulates the effects of the Id family of proteins on the transferrin promoter. All three Id proteins alone downregulated the transferrin promoter activity relative to ITF (Fig. 6) (Chaudhary et al., 2001). As shown in Figure 5, the basal untreated control and ITF levels of transferrin promoter activity are the same in the absence of hormones, such that relative expression in Figure 6 was based on ITF alone. Transferrin promoter activity was restored when ITF2 was co-expressed with Id1, Id2, or Id3 (Fig. 6). The ability of ITF2 to reverse the inhibitory activity of Id was highest for Id1, intermediate for Id2, and lowest for Id3. Overexpression analysis suggests that ITF2 in part influences Sertoli cell differentiation by regulating the inhibitory effects of the Id family of proteins on Sertoli cell differentiated gene products.

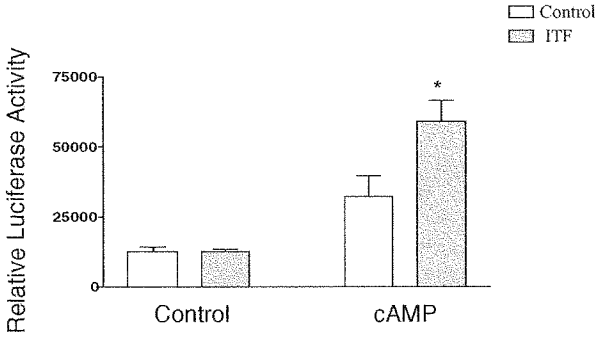


**Fig. 5.** Constitutive overexpression of ITF2 in cultured Sertoli cells. Cultured Sertoli cells were transfected with the proximal 600 bp mouse transferrin promoter-luciferase construct. Overexpression of ITF2 involves the presence of 2 μg of pCMV-ITF2 construct. Basal expression vector (pCMV-myc) is used as a control. The cells were challenged with dibutryl cAMP following the transfection for 72 hr. Data are mean ± SEM from three separate experiments with three replicates per experiment. Asterisk indicates a significant difference between control (pCMV-myc) and ITF2 at $P < 0.01$ by Student's $t$-test.

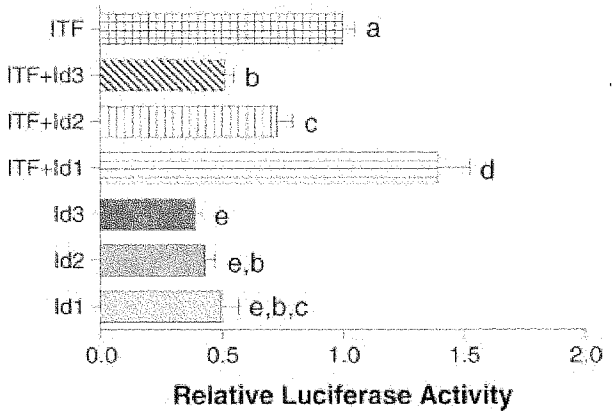


**Fig. 6.** ITF2 effect on Id actions on the transferrin promoter in Sertoli cells. Cultured Sertoli cells were transfected with the transferrin promoter-luciferase construct with Id1, Id2, or Id3 expression plasmids, an ITF2 expression plasmid, or combination of Id and ITF2. The cells were treated with dibutryl cAMP following transfection for 72 hr. The difference between Id1, Id2, or Id3, and ITF2 co-transfections with the corresponding Id proteins presented as relative luciferase activity associated with the transferrin promoter activity with ITF alone. Data is a representative of five different experiments and all treatments with different superscript letters are statistically different from each other, $P < 0.05$ with ANOVA.

### ITF2 Binding Partners

ITF2 functions by binding to other bHLH transcription factors. bHLH heterodimers with ITF2 appear to be involved in the regulation of Sertoli cell differentiated gene expression. To identify possible binding partners, a yeast two-hybrid system was used to verify ITF2 interaction with other known bHLH or HLH proteins expressed in Sertoli cells (Table 1). The Gal4 activation or binding domain and full-length ITF2 were expressed as a fusion protein in yeast cells to analyze ITF2 interaction with other bHLH or HLH transcription

**TABLE 1. ITF2 Interaction With Other bHLH and HLH Transcription Factors**

| | Bait | Prey | -Trp, -Leu, -His, -Ade | β-galactosidase activity |
|---|---|---|---|---|
| 1 | p53 | pSV40 | + | + |
| 2 | E47 | pSV40 | – | – |
| 3 | E47 | ITF2 | – | – |
| 4 | ITF2 | Scleraxis | + | +++ |
| 5 | Id1 | ITF2 | + | + |
| 6 | ITF2 | Id2 | + | + |
| 7 | ITF2 | Id3 | + | ++ |

A (+) in the -Trp, -Leu, -His-, Ade column represents growth of yeast on amino acid selection plates. A (+) in the β-galactosidase activity column represents a minimum of twofold increase in activity, ++ represents a minimum of fourfold increase in activity, and +++ represents a minimum of sixfold increase in activity as compared to the negative control (E47 (bait) and pSV40 (prey)). Negative control is set at 1. Data is representative of a minimum of three different experiments.

factors. The interacting partners, E47, scleraxis, Id1, Id2, and Id3 were fused to the Gal4 activation or binding domain. ITF2 fusion vector was co-transformed with each of the interacting partners. Survival of the double transfected yeast in selection media and the expression of the LacZ reporter gene were used to verify a positive interaction. The pSV40 and p53 constructs were used as a positive control (Table 1). The pBDgal4-E47 and pSV40 were used as the negative control and also excludes the E47 constructs ability to autoactivate the reporter genes (Table 1). Similar controls were conducted for all constructs to exclude the possibility of autoactivation (data not shown). A positive interaction is observed when ITF2 is co-transformed with Id1, Id2, Id3, and scleraxis (Table 1). No interactions were observed when ITF2 was co-transformed with E47. The binding interactions confirm previous observations for ITF2 interactions using different types of procedures such as co-immunoprecipitation (Lassar et al., 1991; Skerjanc et al., 1996; Chen and Lim, 1997; Furumura et al., 2001; Parrinello et al., 2001). The observations suggest that ITF2 may interact with the Id family of proteins and can also interact with select bHLH factors (e.g., scleraxis) known to promote Sertoli cell differentiated functions (Muir et al., 2005).

## DISCUSSION

Sertoli cell differentiation is initiated at the time of testis determination in response in part, to the expression of SRY. After the onset of Sertoli cell differentiation, the cells proliferate until the time of puberty when the cells become post-mitotic and terminally differentiated. During pubertal development, Sertoli cell expression of differentiated gene products increases due to the increase of circulatory FSH levels. Many of these Sertoli cell genes are required for the maintenance and support of spermatogenesis. Identification of bHLH proteins involved in regulating Sertoli cell differentiation is the focus of the current study. Id1 binding partners (i.e., ITF2) were identified using a yeast two-hybrid screen of a 20-day-old rat Sertoli cell cDNA expression library. ITF2 is a ubiquitously expressed bHLH transcription factor involved in differentiation and proliferation of a variety of cell lineages such as mammary epithelial cells (Parrinello et al., 2001), myeloid (Furumura et al., 2001), neuronal cells (Persson et al., 2000; Jogi et al., 2002), trophoblast (Scott et al., 2000), and myogenic cells (Skerjanc et al., 1996). In the current study, the ITF2 transcript was detected in the brain, heart, kidney, lung, muscle, spleen, liver, prostate, ovary, and testis of 20-day-old control of rats. The presence of ITF2 in a variety of adult tissues suggests that it likely has a role in the cellular function of most cells. The current study demonstrates that ITF can enhance FSH actions through suppressing inhibitory actions of the Id family of proteins and increasing the actions of stimulatory proteins (i.e., scleraxis) in Sertoli cells.

The expression of ITF2 during pubertal development was investigated with the pre-pubertal 10-day, mid-pubertal 20-day, and adult 65-day stages of development analyzed. The onset of puberty occurs at 10 days in the rat. At 15 days, the seminiferous tubules form and around 15 days of postnatal development spermatogenesis is initiated. Sertoli cells become post-mitotic and terminally differentiated around 15 days of development. As puberty progresses, ITF2 mRNA levels increase by eightfold when compared with the levels at the onset of puberty (P10). In the adult (P65), ITF2 mRNA levels continue to be significantly higher as compared to the onset of puberty (P10), but are twofold less than that observed at mid-puberty (P20). The onset of puberty is in part controlled by FSH promoting Sertoli cell differentiation. A rise in ITF2 expression is observed during puberty and may be involved in regulating Sertoli cell differentiation during the early stages of puberty. A similar pattern of expression was seen in mouse myeloid cells. ITF2 was shown to be upregulated during the switch to pheomelanogenesis and was able to modulate the transcription of melanogenic genes (Furumura et al., 2001). The increase in ITF2 expression may be necessary for Sertoli cells to make the switch between a mitotic to a post-mitotic cell type. ITF2 may interact with Id proteins to ensure that Sertoli cells exit the cell cycle and to allow cellular differentiation to occur (Chaudhary et al., 2005).

The gonadotropin follicle-stimulating hormone (FSH) and androgens regulate the majority of Sertoli cell functions (Skinner et al., 1989). Studies with cultured Sertoli cells revealed that after treatment with FSH or cAMP ITF2 expression decreases as compared with the untreated control cells. As the Sertoli cell transitions from a mitotic to a post-mitotic cell type, the ITF2 expression declines and may be due to the increased actions of FSH on Sertoli cells. However, ITF2 expression is maintained at high levels in the adult (i.e., P65 vs. P10) so will continue to be necessary to modulate the transcription of Sertoli cell differentiated gene products. The maintenance of high ITF2 levels in the adult Sertoli cells correlates with the long-term effects of FSH to potentially increase ITF2 expression.

Transferrin is a Sertoli cell differentiated gene product that is highly expressed at the onset of puberty and in the adult. Transferrin expression is low early in postnatal development and begins to increase when FSH production increases during puberty (Skinner and Griswold, 1982). The increase in transferrin correlates with the progression of pubertal Sertoli cell differentiation. Therefore, transferrin is a useful marker for Sertoli cell differentiation. Constitutive expression of ITF2 in cultured Sertoli cells enhanced the ability of hormones to stimulate the transferrin promoter suggesting ITF2 can regulate Sertoli cell differentiated gene products. Future studies could use RNAi or dominant negative forms of ITF2 to determine the effects of inhibiting ITF2 actions on Sertoli cells. Previous studies have shown that ITF2 may be a regulator of Id functions in cells. In a mammary epithelial cell line, ITF2 was able to reverse the phenotype induced by constitutive Id1 expression and allowed the expression of differentiated gene products (Parrinello et al., 2001). Id proteins negatively

regulate bHLH transcription factors and inhibit the differentiation of a variety of cells such as trophoblasts (Scott et al., 2000), erythroid cells (Lister et al., 1995), lymphocytes (Bergqvist et al., 2000; Sigvardsson, 2000), myeloid cells (Kreider et al., 1992), and mammary epithelial cells (Desprez et al., 1995). Id proteins promote post-mitotic terminally differentiated Sertoli cells to re-enter the cell cycle and proliferate with a reduction in differentiation (Chaudhary et al., 2005), Figure 7. The current study demonstrates the ability of ITF2 to reverse the inhibitory effects of Id proteins on the transferrin promoter in Sertoli cells. ITF2 was most effective in reversing the inhibitory effects of Id1 and less with Id3 suggesting different binding characteristics between the various Id isoforms and ITF2. Observations suggest ITF2 may be regulating Sertoli cell differentiation by sequestering the inhibitory effects of Id proteins (Fig. 7). In contrast, ITF2 was found to directly dimerize with the positive acting bHLH proteins in Sertoli cells such as scleraxis (Muir et al., 2005). Direct stimulatory actions of ITF2 may occur through heterodimers with bHLH proteins like scleraxis (Muir et al., 2005), Figure 7.

In summary, ITF2 appears to be an important bHLH protein involved in Sertoli cell differentiation. Constitutive expression of ITF2 in cultured Sertoli cells promoted the activation of the transferrin promoter and was able to sequester the inhibitory effects of the Id family of proteins. A rise in ITF2 expression is observed during puberty when Sertoli cells are making the transition from a mitotic to a post-mitotic terminally differentiated cell type. This rise in expression of ITF2 may be necessary to regulate the Id proteins and facilitate the change from a proliferating cell type to a differentiated state. Once the transition is over, ITF2 may be required to interact with other bHLH factors (e.g., scleraxis) to promote Sertoli cell differentiated functions. Future studies using conditional knockout ITF2 models can further elucidate the role of ITF2 in Sertoli cells.

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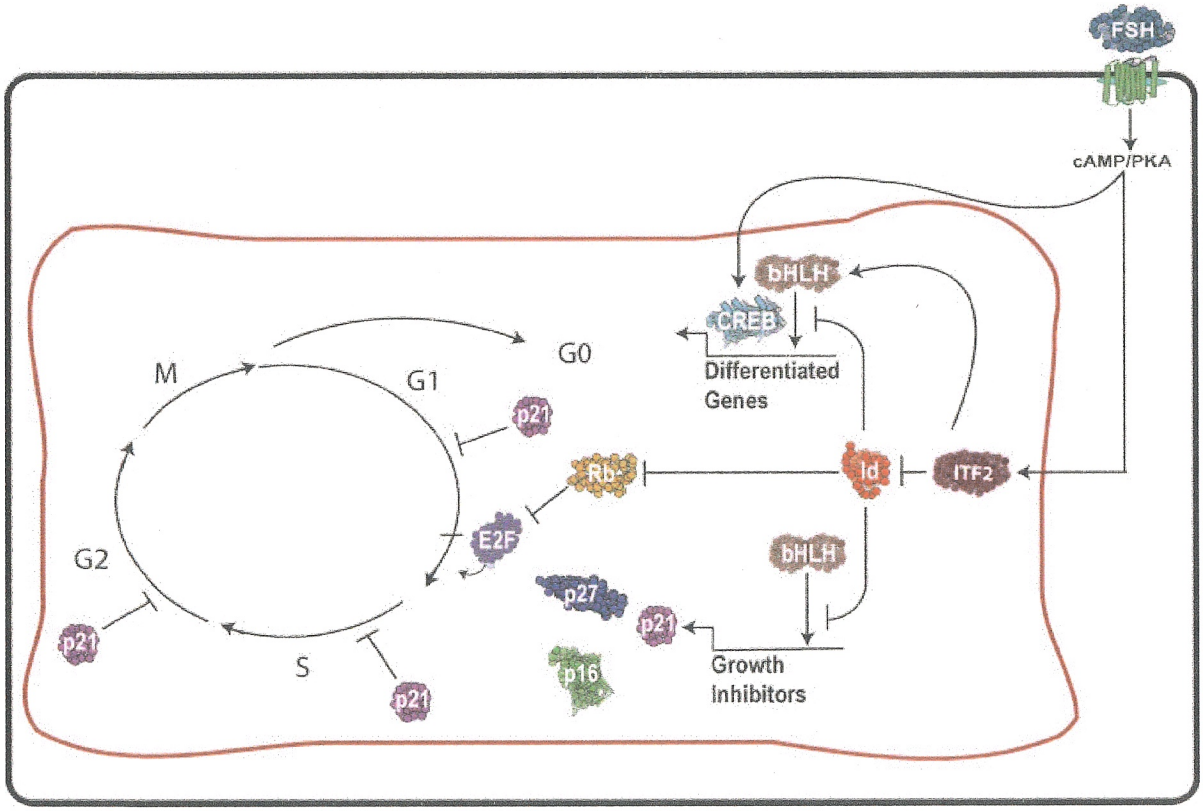


**Fig. 7.** Schematic of proposed ITF2 and Id interactions with cell cycle proteins and cell differentiation proteins in the Sertoli cell. FSH acting at the FSH receptor activates cAMP and protein kinase A (PKA) that regulates ITF2 expression that activates Sertoli cell differentiated genes and inhibits Id actions. ITF2 blocks the ability of Id to bind retinoblastoma protein (Rb) that allows E2F to promote the cell cycle. ITF2 inhibits the ability of Id to block bHLH proteins needed for growth inhibitors pl6, p21, and p27 expression so decreases growth inhibition. ITF also heterodimerizes with bHLH proteins to activate differentiated genes. [See color version online at www.interscience.wiley.com.]

## REFERENCES


Anway MD, Wright WW, Zirkin BR, Korah N, Mort JS, Hermo L. 2004. Expression and localization of cathepsin k in adult rat sertoli cells. Biol Reprod 70:562–569.

Begley CG, Apian PD, Denning SM, Haynes BF, Waldmann TA, Kirsch IR. 1989. The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA-binding motif. Proc Natl Acad Sci USA 86:10128–10132.

Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. 1990. The protein Id: A negative regulator of helix-loop-helix DNA binding proteins. Cell 61:49–59.

Bergqvist I, Eriksson M, Saarikettu J, Eriksson B, Corneliussen B, Grundstrom T, Holmberg D. 2000. The basic helix-loop-helix transcription factor E2-2 is involved in T lymphocyte development. Eur J Immunol 30:2857–2863.

Chaudhary J, Skinner MK. 1999. Basic helix-loop-helix proteins can act at the E-box within the serum response element of the c-fos promoter to influence hormone-induced promoter activation in Sertoli cells. Mol Endocrinol 13:774–786.

Chaudhary J, Cupp AS, Skinner MK. 1997. Role of basic-helix-loop-helix transcription factors in Sertoli cell differentiation: Identification of an E-box response element in the transferrin promoter. Endocrinology 138:667–675.

Chaudhary J, Kim G, Skinner MK. 1999. Expression of the basic helix-loop-helix protein REBalpha in rat testicular Sertoli cells. Biol Reprod 60:1244–1250.

Chaudhary J, Mosher R, Kim G, Skinner MK. 2000. Role of winged helix transcription factor (WIN) in the regulation of Sertoli cell differentiated functions: WIN acts as an early event gene for follicle-stimulating hormone. Endocrinology 141:2758–2766.

Chaudhary J, Johnson J, Kim G, Skinner MK. 2001. Hormonal regulation and differential actions of the helix-loop-helix transcriptional inhibitors of differentiation (Idl, Id2, Id3, and Id4) in Sertoli cells. Endocrinology 142:1727–1736.

Chaudhary J, Sadler-Riggleman I, Ague JM, Skinner MK. 2005. The helix-loop-helix inhibitor of differentiation proteins induce post-mitotic terminally differentiated sertoli cells to re-enter the cell cycle and proliferate. Biol Reprod 72:1205–1217.

Chen B, Lim RW. 1997. Physical and functional interactions between the transcriptional inhibitors Id3 and ITF-2b. Evidence toward a novel mechanism regulating muscle-specific gene expression. J Biol Chem 272:2459–2463.

Corneliussen B, Thornell A, Hallberg B, Grundstrom T. 1991. Helix-loop-helix transcriptional activators bind to a sequence in glucocorticoid response elements of retrovirus enhancers. J Virol 65: 6084–6093.

Daggett MA, Rice DA, Heckert LL. 2000. Expression of steroidogenic factor 1 in the testis requires an E box and CCA AT box in its promoter proximal region. Biol Reprod 62:670–679.

Desprez PY, Hara E, Bissell MJ, Campisi J. 1995. Suppression of mammary epithelial cell differentiation by the helix-loop-helix protein Id-1. Mol Cell Biol 15:3398–3404.

Don J, Stelzer G. 2002. The expanding family of CREB/CREM transcription factors that are involved with spermatogenesis. Mol Cell Endocrinol 187:115–124.

Einarson MB, Chao MV. 1995. Regulation of Idl and its association with basic helix-loop-helix proteins during nerve growth factor-induced differentiation of PC 12 cells. Mol Cell Biol 15:4175–4183.

Fakunding JL, Tindall DJ, Dedman JR, Mena CR, Means AR. 1976. Biochemical actions of follice-stimulating hormone in the Sertoli cell of the rat testis. Endocrinology 98:392–402.

Fawcett DW. 1975. The ultrastructure and functions of the Sertoli Cell. In: Greep R, Hamilton E, editors. Handbook of physiology. Washington D.C.: American Physiology Society, pp 21–55.

Fields S, Song O. 1989. A novel genetic system to detect protein-protein interactions. Nature 340:245–246.

Furumura M, Potterf SB, Toyofuku K, Matsunaga J, Muller J, Hearing VJ. 2001. Involvement of ITF2 in the transcriptional regulation of melanogenic genes. J Biol Chem 276:28147–28154.

Goetz TL, Lloyd TL, Griswold MD. 1996. Role of E box and initiator region in the expression of the rat follicle-stimulating hormone receptor. J Biol Chem 271:33317–33324.

Griswold MD. 1988. Protein secretions of Sertoli cells. Int Rev Cytol 110:133–156.

Griswold MD. 1995. Interactions between germ cells and Sertoli cells in the testis. Biol Reprod 52:211–216.

Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL. 1993. Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell 75:463–476.

Hasty P, Bradley A, Morris JH, Edmondson DG, Venuti JM, Olson EN, Klein WH. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature 364:501–506.

Henthorn P, Kiledjian M, Kadesch T. 1990. Two distinct transcription factors that bind the immunoglobulin enhancer microE5/kappa 2 motif. Science 247:467–470.

Hu JS, Olson EN, Kingston RE. 1992. HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding ability of myogenic regulatory factors. Mol Cell Biol 12:1031–1042.

Idzerda RL, Behringer RR, Theisen M, Huggenvik JI, McKnight GS, Brinster RL. 1989. Expression from the transferrin gene promoter in transgenic mice. Mol Cell Biol 9:5154–5162.

Jan YN, Jan LY. 1993. HLH proteins, fly neurogenesis, and vertebrate myogenesis. Cell 75:827–830.

Javaux F, Donda A, Vassart G, Christophe D. 1991. Cloning and sequence analysis of TFE, a helix-loop-helix transcription factor able to recognize the thyroglobulin gene promoter in vitro. Nucleic Acids Res 19:1121–1127.

Jen Y, Weintraub H, Benezra R. 1992. Overexpression of Id protein inhibits the muscle differentiation program: In vivo association of Id with E2A proteins. Genes Dev 6:1466–1479.

Jogi A, Persson P, Grynfeld A, Pahlman S, Axelson H. 2002. Modulation of basic helix-loop-helix transcription complex formation by Id proteins during neuronal differentiation. J Biol Chem 277:9118–9126.

Kent J, Wheatley SC, Andrews JE, Sinclair AH, Koopman P. 1996. A male-specific role for SOX9 in vertebrate sex determination. Development 122:2813–2822.

Kingston RE, Chen CA, Okayama H. 1993. Calcium phosphate transfection. In: Ausabel FA, Brent R, Kinston RE, Moore DD, Sidman JG, J.A S, Struhl K, editors. Current protocols in molecular biology. New York: Greene and Wiley Interscience. pp 9.1.1–9.1.3.

Kreider BL, Benezra R, Rovera G, Kadesch T. 1992. Inhibition of myeloid differentiation by the helix-loop-helix protein Id. Science 255:1700–1702.

Langlands K, Yin X, Anand G, Prochownik EV. 1997. Differential interactions of Id proteins with basic-helix-loop-helix transcription factors. J Biol Chem 272:19785–19793.

Lassar AB, Buskin JN, Lockshon D, Davis RL, Apone S, Hauschka SD, Weintraub H. 1989. MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. Cell 58:823–831.

Lassar AB, Davis RL, Wright WE, Kadesch T, Murre C, Voronova A, Baltimore D, Weintraub H. 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell 66:305–315.

Lim K, Hwang BD. 1995. Follicle-stimulating hormone transiently induces expression of protooncogene c-myc in primary Sertoli cell cultures of early pubertal and prepubertal rat. Mol Cell Endocrinol 111:51–56.

Lister J, Forrester WC, Baron MH. 1995. Inhibition of an erythroid differentiation switch by the helix-loop-helix protein Id1. J Biol Chem 270:17939–17946.

Liu YP, Burleigh D, Durning M, Hudson L, Chiu IM, Golos TG. 2004. Id2 is a primary partner for the E2-2 basic helix-loop-helix transcription factor in the human placenta. Mol Cell Endocrinol 222:83–91.

Ma Q, Kintner C, Anderson DJ. 1996. Identification of neurogenin, a vertebrate neuronal determination gene. Cell 87:43–52.

Miyagishi M, Hatta M, Ohshima T, Ishida J, Fujii R, Nakajima T, Fukamizu A. 2000. Cell type-dependent transactivation or repression of mesoderm-restricted basic helix-loop-helix protein, POD-1/Capsulin. Mol Cell Biochem 205:141–147.

Moore A, Krummen LA, Mather JP. 1994. Inhibins, activins, their binding proteins and receptors: Interactions underlying paracrine activity in the testis. Mol Cell Endocrinol 100:81–86.

Morohashi KI, Omura T. 1996. Ad4BP/SF-l, a transcription factor essential for the transcription of steroidogenic cytochrome P450 genes and for the establishment of the reproductive function. FASEB J 10:1569–1577.

Muir T, Sadler-Riggleman I, Skinner MK. 2005. Role of the basic helix-loop-helix transcription factor, scleraxis, in the regulation of sertoli cell function and differentiation. Mol Endocrinol 19:2164–2174.

Murre C, McCaw PS, Baltimore D. 1989a. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell 56:777–783.

Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB, et al. 1989b. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58:537–544.

Nabeshima Y, Hanaoka K, Hayasaka M, Esumi E, Li S, Nonaka I. 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature 364:532–535.

Olson EN, Klein WH. 1994. bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. Genes Dev 8:1–8.

Parrinello S, Lin CQ, Murata K, Itahana Y, Singh J, Krtolica A, Campisi J, Desprez PY. 2001. Id-1, ITF-2, and Id-2 comprise a network of helix-loop-helix proteins that regulate mammary epithelial cell proliferation, differentiation, and apoptosis. J Biol Chem 276: 39213–39219.

Persson P, Jogi A, Grynfeld A, Pahlman S, Axelson H. 2000. HASH-1 and E2-2 are expressed in human neuroblastoma cells and form a functional complex. Biochem Biophys Res Commun 274:22–31.

Rannikko A, Penttila TL, Zhang FP, Toppari J, Parvinen M, Huhtaniemi I. 1996. Stage-specific expression of the FSH receptor gene in the prepubertal and adult rat seminiferous epithelium. J Endocrinol 151:29–35.

Rigolet M, Rich T, Gross-Morand MS, Molina-Gomes D, Viegas-Pequignot E, Junien C. 1998. cDNA cloning, tissue distribution and chromosomal localization of the human Id4 gene. DNA Res 5:309–313.

Saxlund MA, Sadler-Riggleman I, Skinner MK. 2004. Role of basic helix-loop-helix (bHLH) and CREB transcription factors in the regulation of sertoli cell androgen-binding protein expression. Mol Reprod Dev 68:269–278.

Scott IC, Anson-Cartwright L, Riley P, Reda D, Cross JC. 2000. The HAND1 basic helix-loop-helix transcription factor regulates trophoblast differentiation via multiple mechanisms. Mol Cell Biol 20: 530–541.

Sigvardsson M. 2000. Overlapping expression of early B-cell factor and basic helix-loop-helix proteins as a mechanism to dictate B-lineage-specific activity of the Iambda5 promoter. Mol Cell Biol 20:3640–3654.

Skerjanc IS, Truong J, Filion P, McBurney MW. 1996. A splice variant of the ITF-2 transcript encodes a transcription factor that inhibits MyoD activity. J Biol Chem 271:3555–3561.

Skinner MK, Griswold MD. 1980. Sertoli cells synthesize and secrete transferrin-like protein. J Biol Chem 255:9523–9525.

Skinner MK, Griswold MD. 1982. Secretion of testicular transferrin by cultured Sertoli cells is regulated by hormones and retinoids. Biol Reprod 27:211–221.

Skinner MK, Schlitz SM, Anthony CT. 1989. Regulation of Sertoli cell differentiated function: Testicular transferrin and androgen-binding protein expression. Endocrinology 124:3015–3024.

Sun XH, Copeland NG, Jenkins NA, Baltimore D. 1991. Id proteins Idl and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. Mol Cell Biol 11:5603–5611.

Tung PS, Skinner MK, Fritz IB. 1984. Cooperativity between Sertoli cells and peritubular myoid cells in the formation of the basal lamina in the seminiferous tubule. Ann NY Acad Sci 438:435–446.

wain A, Narvaez V, Burgoyne P, Camerino G, Lovell-Badge R. 1998. Daxl antagonizes Sry action in mammalian sex determination. Nature 391:761–767.

Waites GM, Gladwell RT. 1982. Physiological significance of fluid secretion in the testis and blood-testis barrier. Physiol Rev 62:624–671.

Walker WH, Fucci L, Habener JF. 1995. Expression of the gene encoding transcription factor cyclic adenosine 3′,5′-monophosphate (cAMP) response element-binding protein (CREB): Regulation by follicle-stimulating hormone-induced cAMP signaling in primary rat Sertoli cells. Endocrinology 136:3534–3545.

Weintraub H. 1993. The MyoD family and myogenesis: Redundancy, networks, and thresholds. Cell 75:1241–1244.

Whaley PD, Chaudhary J, Cupp A, Skinner MK. 1995. Role of specific response elements of the c-fos 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 136:3046–3053.