

**USF1 AND USF2: MOLECULAR REGULATORS OF THE SWITCH BETWEEN
PROLIFERATION AND DIFFERENTIATION IN POSTNATAL RAT SERTOLI CELLS**

by

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Sertoli cells provide nutrients and growth factors for developing germ cells. Each Sertoli cell can support a finite number of developing germ cells. During development of the testis, cessation of Sertoli cell proliferation and the onset of differentiation establishes the final number of Sertoli cells and hence the number of sperm that can be produced. Studies in this dissertation explore the hypothesis that the transition from proliferation to differentiation is facilitated by E-box transcription factors that induce the expression of differentiation-promoting genes. The relative activities of E-box proteins were studied in primary Sertoli cells isolated from 5, 11, and 20 day-old rats, representing proliferating, differentiating, and differentiated cells, respectively. DNA binding by E-box proteins is nearly undetectable at 5 days after birth but peaks with initiation of differentiation at 11 days after birth and remains elevated. Upstream Stimulatory Factors 1 and 2 (USF1, USF2) were the predominant E-box proteins present within DNA-protein complexes formed after incubating E-box containing probes with nuclear extracts from developing Sertoli cells. Increased USF binding activity corresponded with elevated *Usf1* mRNA and USF1 protein levels 11 days after birth. The potentiator of Sertoli cell differentiation, thyroxine, induces USF DNA binding in Sertoli cells prior to differentiation. Decreased nuclear expression of ID proteins may permit increased USF DNA binding during Sertoli cell differentiation. Several genes required for Sertoli cell differentiation and differentiated Sertoli cell functions have USF binding sites within their promoters. Two potential USF target genes, *Nr5a1* and *Shbg*, were induced in

11 day-old Sertoli cells when compared with 5 day-old Sertoli cells. DNA binding studies of *Nr5a1* and *Shbg* promoter sequences determined that USF1 and USF2 binding to E-box motifs increased during differentiation. *In vivo* binding assays confirmed that USF1 and USF2 occupy the E-box within the *Nr5a1* promoter. These data support the ideas that increased USF protein expression induces differentiation-associated gene expression and that USF-mediated alterations in gene transcription are responsible for the onset of differentiation in developing Sertoli cells. These USF-mediated processes would then determine the final number of Sertoli cells present within the testes and the upper limit of fertility.

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PREFACE

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1.0 THE SERTOLI CELL

1.1 STRUCTURAL ORGANIZATION OF THE TESTIS

The major reproductive organ within the mammalian male is the testis (Figure 1A). The testes are contained within the scrotal sac and are surrounded by a thick capsule, the tunica albuginea (reviewed in [1]). Within the testis, sperm are produced in the seminiferous tubules (Figure 1B). Seminiferous tubules are surrounded by layers of peritubular cells [2, 3]. The interstitial space between the seminiferous tubules contains fibroblasts, nerves, blood vessels, lymphatics and clusters of Leydig cells that secrete the steroid hormone, testosterone (T) [3]. Seminiferous tubules contain the germ cell progenitors, and spermatogonia that lie at the basement membrane of the seminiferous tubule [1, 4]. Sertoli cells, non-dividing somatic cells of the seminiferous tubules, also lie on the basement membrane and support these progenitors outside the blood-testis barrier in the germinal epithelium [5] (Figure 1C). Sertoli cells extend the radius of the tubule with irregular projections of cytoplasm that envelope developing germ cells [1, 4, 6]. Sertoli cells surround germ cells throughout their development and provide necessary maturation factors. This close association allows nutrients and growth factors to be supplied by Sertoli cells to the germ cells.

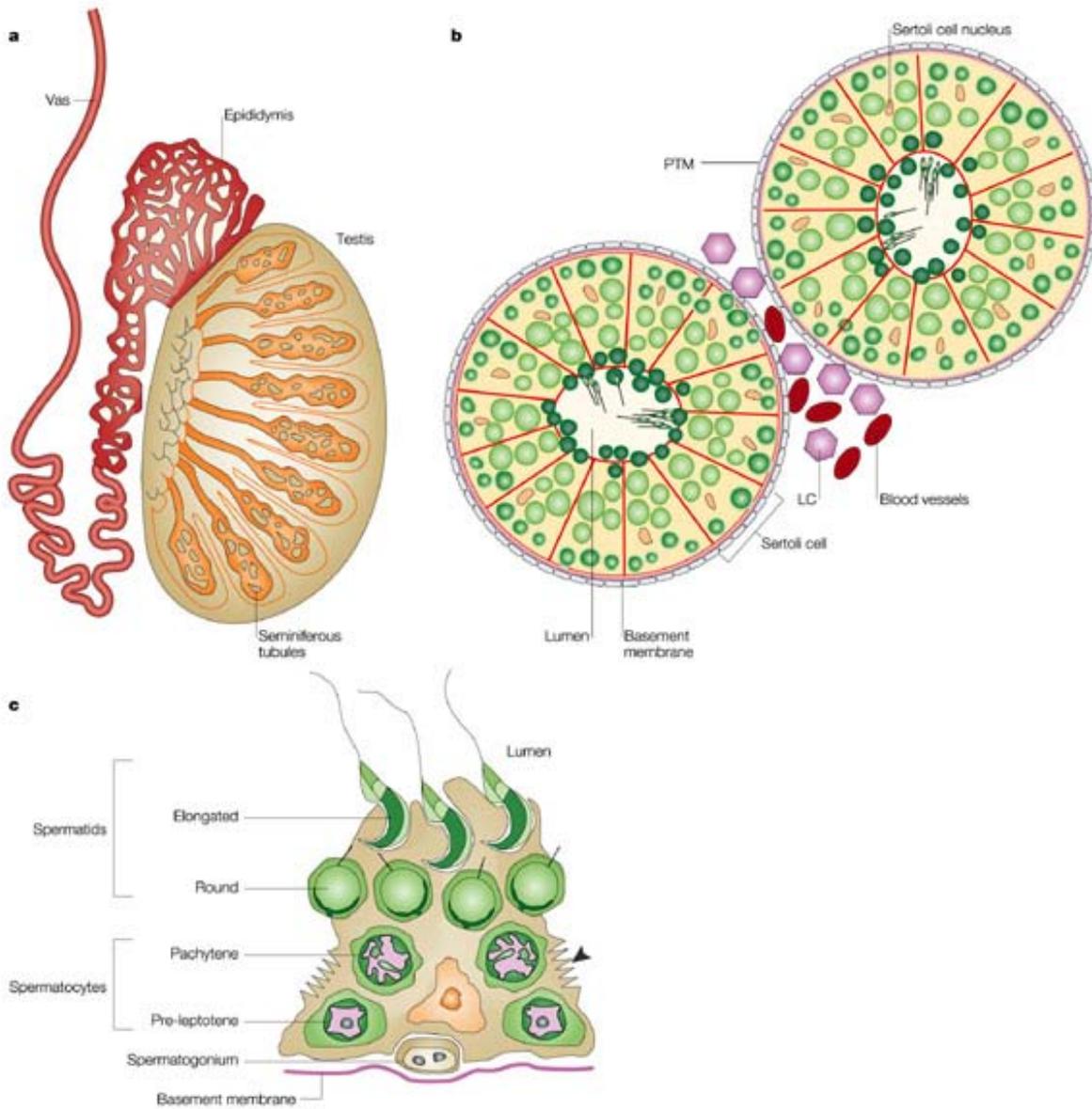


Figure 1. Organization of the Testis.

A) A cross-section through a testis, showing the location of the seminiferous tubules, the vas deferens and the epididymis. B) A diagrammatic cross-section through a testicular tubule, showing the germ cells (green) at different stages of maturation developing embedded in somatic Sertoli cells (each Sertoli cell is outlined in red). Leydig cells (LC), where testosterone is synthesized, are present in the interstitium. Maturing sperm are shown in the lumen of the tubules. PTM, peritubular myoid cell. C) A single Sertoli cell with its associated germ cells. Note that tight junctions between Sertoli cells (arrowhead) define two compartments: the stem cells and the pre-meiotic cells (spermatogonia) are found on one side of the junction, whereas the meiotic (spermatocytes) and the post-meiotic (round and elongating spermatids) cells are found organized in strict order of maturation towards the lumen (cytoplasm is shown in dark green, DNA is shown in pink, Sertoli cell nucleus is shown in orange). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics [7], Copyright 2002.

1.2 SPERMATOGENESIS

As spermatogonia enter meiosis, they move through the blood-testis barrier created by the Sertoli cells [1]. Germ cells migrate toward the center of the tubule as they progress through the stages of spermatogenesis and spermiogenesis. Spermatogenesis is the process during which diploid germ cells undergo meiosis in order to become haploid round spermatids [8]. The spermatids then undergo cytoplasmic reduction, nuclear condensation, and flagella formation in the process of spermiogenesis [8]. Mature spermatozoa are then released, through the process of spermiation, into the lumen of the seminiferous tubules [4, 8]. Once released, mature spermatozoa travel down the seminiferous tubules into the rete testes and then to the epididymis where sperm maturation is completed [1, 4].

The process of spermatogenesis is a complex and highly ordered process to produce mature spermatozoa from spermatogonia. Spermatogenesis occurs in a regular repeating pattern that can be arranged into stages. Each stage has a unique complement of developing germ cells associated with the Sertoli cells and each other at a given time. In the rat, the complement of germ cells can be classified into 14 stages (I through XIV) that create one cycle of the seminiferous epithelium [8]. Complete maturation of spermatozoa from spermatogonia requires 4.5 cycles of the seminiferous epithelium [8, 9]. Spermatogenesis requires a variety of hormonal signals in a stage-specific manner; therefore, multiple cycles allow proper hormonal signaling at key points in germ cell development [9]. T and follicle stimulating hormone (FSH) are the main regulators of spermatogenesis [1, 10]. Testicular T levels remain relatively constant throughout spermatogenesis but T actions are regulated through the availability of the androgen receptor (AR). T is essential for initiation and maintenance of spermatogenesis [11]. During spermatogenesis in the rat, AR levels in the Sertoli cell are maximal at stages VII-VIII after

which time the AR levels decrease [12, 13]. During stages VII and VIII, T is required for the meiotic progression from preleptotene to pachytene spermatocytes and to maintain step 8 and 9 round spermatids during spermiogenesis [14, 15]. T is not required for spermatogonial development [16]. FSH is necessary for quantitatively normal spermatogenesis [17]. FSH binding to Sertoli cells and FSH receptor (*Fshr*) mRNA in Sertoli cells is highest in stages XIII, XIV, and I of the seminiferous epithelium while FSH binding and *Fshr* mRNA levels are lowest in stages VII and VIII [16, 18-21]. The regulation of spermatogenesis by T and FSH has been extensively reviewed elsewhere [1, 16, 22].

1.3 SERTOLI CELL FUNCTION

Fully-differentiated Sertoli cells carry out a variety of functions within the testis. Sertoli cells maintain tight junctions with each other to sustain the blood-testis barrier [5]. Formation of the blood-testis barrier by mature Sertoli cells allows the secretion of tubular fluid that allows nourishment and transport of sperm throughout the rest of the excurrent duct system [23]. The blood-testis barrier functions to separate the seminiferous tubule into a basal and luminal compartment [5]. The basal compartment contains spermatogonia and is exposed to components from the blood supply including plasma proteins, blood-borne antibodies, and potentially harmful chemicals [24]. The luminal compartment contains germ cells undergoing the progressive stages of spermatogenesis.

The main function of Sertoli cells is to support and protect the developing germ cells. Sertoli cells provide key metabolites to developing germ cells in the form of inositol [25], pyruvate [26], lactate [26, 27]. Sertoli cells secrete several binding proteins for the transport of

key vitamins and ions to germ cells. These binding proteins include transferrin for iron [28], ceruloplasmin for copper [29], and retinoid binding protein for vitamin A [30]. Sertoli cells also secrete Kit ligand, which is necessary for germ cell viability and migration [31] as well as glial cell-derived neurotrophic factor that acts on spermatogonia to promote proliferation and development [32].

Sertoli cells maintain constant contact with developing germ cells through specialized junctions and are thought to aid in the shaping of developing germ cells [33]. The attachment of Sertoli cells to germ cells and the release of spermatozoa into the lumen of the seminiferous tubule is controlled through the actions of T through AR (reviewed in [34]). Sertoli cells also secrete plasminogen activators (serine proteases) in a stage-dependent manner to aid in germ cell morphological changes [33]. Excess cytoplasm that is shed by germ cells as residual bodies is phagocytosed by Sertoli cells. [35].

1.4 HORMONAL CONTROL OF SERTOLI CELL FUNCTIONS

Appropriate control of testicular function and spermatogenesis requires a tightly regulated feedback mechanism regulated by the hypothalamo-pituitary-testicular axis. Gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile manner from GnRH neurons within the hypothalamus [36, 37]. GnRH travels through the blood via a portal system to the pituitary where it can bind to GnRH receptors (GnRHR) on gonadotrophs within the anterior pituitary [4, 38]. Gonadotrophs are capable of secreting the gonadotropins, FSH and luteinizing hormone (LH) [4]. GnRH secreted at a high pulse frequency (30 minute intervals) stimulates LH and α gonadotropin subunit biosynthesis and secretion [39, 40]. *Lhb* and *Gnrhr* mRNAs can only

be induced when the amplitude of GnRH is between 10 and 75 ng/pulse [41-43]. GnRH secreted at a low pulse frequency (2 hour intervals) tends to favor FSH biosynthesis and secretion [39, 40]. *Fshb* mRNA increases with all pulse amplitudes of GnRH [44]. These gonadotropins are then carried by the blood to their site of action within the testis.

The site of FSH action within the testis has been localized to Sertoli cells [4]. One report suggests that FSHR is located on spermatogonia [45], but this result was not confirmed in other studies [18, 46]. FSH binding to FSHR can lead to the stimulation of adenylyl cyclase to increase cyclic adenosine monophosphate (cAMP) concentrations within the cell [4]. Prolonged FSH stimulation (≥ 24 hours) results in the down-regulation of FSHR, cAMP, and the up-regulation of phosphodiesterase inhibitors [4, 47-49]. FSHR down-regulation is discussed in greater detail in Chapter 8. Also, FSH binding to FSHR activates calcium channels within the cell membrane to up-regulate intracellular calcium concentrations [4, 50]. FSH stimulation leads to an increase in glucose transport [51] and the conversion of glucose to lactate [52], a source of energy for germ cells [4]. The specific genes and processes under the control of FSH to support developing germ cells are not fully understood.

LH is secreted in an episodic and pulsatile manner in accordance with GnRH secretions [4, 53]. The LH receptor (LHR) is found on Leydig cells within the testis [54] and is essential for the synthesis of T through the regulation of necessary steroidogenic enzymes [55]. An increase in pulse frequency of LH leads to an increase in T production [56]. Excess LH can act through negative feedback to down-regulate LHR on the surface of Leydig cells [54]. T can feedback to act at the level of the hypothalamus to decrease LH pulse frequency without changing the pulse amplitude [4].

Sertoli cells also secrete the hormones inhibin [57] and activin [58], which are also involved in the feedback control of hormone production in the pituitary [4, 59]. Inhibin decreases FSH production in pituitary, whereas activin can stimulate FSH production [4, 35, 59-61]. Inhibin down-regulates *Fshb* mRNA within 2 hours [61]. Inhibin does not regulate GnRH [4, 37]; however, inhibin has been suggested to act on GnRHR number [62]. In contrast, activin induces *Fshb* mRNA production [60, 61]. Additionally, estrogen produced by Sertoli cells during development or in Leydig cells in the adult may suppress *Fshb* gene transcription [63]. The ability of T to inhibit FSH secretion is difficult to distinguish from the effects of inhibin [4]. In rat, low doses of T inhibit *Fshb* through the suppression of GnRH release [43]. A high dose of T can activate *Fshb* through direct action on the pituitary [43]. This tightly regulated mechanism of hormonal feedback allows proper control of germ cell development.

1.5 PRENATAL SERTOLI CELL DIFFERENTIATION

Testis development begins in the early embryo. Development of the prenatal testis and Sertoli cell differentiation has been elucidated predominantly by studies in mice although similar events occur in other species albeit on a different time scale (Table 1) [64]. The genital ridge begins as a single layer of coelomic epithelium around 9-9.5 days post coitus (dpc) in mouse [65]. During this time (9-10 dpc), primordial germ cells migrate to colonize the urogenital ridge forming the bipotential gonad [66]. Sex determination in the male requires the expression of sex determining region Y (SRY) around 11 dpc to allow the pre-Sertoli cells to differentiate, proliferate, and aggregate with primordial germ cells [67, 68]. Pre-Sertoli cells originate from the coelomic epithelium between 11.2 and 12.5 dpc [69]. During the peak of *Sry* gene expression

[70], pre-Sertoli cells double in number. Pre-Sertoli cells produce anti-mullerian hormone (AMH) to inhibit Mullerian ducts, the precursors to the female reproductive tract (11-13 dpc) [71]. Mesonephric cells, pre-peritubular cells, migrate into the developing gonad and enclose pre-Sertoli cell and primordial germ cell aggregates to form seminiferous cords [72]. During the development of the seminiferous cords, pre-Sertoli and primordial germ cells cease to proliferate [64]. During cord formation, pre-Sertoli cells undergo morphological changes from a mesenchymal cell type to an epithelial cell type and must adhere to each other and the surrounding extracellular matrix [73, 74]. Immature Leydig cells differentiate and, in rat, steroidogenesis begins at approximately 14.5 dpc [75]. Steroidogenesis allows the production of testosterone to stabilize the Wolffian duct for further differentiation of the male excurrent duct system [76, 77]. Once cord formation is complete, pre-Sertoli cells again proliferate. Pre-Sertoli cell proliferation occurs at its greatest rate from 18 dpc to birth in the rat and continues until puberty (Figure 2) [78].

Table 1. Prenatal Testicular Developmental Time Points.

Species	Genital Ridge Formation	Bipotential Gonad Formation	Cord Formation	<i>Sry</i> expression
Human	5 weeks	6 weeks	7-8 weeks	7 weeks
Rat	10-11 dpc	11.5-12.5 dpc	13.5-14 dpc	12 dpc
Mouse	9-10 dpc	10-11.5 dpc	11.5-12.5 dpc	10.5 dpc

Adapted from A.S. Cupp and M.K. Skinner (2006) [64].

1.6 POSTNATAL SERTOLI CELL DIFFERENTIATION

1.6.1 Proliferation of Postnatal Sertoli Cells

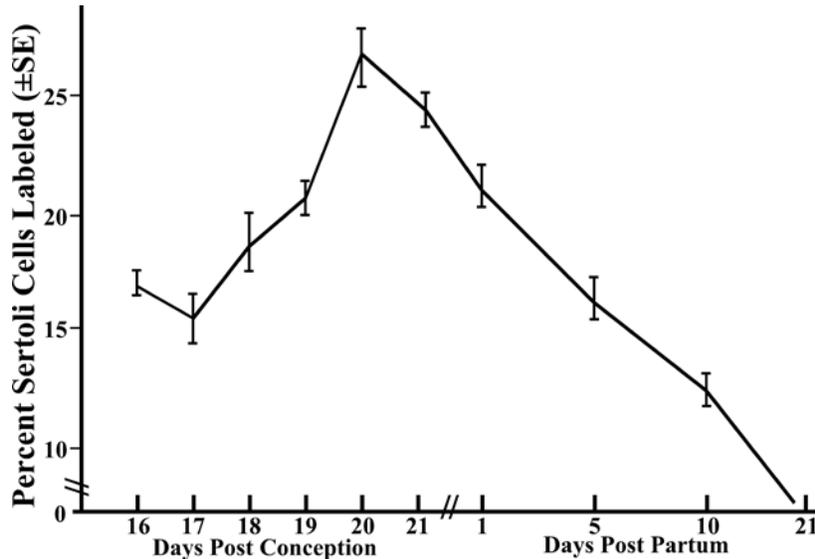


Figure 2. Proliferation of Sertoli Cells in Fetal and Postnatal Rats.

Graph is recreated from J. Orth (1982) [78] who determined the percentages of Sertoli cell nuclei that had incorporated ^3H -thymidine and were labeled in autoradiographs. Each point is a mean of six animals from two litters ($\pm\text{SE}$). Significant increases occurred prior to birth and decreased during the postnatal period. No labeled Sertoli cells were found on day 21 after birth.

Each Sertoli cell is only capable of supporting a finite number of germ cells and the number of Sertoli cells in the adult is established after puberty [79]. Thus, the final number of Sertoli cells sets the upper limit for testicular sperm production and influences the level of male fertility [1, 80]. The

number of Sertoli cells is primarily determined by the expansion of Sertoli cells prior to the completion of puberty [78, 81]. Apoptosis of Sertoli cells is rare [81]. In rodents, Sertoli cells begin to proliferate during fetal development, but after birth the rate of Sertoli cell proliferation decreases steadily in rats and mice from 5 to 15 days after birth with mitotic activity ceasing after days 15 to 21, depending on the strain studied (Figure 2) [78, 82-84]. The decrease in Sertoli cell proliferation during the differentiation period has been well documented in rats [78, 82, 85] and mice [86]. The percentage of thymidine labeled Sertoli cells is 33% at birth and 32% 5 days after birth in the rat, but decreases to 18% and 7% at 10 and 11 days, respectively (Table 2) [82]. By 16 days after birth in the rat, labeled Sertoli cells can no longer be detected [8, 82].

During the period in which proliferation ceases, the Sertoli cell undergoes a developmental process that results in a fully-differentiated, non-proliferating cell that is capable of supporting spermatogenesis.

Table 2. Percentage of Postnatal Rat Sertoli Cells with ³H-Thymidine Incorporation.

Age of Rat (Days)	% Labeled Sertoli cells	Age of Rat (Days)	% Labeled Sertoli cells
0-1	33 ± 5	11	7.0 ± 2
2	32 ± 4	12	4.0 ± 1
3	29 ± 6	13	3.5 ± 2
4	30 ± 6	14	2.5 ± 2
5	32 ± 5	15	1.0 ± 1
6	31 ± 6	16	0
7	23 ± 4	25	0
8	20 ± 3	35	0
9	17 ± 5	45	0
10	18 ± 4	60	0

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1.6.2 Hormonal Control of Postnatal Sertoli Cell Differentiation

Several hormones are involved in the onset of Sertoli cell differentiation. FSH induces Sertoli cell proliferation postnatally [87, 88]. FSH with activin stimulates the prepubertal proliferation of Sertoli cells and gonocyte proliferation [35, 89]. Estrogens are produced within Sertoli cells due to the expression of aromatase prepubertally, which may also function to inhibit prepubertal androgen production [35]. The expression of aromatase in Sertoli cells decreases during development although aromatase continues to be expressed by Leydig cells [35]. FSH can also inhibit estrogen production [90]. Despite the maintenance of FSH expression throughout puberty and into adulthood, Sertoli cells stop dividing [88]. FSH actions in fully-differentiated Sertoli cells are described in Chapter 8. T can suppress proliferation and induce differentiation in

Sertoli cells (reviewed in [87]). Thyroid hormone actions during Sertoli cell proliferation and differentiation are discussed in detail in Chapter 6.

1.6.3 Molecular Markers of Postnatal Sertoli Cell Differentiation

Once maturation of Sertoli cells ensues, the progression of differentiation can be characterized by the levels of proteins expressed at a given time. Genes expressed during the proliferative phase of Sertoli cell development include *Amh* and cytokeratin 18 (*Loc681147*) [91, 92]. AMH is expressed in premature Sertoli cells, starting 15 dpc and continues to be expressed until the onset of puberty [91, 93]. AMH expression declines as meiotic germ cells appear and as Sertoli cells gain increased androgen sensitivity [94-97]. FSH is thought to lead to the down-regulation of the *Amh* gene during puberty [35]. *Loc681147* mRNA is expressed beginning in fetal life and decreases throughout postnatal development, disappearing by 14 days after birth [98]. Differentiation markers for Sertoli cells include sex hormone binding globulin [SHBG, also known as androgen binding protein (ABP)], FSHR, AR, transferrin (TRF), and cell cycle inhibitors [99, 100]. Sertoli cells secrete SHBG that functions to maintain a high concentration of testosterone within the testis and throughout the rest of the excurrent duct system [101]. SHBG is present 14 days after birth and levels continue to rise until 40 days after birth when they plateau; however, the greatest increase in SHBG levels is between the second and third weeks after birth [102-104]. FSHR is present prior to birth but levels do not peak in Sertoli cells until the initiation of spermatogenesis, between 7 and 21 days after birth in the rat [18, 105-107]. AR is not detectable during the fetal or neonatal life of rats; however, Sertoli cells do express AR starting 4 to 8 days after birth, prior to the end of proliferation [13]. TRF, a common marker for Sertoli cell differentiation, is present 10 days after birth and its levels continue to rise into adulthood [28,

108]. Cell cycle inhibitors also inhibit proliferation within Sertoli cells; therefore, the onset of their expression coincides with the onset of differentiation [109]. In addition to components of the basement membrane and the blood-testis barrier, these proteins provide ideal molecular markers that can be utilized to study the progression of Sertoli cell development.

1.7 CONCLUSION

In the testis, Sertoli cells are the targets of hormonal signals that regulate spermatogenesis. Sertoli cells translate hormonal signals into the production of factors that are required by germ cells. The cytoplasm of a Sertoli cell surrounds germ cells during all stages of germ cell development and this close association allows nutrients and growth factors to be supplied by Sertoli cells to the germ cells. Sertoli cells only divide until the onset of puberty and then never divide again [1, 100]. Sertoli cell death is also rare. Therefore, after puberty, the number of Sertoli cells in the adult testis is fixed. Each Sertoli cell can support only a given number of germ cells [79]. Thus, the number of Sertoli cells establishes the total number of developing sperm that can be supported and the upper level of male fertility [1, 100]. The goal of this dissertation is to understand the mechanism by which Sertoli cells regulate the timing of their differentiation and establish their final number.

2.0 MODEL SYSTEMS TO STUDY SERTOLI CELLS

The choice of an appropriate research model, whether it is animal or cell based, can alter the application and relevance of the research. In studies of Sertoli cells, the appropriate model is dependent on the aspect of Sertoli cell physiology in question. Primate models are the closest animal model to humans. In primates and humans, Sertoli cells divide during the neonatal period and then proliferation slows in the absence of gonadotropins during the juvenile period [110]. Proliferation of Sertoli cells resumes during puberty when gonadotropin secretion increases to obtain the final number of Sertoli cells prior to differentiation [110]. However, primate models have challenges. First, the animals are expensive to raise and house. Secondly, primates have a long lifespan extending experiments over months or years.

Rodents have the advantages of growing rapidly, reproducing frequently, are easy to handle, and are relatively inexpensive. The most striking difference between the use of a rodent model and that of a primate is the absence of the juvenile hiatus of gonadotropin secretion in the rodent model [111, 112]. In rats, the neonatal, juvenile, and pubertal periods of development occur within a two week period in a relatively constant gonadotropin environment [112]. In rats, Sertoli cells divide throughout the postnatal period until approximately 15 to 20 days after birth [78, 82]. Rat testes descend into the scrotal sac approximately 15 days after birth, around the time of seminiferous tubule maturation [112]. In the rat, the first spermatozoa can be detected

between 40 and 45 days after birth [9]. The first mature sperm reach the vas deferens between 54 and 59 days after birth [113].

The onset of Sertoli cell differentiation and thus the onset of puberty occur in both primates and rodents in the presence of gonadotropins. Studies presented in this dissertation are concerned with establishment of the final number of Sertoli cells during the pubertal period when gonadotropins are present. Therefore, the rat is a suitable model for study of the onset of Sertoli cell differentiation because the endocrine background in both rat and primate are similar during this period of Sertoli cell development.

2.1 SERTOLI CELL CULTURES

Studying any cell outside of the organism can pose a challenge to recreating an artificial environment reflecting the cell's *in vivo* environment. However, the use of cell culture allows the study of direct actions of substances on the cells without interference of other cell types. Results from cultured Sertoli cells should be carefully interpreted as “what the cell is capable of doing” rather than what it is doing *in vivo* [114]. The development of defined culture media and specialized support matrices enable the study of cells in “*in vivo*”-like conditions.

The study of Sertoli cells has traditionally been performed using a variety of culture methods. Several isolation methods have been reviewed elsewhere [114-117]. First, the testes must be removed from the body and then be decapsulated to expose the seminiferous tubules. Next, the tubules are incubated in an enzyme solution to dissociate the cells of the interstitium from the tubules which are removed through differential sedimentation at unit gravity. The tubules are then incubated with a second enzymatic solution to disperse the cells of the

seminiferous tubules. Isolated cells are seeded on glass or plastic surfaces coated with an extracellular matrix component. Cells are maintained in a serum-free media containing essential vitamins, amino acids, and hormones. Hypotonic shock or incubation at 37 C can be used to remove germ cells from culture [118]. Cultured Sertoli cells can be maintained for several weeks but may lose responsiveness to known stimuli, such as T, after 6-10 days in culture due to the loss of expression of the Sertoli cell genes (i.e. *Ar*) with prolonged culture [114].

2.2 CAVEATS TO SERTOLI CELL CULTURE

Sertoli cell isolations present specific challenges. Because the Sertoli cell cytoplasm has many extensions to maintain contact with all stages of developing germ cells, it is common to get breakage of these cytoplasmic processes during isolation [119, 120]. The close association of Sertoli cells with germ cells also makes it difficult to obtain a pure culture of cells. As the animal ages to adulthood, more germ cells are present and the junctions between the germ cells and the Sertoli cells become more developed [121]. For this reason, most studies of cultured Sertoli cells involve cells isolated between 15 and 20 days after birth. At this age, the percentage of germ cells is minimal and the Sertoli cells have acquired many of their adult characteristics [116]. Some labs have developed methods to remove germ cells through the use of nylon mesh [122]. Another factor that may inhibit complete dissociation of Sertoli cells from one another are components of the blood-testis barrier. The tight junctions between Sertoli cells may result in isolation of clusters of Sertoli cells rather than a single cell suspension [121]. Despite these problems, Sertoli cells can be isolated regularly with 90% or greater purity from mature animals [122, 123].

Sertoli cells cultured on glass or plastic form a flat “dedifferentiated” morphology. In an attempt to maintain Sertoli cell structure in culture cells are normally maintained on a coating of extracellular matrix components (i.e. Matrigel) [115, 122]. Alternatively, bicameral chamber systems have been used to allow growth and polarization of Sertoli cells on a solid support while maintaining two different media conditions for the luminal and basolateral aspects of the Sertoli cell [115]. These methods are of particular use when studying Sertoli cell secretion or effects of pulsatile hormone release on the Sertoli cell. Any of these methods can be used to study Sertoli cell-germ cell interactions through co-culture of germ cells onto Sertoli cells [124]. Transillumination techniques were developed to study adult Sertoli cell-germ cell interactions specific to a stage of spermatogenesis [125, 126]. Seminiferous tubule fragments containing specific stages isolated from transillumination have also been used in explant cultures to maintain the tubule integrity [126]. To the author’s knowledge, only one group, Anway *et al.* (2003), has isolated Sertoli cells directly from an adult animal without culturing cells [127]. Using this method of isolation, an 80% pure population of adult Sertoli cells was obtained [127]. This method allows the study of Sertoli cells without changes that may occur in culture.

Studies of pre-Sertoli cells from the developing embryo [128] and pre-pubertal Sertoli cells are not as common as those from pubertal animals. Although similar methods are used, the number of cells obtained is more limited despite evidence that immature Sertoli cells are able to divide in culture [129]. A recent paper suggests that even adult Sertoli cells removed from the intact tubule result in the appearance of dividing cells in culture [130]. The predominant challenge with culturing Sertoli cells from neonate and immature rats is the lack of starting material due to the limited size of the undeveloped testis. As a result, a large number of animals must be used to obtain enough Sertoli cells to conduct biochemical assays.

2.3 ISOLATION OF SERTOLI CELLS FROM POSTNATAL RATS FOR IMMEDIATE BIOCHEMICAL ANALYSIS

To examine Sertoli cell biochemical properties as close to *in vivo* conditions as possible, enriched fractions of Sertoli cells were assayed immediately after isolation from the rat testes using a protocol adapted from Anway and colleagues [127]. Sertoli cells were isolated from the testes of 5 and 11 day-old rats representative of proliferating and differentiating Sertoli cells, respectively. Using this protocol, approximately 1.7×10^7 cells were obtained from ten 5 day-old rat pups and 9.0×10^7 cells from ten 11 day-old rat pups. Imaging analysis confirmed that the isolation procedure resulted in highly enriched Sertoli cell fractions (Figure 3A). Approximately $85 \pm 2\%$ of the cells were positive for the Sertoli cell-specific marker vimentin (Figure 3B). Approximately $9 \pm 3\%$ were positive for the peritubular cell marker α -smooth muscle actin and $2.5 \pm 2.0\%$ to $4.3 \pm 3.1\%$ (5 and 11 days after birth respectively) stained positive for the Leydig cell marker Oil Red O (Figure 3B). The remaining $1.5 \pm 1.7\%$ of cells were not labeled and are most likely immature germ cells.

To facilitate studies of factors that may regulate Sertoli cell differentiation, Sertoli cells from 5 and 11 day-old rats were studied after being placed in culture. In addition, fully-differentiated Sertoli cells from 20 day-old rats were cultured. By using a gentler isolation procedure, lacking a hypotonic shock, and maintaining the Sertoli cells in culture for three days prior to manipulation, cell survival was increased compared to the Anway protocol [127]. Culture of the Sertoli cells for three days allows the detachment of germ cells from Sertoli cells, resulting in a purer population of Sertoli cells. Previous studies have shown that Sertoli cell populations are greater than 95% pure four days after initiating the cultures [123].

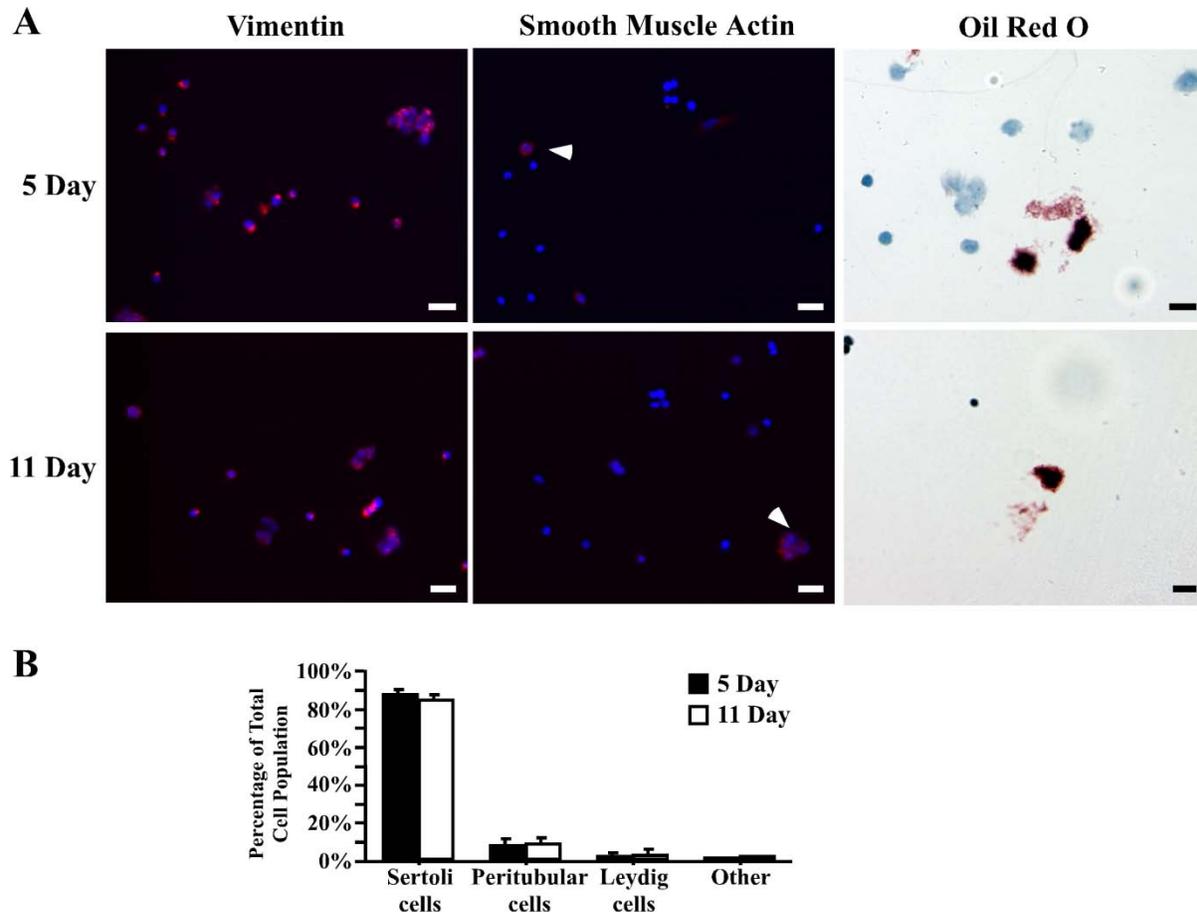


Figure 3. Enriched Fractions of Sertoli Cells Obtained Immediately after Isolation.

Cell suspensions obtained immediately after enzymatic digestion of rat testes were cultured on coverslips for 2 h and then fixed. A) Sertoli cells were detected using antisera against vimentin (red, left column), peritubular cells were detected by antisera against alpha smooth muscle actin (red, white arrows, middle column), and nuclei were stained with Hoechst dye (blue). Leydig cells were stained with the lipid stain Oil Red O (red stain, right column). Bar = 20 μ m. B) Quantitation of the mean (\pm SE) percentage of cell-specific markers of 10 random fields from multiple coverslips is provided.

2.4 CONCLUSION

Studies in rat Sertoli cells are an appropriate model for the studies conducted in this dissertation because the establishment of the final number of Sertoli cells during the pubertal period occurs in both rats and primates when gonadotropins are present to promote the onset of puberty. To study processes most closely to the *in vivo* situation, Sertoli cells will be assayed

immediately after isolation using a modified protocol from Anway, *et al.* [127]. Additionally, cultured Sertoli cells will be used for studies whose goal is to determine protein localization or for studies investigating hormonal regulators of Sertoli cell differentiation. Sertoli cells will be isolated from 5, 11, and 20 day-old rats, representative of proliferating, differentiating, and differentiated cells, respectively.

3.0 BASIC HELIX-LOOP-HELIX PROTEINS: IDENTIFICATION OF USF1 AND USF2 AS POTENTIAL MEDIATORS OF SERTOLI CELL DIFFERENTIATION

Hypothesis: The DNA binding of bHLH proteins increases during Sertoli cell differentiation.

3.1 INTRODUCTION

3.1.1 Basic Helix-Loop-Helix Proteins

Basic helix-loop-helix (bHLH) proteins function in cell differentiation, growth control, and transcription regulation (reviewed in [131]). bHLH proteins are evolutionarily conserved transcription factors that contain a basic DNA binding motif and a helix-loop-helix protein binding motif [132, 133]. The HLH domain contains two amphipathic helices with conserved hydrophobic residues on one side of the helix to facilitate dimerization between bHLH proteins [132]. A loop between the two helices is essential for proper spacing between the helical domains [132, 134-136]. Mutation of hydrophobic residues or substitution of helical domains from other bHLH proteins can eliminate proper dimer formation [133]. The bHLH transcription factors bind to specific DNA sequence motifs known as E-boxes (CANNTG) [137, 138] and closely related N boxes (CACNAG) [139, 140]. Flanking DNA sequences on either side of the consensus sites confirm specificity among proteins that share binding sites [131]. Conservation

of arginines within the DNA binding domain of E-box proteins is critical for proper interaction with the major groove of the DNA [135]. Contacts with the phosphate backbone also stabilize the interactions of the dimer with the DNA [132, 134-136]. Although bHLH proteins bind to E-boxes with relatively low affinity, they act with great biological specificity suggesting a need for additional regulators to facilitate their actions. Several bHLH proteins contain additional protein binding motifs that are always positioned carboxy-terminal to the HLH portion of the protein (reviewed in [141]).

bHLH proteins were originally classified according to their functions [131] (Table 3). Group I contained ubiquitously expressed proteins that could form homodimers or heterodimers with those members in the tissue-restricted Group II [131]. Group I proteins included transcription factor E2A (TCFE2A; E12 and E47) [142, 143] and transcription factor 4 (TCF4) [144] which are involved in B cell-, muscle-, and pancreatic-specific gene regulation. Another

Table 3. Classification of bHLH Proteins.

Phylogenetic Group	Preferential Binding Site	Additional Protein Interaction Domains	Original Classification	Examples of Classified Proteins
A	CAGCTG or CACCTG		I, II	MYOD1 , Twist, Net, TCFE2A (E12 and E47), TCF4, HEB, daughterless, myogenin, MYF5
B	CACGTG or CATGTTG	bZIP	III, IV	MAX, MXD1, USF1, USF2, TFE3, MYC, TFEB
C	ACGTG or GCGTG	PAS		Arnt, HIF, Clock, single-minded
D	Lack a basic domain and hence do not bind DNA		V	ID, emc
E	CACGCG or CACGAG (N-box sequences)	Orange domain, WRPW peptide	VI	Hairy, enhancer of split
F		COE domain		Coe (Col/Olf-1/EBF)

Adapted from C. Murre, *et al.* (1994) [131] and S. Jones (2004) [141].

member, daughterless, is involved in sex determination and neurogenesis in *Drosophila melanogaster* [145-147]. Group II proteins included myogenic differentiation 1 (MYOD1), myogenin, and myogenic factor 5 (MYF5), all involved in muscle development [148-150], and the achaete-scute complex of *Drosophila*, which is involved in formation of peripheral nerves [151, 152]. Group III contained myelocytomatosis oncogene (MYC)-related proteins involved in growth control: neuroblastoma derived MYC (MYCN), lung carcinoma derived MYC (MYCL1), transcription factor binding to immunoglobulin heavy chain enhancer 3 (TFE3), and transcription factor EB (TF-EB) [153]. Group IV contained the MYC interactors MYC-associated factor X (MAX) and MAX dimerization protein 1 (MXD1) [154]. Members of Group V lacked a basic DNA binding domain and included inhibitor of DNA binding (ID) proteins [155] and the *Drosophila* extra macrochaetae (emc) protein [156, 157] whereas Group VI members contained proline residues within their basic DNA binding domain (hairy, enhancer of split) [139, 158].

The bHLH proteins were reclassified according to evolutionary conservation of the genetic sequence and accounted for the consensus sequence bound by the proteins as well as any additional protein dimerization domains that may be present (reviewed in [141]) (Table 3). Class A contains proteins bind to CAGCTG or CACCTG motifs and comprise of members from the old Groups I and II [159]. Class B contains proteins that bind CACGTG or CATGTTG motifs and are comprised of members from the old groups III and IV [159]. Many members of Class B, including upstream stimulatory factors 1 and 2 (USF1 and USF2), also contain additional leucine zipper motifs [159, 160]. Class C members bind to ACGTG or GCGTG domains and contain an additional Per/Arnt/Sim (PAS) protein binding domain [160]. This class includes the proteins single-minded, Arnt, Hypoxia inducible factor (HIF), and Clock [159, 160]. Class D members

lack a basic DNA binding domain and include the old Group V members [159]. Class E members bind to closely related N-boxes, CACGCG or CACGAG [160]. Additionally, Class E proteins contain an orange protein binding domain and a WRPW peptide [160]. The members of this group originally fell into Group VI; as such they contain proline residues in their DNA binding motif. The final class, Class F, contain proteins with a COE (Col/Olf-1/EBF) domain that is involved in dimerization and DNA binding [160]. This currently accepted bHLH classification allows room for newly discovered proteins and is not restrictive to new members based on location or function of the protein.

3.1.2 Basic Helix-Loop-Helix Proteins in Sertoli Cells

The bHLH transcription factors previously shown to be expressed in Sertoli cells include TCF4 (also known as ITF2 or E2-2)[161], scleraxis (SCX) [162], USF1, USF2 [163], REB α (the rat homologue of the human HeLa E-box binding protein, HEB (TCF12)) [164], and E47 but not E12 proteins that are derived from the *Tcfe2a* gene (formerly known as E2A) [165]. Additionally, ID proteins, members of Class D, are also expressed within Sertoli cells and will be discussed in Chapter 5 [166]. Class A bHLH proteins bind to CAGCTG motifs and contain E47, ITF2, and REB α [167, 168]. The E47 E-box protein has been implicated in numerous activities in Sertoli cells, including FSH-induced regulation of *Trf* and the influencing of hormone-induced activation of the *Fos* gene promoter through the serum response element [168, 169]. TRF is a marker of Sertoli cell differentiation and is essential for differentiated Sertoli cell function. Class B proteins include USF1, USF2, and MYC and bind to CACGTG motifs [170]. Class B E-box motifs are known to regulate the promoters of the steroidogenic factor 1 (*Nr5a1*) and the *Fshr* genes [109, 171-173]. Both of these genes are critical for Sertoli cell differentiation; however,

only *Fshr* is Sertoli cell-specific. Due to the importance of transferrin and FSHR expression within differentiated Sertoli cells, consensus binding sequences for both the Class A and Class B bHLH proteins were used to study the activities of the bHLH proteins during postnatal Sertoli cell differentiation.

3.2 RESULTS

3.2.1 DNA Binding of the E47 E-box Protein Is Low in 5 and 11 Day-old Sertoli Cells

To investigate the binding activity of E47 and the related E12 E-box factors in Sertoli cells during differentiation, electrophoretic mobility shift assays (EMSA) were performed. Nuclear extracts from Sertoli cells immediately after isolation from 5 and 11 day-old rats were incubated with probes that were previously shown to bind E47, including a consensus binding site for E47 (Figure 4A), the *Trf* promoter E-box (Figure 4B), or the E-box region from the *Id2* promoter (Figure 4C) [168, 169, 174]. Incubation of the E47-selective probes with Sertoli cell nuclear extracts did not result in detection of any DNA-protein complexes. In contrast, all the probes formed complexes with nuclear extracts derived from splenic origin CD19⁺ B cells, activated with lipopolysaccharide, that are known to contain high levels of E47 and E12 binding activity (Figure 4A-D) [132, 144, 175]. In addition, E47 was over-expressed in COS7 cells and incubation of these extracts with a probe containing the E-box region of the *Trf* promoter resulted in a DNA-protein complex (Figure 4E, lane 2). The DNA-protein complex formed with the transfected COS7 cells was disrupted by an anti-E47 antibody (Figure 4E, lane 4). The protein binding activity of the Sertoli nuclear extracts was confirmed by the finding that DNA-

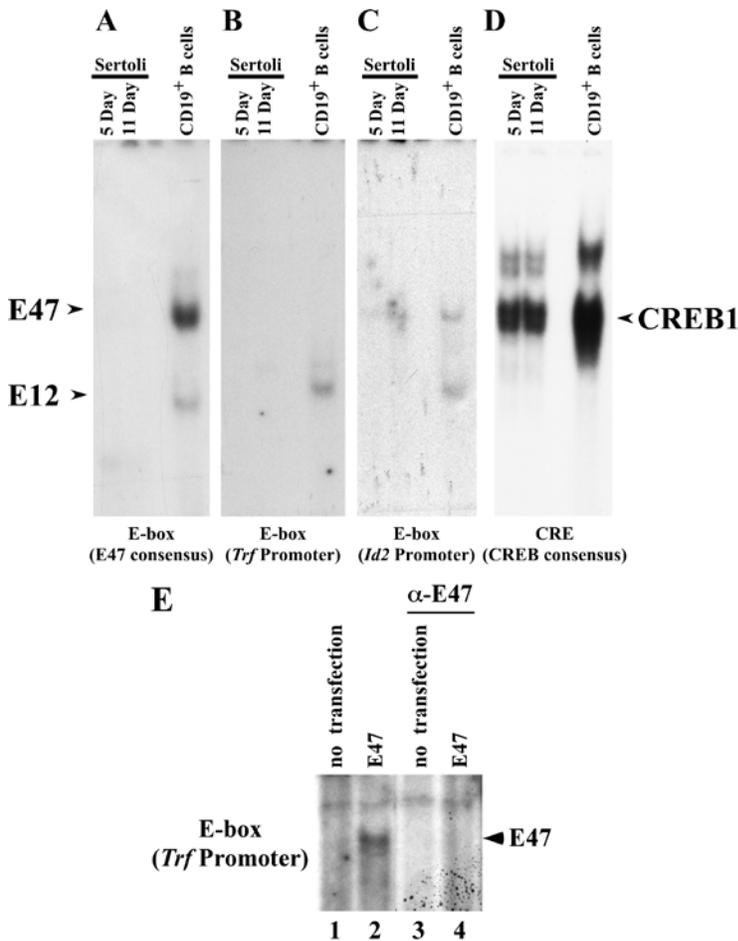


Figure 4. E47 DNA Binding is Not Detected in Proliferating or Differentiating Sertoli Cells.

EMSAs using probes containing a consensus E-box selective for E47 binding (A), the *Trf* promoter E-box (B), the *Id2* promoter E-box (C), or a CREB-binding site (D) were incubated with nuclear extracts from Sertoli cells assayed immediately after isolation from 5 and 11 day-old testis or with CD19⁺ B cells isolated from mouse spleen. The DNA-protein complexes formed by E47, E12, and CREB1 are indicated. The complexes were identified from the results of separate supershift assays using E47- and E12-selective antisera (data not shown). Assays for E47-selective binding probes were exposed for 7 days, while CREB1 binding complexes were detected after 12 h. (E) EMSAs using a probe containing the E-box region of the *Trf* promoter was incubated with 5 mg of COS7 cell extracts without (lanes 1 and 3) or with (lanes 2 and 4) over-expressed E47. Supershift analysis indicated that the detected band was E47 (lanes 3 and 4). Free probe was run off of the gels.

MYC, EMSA studies were again conducted with nuclear extracts of Sertoli cells assayed immediately after isolation from 5 and 11 day-old rats. For probes containing either a consensus USF binding site or a region of the *Fshr* promoter containing an E-box known to bind USF [163,

protein complexes were formed using a probe containing a binding site for the CREB1 transcription factor (Figure 4D). CREB binding is generally stable under most conditions and is not altered during differentiation [176]. Together, these studies indicate that E47 binding activity is below detectable limits in the nuclei of 5 and 11 day-old Sertoli cells.

3.2.2 The DNA Binding of USF1 and USF2 Increase During Sertoli Cell Differentiation

To investigate the binding of Class B E-box factors, including USF1, USF2, and

177], protein binding was low or undetectable in extracts from 5 day-old Sertoli cells but binding activity increased at least 2-fold at 11 days (Figure 5A). As expected, CREB1 binding to a consensus CREB binding motif (CRE) was unchanged at 5 and 11 days (Figure 5A).

To identify E-box binding proteins that are responsible for forming the DNA-protein complexes, supershift studies were performed. Sertoli cell nuclear extracts assayed immediately after isolation from 11 day-old testes were preincubated with non-immune sera or antisera against USF1, USF2, E47, E2A, or MYC before incubation with the E-box region of the *Fshr* promoter (Figure 5B). The supershift assay demonstrated that both USF antisera partially supershifted and disrupted DNA-protein complexes, whereas the E47, E2A, and MYC antisera had little effect. These data suggest that USF1 and USF2 proteins are the major E-box factors

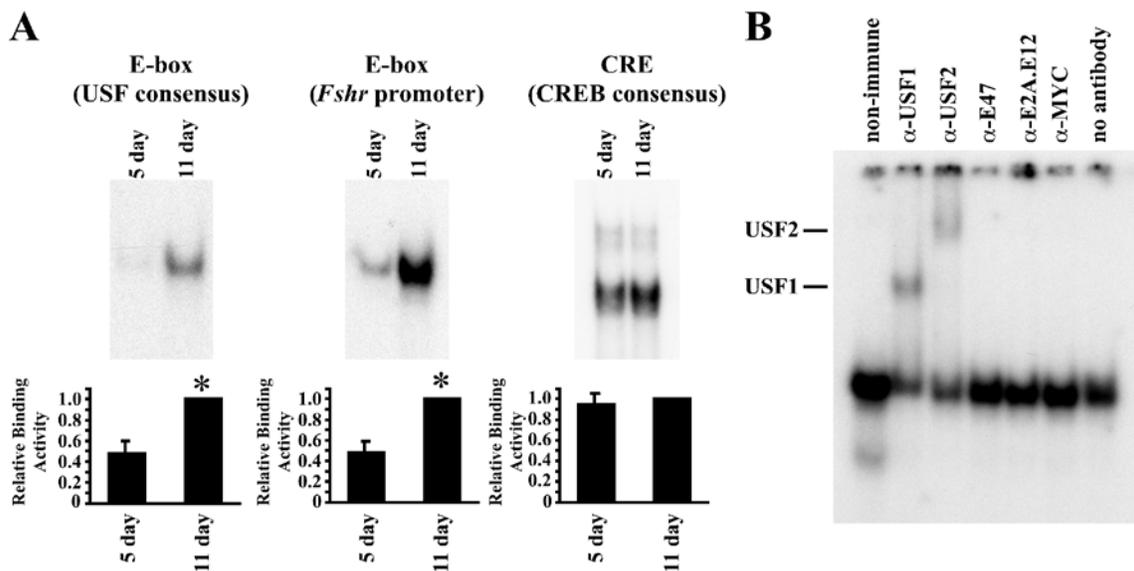


Figure 5. USF DNA Binding Increases During Differentiation in Nuclear Extracts Prepared from Sertoli Cells Immediately After Isolation.

Nuclear extracts isolated from Sertoli cells were immediately assayed for DNA-protein interactions. A) In EMSAs, radiolabeled probes containing 1) a consensus E-box that is selective for USF binding, 2) a region of the *Fshr* promoter containing an E-box known to bind USF proteins, or 3) a CREB-binding site (CRE) were incubated with Sertoli cell nuclear extracts from 5 and 11 day-old rats. Representative images of the resulting DNA-protein complexes are shown, and quantitation of the mean (\pm SE) of five independent experiments is provided. The relative binding for each probe is normalized to that of 11 day-old Sertoli cells (=1). Statistically significant differences ($p < 0.05$) are indicated by an asterisk (*). B) Nuclear extracts of enriched Sertoli cells from 11 day-old rats were incubated with non-immune sera or antisera against USF1, USF2, E47, E2A, or MYC. Supershifted DNA-protein complexes containing USF1 or USF2 are indicated. Exposure times for all complexes are less than 48 h. Free probe was run off of the gels.

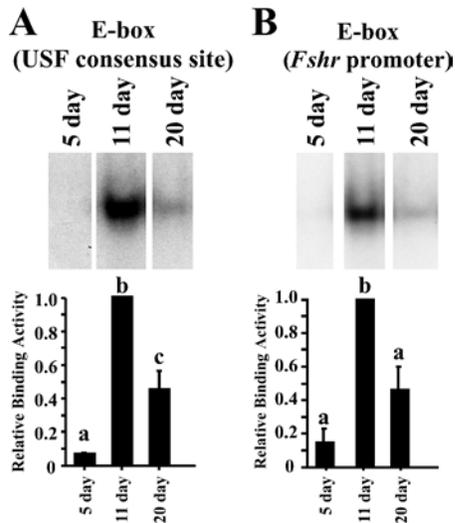


Figure 6. USF DNA Binding Increases in Cultured Sertoli Cells During Differentiation.

In EMSAs, radiolabeled probes containing a consensus E-box that is selective for USF binding (A) or a region of the *Fshr* promoter containing an E-box known to bind USF proteins (B) were incubated with nuclear extracts isolated from cultured 5, 11, and 20 day-old Sertoli cells. Representative images of the resulting DNA protein complexes are shown, and quantitation of the mean (\pm SE) of three independent experiments is provided. Free probe was run off the gel. All bands shown for each age were resolved on the same gel. The relative binding for each condition is normalized to that of 11 day-old Sertoli cells ($=1$). Values with different lowercase letters differ significantly ($p < 0.05$).

promoter E-box probes respectively. In agreement with the results obtained from Sertoli cells assayed immediately after isolation (Figure 4A-C), E47 DNA binding was below the level of detection using three E47 selective probes and extracts from cultured 5, 11, or 20 day-old Sertoli cells (data not shown). Together, these data confirm that the developmental profile of E-box protein binding activities and the prevalence of USF binding activity are similar in Sertoli cells assayed immediately after isolation and after three days in culture.

that account for increased binding to E-box motifs during Sertoli cell differentiation.

3.2.3 USF DNA Binding is Similar in Nuclear Extracts Prepared from Sertoli Cells Immediately After Isolation or from Cultured Sertoli Cells

EMSA analysis of nuclear extracts of cultured Sertoli cells isolated from 5 and 11 day-old rats revealed that the levels of E-box protein binding to the USF consensus and *Fshr* promoter probe were low at 5 days but increased 5- to 10-fold at 11 days (Figure 6A, B). E-box binding activity decreased at 20 days but was still 6.98 ± 0.13 ($p < 0.05$)- or 2.78 ± 0.17 -fold greater than the E-box binding activity present in 5 day-old Sertoli cells at the USF consensus and *Fshr*

3.3 CONCLUSIONS

The DNA binding of bHLH proteins was hypothesized to increase during Sertoli cell differentiation because E-box proteins are known to contribute to differentiation in other cell types and had previously been shown to regulate genes important for Sertoli cell differentiated functions [163, 167, 168, 177-179]. The bHLH protein E47 was previously shown to be important in the regulation of *Trf* and *Id2* genes in Sertoli cells [167, 168, 178]. However, E47 binding to E-box motifs was undetectable between 5 and 11 days after birth in the rat (Figure 4 A-C). The inability to detect E47 binding at any of three E47-selective E-box probes agrees with results from Skinner and colleagues in which E47 binding to the *Trf* promoter E-box was not observed except after stimulation for 48 hours by FSH in 20 day-old rat Sertoli cells [168]. In contrast, binding of USF1 and USF2, key regulators of the *Fshr* gene, increases to E-box motifs between 5 and 11 days after birth (Figure 5A-B). These results are consistent with those of the Heckert laboratory that found USF proteins bound to the *Fshr* promoter E-box in fully-differentiated rat Sertoli cells (27 or 50 days-old) [163, 177, 179]. The ability of USF1 or USF2 antisera to supershift the protein binding complex at the E-box in the *Fshr* promoter is incomplete, but when both antisera are incubated with 20 day-old Sertoli cell extracts, the complex formation is completely ablated [179]. This result supports the idea that both USF1 and USF2 are present in the DNA protein complex present at the *Fshr* promoter E-box. The binding activity of E47, USF1, and USF2 is similar in both Sertoli cells assayed immediately after isolation and in cultured Sertoli cells (Figure 5 vs. Figure 6). In cultured Sertoli cells, USF1 and USF2 binding decreased between 11 and 20 days after birth to levels at or above the levels of binding seen from 5 day-old cells (Figure 6). Together, these data indicate that the binding of USF1 and USF2, but not E47, E-box proteins increases during Sertoli cell differentiation,

supporting the hypothesis and identifying USF1 and USF2 as potential regulators of Sertoli cell differentiation.

The findings that USF proteins are the predominant E-box transcription factors that are able to bind E-box motifs during Sertoli cell differentiation and that USF proteins are the major E-box regulators of the *Fshr* is consistent with previous studies showing that male fertility is compromised in the absence of FSHR and USF2 [180-182]. *Fshr*^{-/-} mice are fertile but present with a smaller testis weight by 20 days after birth through adulthood due to a reduced number of Sertoli cells when compared with wild-type littermates [180, 181]. *Usf2*^{-/-} mice have a distinct growth defect and males have compromised fertility as only one in three males are able to sire a litter [182]. Further analysis of the *Usf2*^{-/-} mice to determine the male fertility deficiency has not been conducted.

The data presented here suggest that between 5 and 11 days after birth the DNA binding of USF proteins increases. It is assumed that the increase in USF DNA binding will promote transcription of genes regulated by USF1 and USF2. Therefore, USF proteins may integrate regulatory signals to ensure that the correct differentiation-associated genes are expressed [163, 177, 179] in a time-course consistent with the onset of differentiation in Sertoli cells. Presently, the molecular mechanisms of USF1 and USF2 regulation in Sertoli cells are unknown.

4.0 EXPRESSION OF USF1 AND USF2 WITHIN PROLIFERATING AND DIFFERENTIATING SERTOLI CELLS

Hypothesis: USF1 and USF2 mRNA and protein expression levels increase during differentiation and correspond with increased USF DNA binding at E-box motifs.

4.1 INTRODUCTION

4.1.1 USF1 and USF2: Properties of the Genes and Proteins

USF proteins were originally identified as regulators of the adenoviral major late promoter (MLP). These 43 and 44 kilodalton (kD) proteins, USF1 and USF2 respectively, were found to bind to the MLP equally well and at an optimum of 75 mM KCl [183]. The 44 kD USF2 was originally thought to be a splice variant or regulator of the 43 kD USF1 form as the two proteins were immunologically distinct but were routinely associated with one another [184]. Purified 43 kD USF was found to reside in solution as a dimer and was efficient in inducing MLP activity in the absence of the 44 kD form [184].

E	Human	MDMLDPGLDPAASATAAAAASHDKGPEAEEGVELQEGGDGPGAEEQTAVAITSVQQA AFG 60
	Rat	MDMLDPGLDPASSATAAAAASHDKGPEAEEGVELQEGGDGPGAEEQTAVAIASVQQA AFG 60
	Mouse	MDMLDPGLDPASSATAAAAASHDKGPEAEEGVELQEGGDGPGAEEQTAVAIASVQQA AFG 60 *****:*****:*****
Human	DHNIQYQFRTE TNGGQV TYRVVQV TDGQLD GQGD TAGAVSVVSTAAFAGGQQAVTQVGV D 120	
Rat	DHNIQYQFRTE SNGGQV TYRVVQV TDGQLD GQGD AAGAVSVVSTAAFAGGQQAVTQVGV D 120	
Mouse	DHNIQYQFRTE SNGGQV TYRVVQV TDGQLD GQGD AAGAVSVVSTAAFAGGQQAVTQVGV D 120 *****:*****:*****	
Human	GAAQRPGPAAASVPPGPAAPFLAVIQNPF SNGGSPAAEAVSGEARFAYFPASSVGD TTA 180	
Rat	GAAQRPGPAAASVPTGPAAPFLAVIQNPF SNGGSPAAEAVSGEARFAYFPASSVGD TTA 180	
Mouse	GAAQRPGPAAASVPTGPAAPFLAVIQNPF SNGGSPAAEAVSGEARFAYFPASSVGD TTA 180 *****.*****	
Human	VSVQTTDQSLQAGGQFYVMMTPQDVLQ TGTQRTIAPRTHPYSPKIDGTRTPRDE RRRRAQH 240	
Rat	VSVQTTDQSLQAGGQFYVMMTPQDVLQ TGTQRTIAPRTHPYSPKIDGTRTPRDE RRRRAQH 240	
Mouse	VSVQTTDQSLQAGGQFYVMMTPQDVLQ TGTQRTIAPRTHPYSPKIDGTRTPRDE RRRRAQH 240 *****	
		Basic
Human	NEVERRRRDKINNWIVQLSKIIPDCNADNSKTGASKGGILSKACDYIRELRQTNQRMQET 300	
Rat	NEVERRRRDKINNWIVQLSKIIPDCHADNSKTGASKGGILSKACDYIRELRQTNQRMQET 300	
Mouse	NEVERRRRDKINNWIVQLSKIIPDCHADNSKTGASKGGILSKACDYIRELRQTNQRMQET 300 *****:*****	
		Helix-Loop-Helix
Human	FKEAEFLQMDNELLRQQIEELKNENALLRAQLQQH NLEMVGE GTRQ 346	
Rat	FKEAEFLQMDNELLRQQIEELKNENALLRAQLQQH NLEMVGE STRQ 346	
Mouse	FKEAEFLQMDNELLRQQIEELKNENALLRAQLQQH NLEMVGE STRQ 346 *****.***	
		Leucine Zipper

Crystallization of the USF dimer by Ferre-D'Amare *et al.* [135] revealed detailed information on the formation of the dimer and protein-DNA contacts (Figure 7A). USF contains three functional domains: 1) the basic DNA binding domain, 2) the helix-loop-helix (HLH) domain, and 3) the leucine zipper (ZIP). The basic DNA binding domain specifically interacts with the DNA consensus sequence, GGCCACGTGACC [135, 186]. The HLH motif and loop domain mediates protein-protein interactions and stabilizing the homodimer's DNA binding domain [135]. The presence of the leucine zipper is required for efficient DNA binding to the recognition motif [141, 186]. USF binding to DNA does not induce DNA bending, but the presence of the leucine zippers allows USF to function as a homotetramer that can simultaneously bind two spatially distinct DNA recognition sites, allowing for chromatin looping [135]. Activation of transcription by USF was found to require the HLH-ZIP domain and a small region of 43 kD USF close to the N-terminus, amino acids 93-156 [187].

With the cloning of the gene for the 44 kD USF2 protein in the 1990s, comparisons between the genes and distinct functions of USF1 and USF2 could be ascertained (Figure 7B) [188, 189]. Both genes are closely related and the USF1 and USF2 proteins are highly conserved between mouse, rat, monkey, and human (Figure 7D,E). The structure of the genes are such that small introns separate exons that correspond to functional domains of the proteins (Figure 7C) [190, 191]. Both *Usf1* and *Usf2* lack a TATA box motif, however, an initiator sequence is present in the promoters for both genes [170, 191, 192]. The *Usf1* promoter has regulatory binding sites for the transcription factor 1 SP1 (SP1), GATA factors, YY1 transcription factor, neurofibromatosis 1 (NF1), CCAAT/enhancer binding protein (C/EBP), and transcription factor AP-2 whereas *Usf2* contains two E-box motifs within its promoter suggestive of auto-feedback [191, 192]. Both *Usf1* and *Usf2* are expressed ubiquitously although the ratio of *Usf1* to *Usf2* varies between cell types [193]. *Usf1* is not thought to have functional alternative transcripts, whereas *Usf2* encodes two transcripts due to alternative splicing and alternative poly(A) site usage [190, 191, 193]. The alternatively spliced USF2 (USF2b) lacks exon 4 that encodes a positive regulatory domain and fails to induce transactivation. As such, USF2b can act as a dominant negative dimerization partner [170]. USF1 and USF2 have highly conserved dimerization and DNA binding motifs while varying at the N-terminus [193]. This high level of conservation allows the two proteins to have similar DNA binding properties [193]. The conserved nature of USF1 and USF2 has made it difficult to distinguish between specific actions of each protein.

4.1.2 *Usf* Knockout Mice Suggest an Involvement of USF Proteins in Male Fertility

The Sawadogo laboratory created *Usf* knockout mice to determine the biological role of USF1 and USF2 [182]. *Usf1*^{-/-} mice were viable and fertile. These mice had only slight behavioral abnormalities with excessive trimming behavior resulting in mice lacking whiskers and nasal fur. These mice displayed a dose-dependent elevation of *Usf2* with heterozygotes expressing 24% more *Usf2* and homozygotes expressing 66% more. *Usf1*^{-/-} mice, especially females, had spontaneous epileptic seizures suggesting a role in brain function. *Usf2*^{-/-} mice had an obvious growth defect as these mice were routinely 20-40% smaller than their wild-type or heterozygote littermates. A large percentage of *Usf2*^{-/-} pups died shortly after birth. The mice that survived maintained a small stature throughout postnatal development. The fertility of *Usf2*^{-/-} males was affected with only one third of males able to sire a litter with wild-type females. The males also had a shortened lifespan on average of 2.5-4.5 months and no males have lived longer than 6 months. *Usf1*^{-/-}*Usf2*^{-/-} and *Usf1*^{+/-}*Usf2*^{-/-} are embryonic lethal. These phenotypes suggest that whereas USF2 can compensate for most of USF1's functions, USF1 is unable to compensate for USF2's effects on growth, male fertility, and male lifespan.

4.1.3 USF1 and USF2: Ubiquitous Transcription Factors

USF1 and USF2 are ubiquitously expressed transcription factors. USF binding to DNA recruits general transcription complexes and can enhance the transactivation by SP1 transcription factor and metal regulatory transcription factor-1 [194-196]. Also, USF binding to DNA can recruit chromatin modifying enzymes. USF proteins preferentially interact with nucleosomes containing highly acetylated histone 4 and USF1 is present in complexes with histone

acetyltransferases, histone acetylases, and histone methylases [197, 198]. Additionally, USF2, but not USF1, is capable of initiating transcription of human *Top3a* (topoisomerase DNA III α) contributing to the removal of supercoils within chromatin strands [199]. Finally, USF1 and USF2 have been implicated in cell cycle regulation. USF1 and USF2 regulate the expression of genes required for differentiation in multiple cell types including in osteoclasts [200, 201], osteoblasts [202], adipocytes [203], and trophoblasts [204]. USF proteins are known to regulate the expression of cell cycle inhibitors CDKN1B (p27^{KIP1}) [205, 206], CDKN1A (p21^{CIP1}) [206], and Tp53 [206] as well as CCNB1 (cyclin B1) [206, 207] and cyclin dependent kinases [205, 208]. USF1 and USF2 may function as general transcription factors that are important in cell cycle control and may provide regulation of essential genes in all cells, including Sertoli cells. However, USF1 and USF2 actions in Sertoli cells have not been extensively studied. This chapter will characterize USF1 and USF2 expression in Sertoli cells during postnatal differentiation.

4.2 RESULTS

4.2.1 *Usf1* mRNA Levels Increase Between 5 and 11 Days After Birth in Isolated Sertoli Cells

To determine whether increased USF DNA binding during Sertoli cell differentiation corresponded with increased *Usf* mRNA expression, mRNAs encoding E-box proteins were assayed during Sertoli cell development using quantitative PCR (qPCR) with $\Delta\Delta C_t$ analysis. Assays of mRNA preparations from Sertoli cells immediately after isolation from 5 and 11 day-

old rat testes, revealed a significant 1.73 ± 0.32 -fold ($p < 0.05$) increase in *Usf1* mRNA levels 11 days after birth (Figure 8A). *Usf2* and *Tcfe2a* (E47) mRNA levels also trended higher (1.23 ± 0.19 and 1.27 ± 0.14) but did not reach a level of statistical significance (Figure 8B, C). *Trf* mRNA levels increased 2.88 ± 0.76 -fold ($p < 0.05$) from 5 to 11 days in agreement with previous studies showing that *Trf* mRNA levels increase during differentiation (Figure 8D) [28, 108]. The mRNA levels of the housekeeping gene *Ppia* (peptidylprolyl isomerase A, commonly known as cyclophilin) did not change over the course of differentiation (data not shown) and were used to normalize mRNA expression levels.

The efficiency-corrected ΔCt analysis of qPCR was used to determine the differences in the relative quantity of mRNA present for each gene of interest [209]. Average fold-changes in

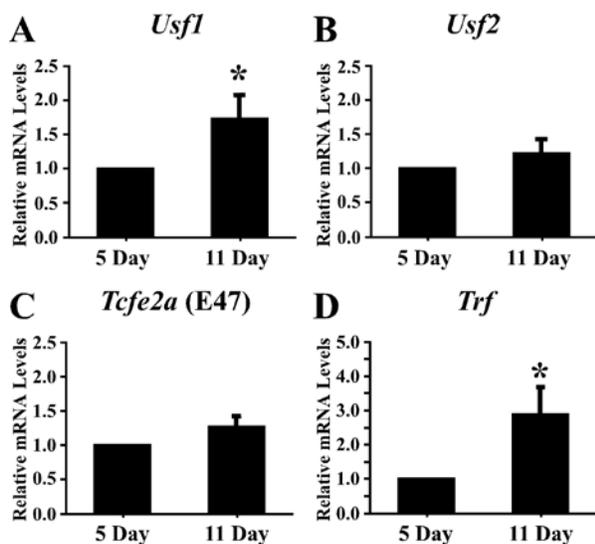


Figure 8. *Usf1* mRNA Levels Increase During Development in Sertoli Cells Assayed Immediately After Isolation.

The mRNAs isolated from 5 and 11 day-old Sertoli cells were analyzed by qPCR using primers for *Usf1* (A), *Usf2* (B), *Tcfe2a* (E47) (C), and *Trf* (D). Data were analyzed using the $\Delta\Delta\text{Ct}$ method and quantitation of the mean ($\pm\text{SE}$) of three individual experiments for each condition is provided for each primer set. The relative mRNA levels were normalized to *Ppia* levels and were made relative to 5 day-old Sertoli cells (=1). Statistically significant differences ($p < 0.05$) are indicated by an asterisk (*).

relative quantity between 5 and 11 days for *Usf1*, *Usf2*, and *Tcfe2a* (E47) were very similar to the relative-fold changes obtained from the $\Delta\Delta\text{Ct}$ analysis; however, the average fold changes in relative quantities did not reach statistical significance (Table 4, Rows 5-6). *Trf* mRNA levels increased 2.66 ± 0.63 ($p < 0.05$) significantly between 5 and 11 days as expected (Table 4, Rows 5-6). *Usf2* mRNA relative quantities in 5 day-old Sertoli cells are 7.31 ± 2.08 -fold ($p < 0.05$) greater than that of *Usf1* mRNA relative quantities (Table 4, Row 7). However, by 11 days after birth, relative

quantities of *Usf1* and *Usf2* are not statistically different (Table 4, Row 8). *Tcfe2a* (E47) and *Trf* are expressed at levels comparable to *Usf1* 5 days after birth and only *Trf* is expressed in greater relative quantities than *Usf1* 11 days after birth (Table 4, Row 8). Together, both qPCR analyses suggest that *Usf1* mRNA levels but not *Usf2* mRNA levels increase during Sertoli cell differentiation and this increase correlates with the increase in USF DNA binding 11 days after birth.

The expression of mRNAs encoding E-box proteins was also assayed by qPCR in cultured Sertoli cells to determine whether mRNA expression patterns during Sertoli cell differentiation were maintained in culture. $\Delta\Delta\text{Ct}$ qPCR analysis of mRNA preparations from cultured Sertoli cells showed statistically significant increases in *Usf1* (1.41 ± 0.03 -fold, $p <$

Table 4. Comparison of bHLH mRNA Levels Using the $\Delta\Delta\text{Ct}$ Method and the Efficiency-corrected ΔCt Method of qPCR Analysis in Rat Sertoli Cells Assayed Immediately After Isolation.

	Sertoli Cell Age	<i>Usf1</i>	<i>Usf2</i>	<i>Tcfe2a</i> (E47)	<i>Trf</i>	Row Number
$\Delta\Delta\text{Ct}$ Relative Fold Change Compared to 5 Day-old Sertoli Cells (Figure 8)*	5 Days	1.00 \pm 0.0	1.00 \pm 0.0	1.00 \pm 0.0	1.00 \pm 0.0	1
	11 Days	1.73 \pm 0.32*	1.23 \pm 0.19	1.27 \pm 0.14	2.88 \pm 0.76*	2
Relative Quantity Using the Efficiency-Corrected ΔCt Method Relative to <i>Ppia</i>	5 Days	0.00862 \pm 0.00198	0.00862 \pm 0.00198	0.01460 \pm 0.00087	0.02360 \pm 0.00055	3
	11 Days	0.01609 \pm 0.00715	0.07138 \pm 0.01540	0.01838 \pm 0.00156	0.06236 \pm 0.01410	4
Average Fold Change in Relative Quantity Compared to 5 Day-old Sertoli Cells*	5 Days	1.00 \pm 0.0 ^a	1.00 \pm 0.0	1.00 \pm 0.0	1.00 \pm 0.0	5
	11 Days	1.76 \pm 0.37	1.21 \pm 0.17	1.48 \pm 0.14	2.66 \pm 0.63*	6
Average Fold Change in Relative Quantity Over <i>Usf1</i>	5 Days	--	7.31 \pm 2.08**	1.81 \pm 0.37	2.92 \pm 0.59	7
	11 Days	--	5.35 \pm 2.10	1.37 \pm 0.37	4.34 \pm 0.77**	8

*Change between 5 and 11 days is significantly different ($p < 0.05$).

**Change between relative quantities when comparing gene to *Usf1* is significantly different ($p < 0.05$).

0.05), *Usf2* (1.42 ± 0.12 -fold, $p < 0.05$), and *Tcfe2a* (E47; 1.59 ± 0.15 -fold, $p < 0.05$) mRNA levels in cells isolated from 11 day-old rats when compared to mRNA levels from 5 day-old Sertoli cells (Figure 9A-C). *Trf* mRNA levels also increased 2.79 ± 0.31 -fold from 5 to 11 days and from 11 to 20 days after birth (Figure 9D) in agreement with studies in Sertoli cells assayed immediately after isolation showing that *Trf* mRNA levels increase in cells isolated during three key time periods in Sertoli cell differentiation (Figure 8D). The mRNA levels of the housekeeping gene *Ppia* did not change over the course of differentiation (data not shown).

Efficiency-corrected ΔCt analysis of mRNA levels in cultured Sertoli cells revealed

similar results. The relative quantities of *Usf1*, *Usf2*, and *Trf* increased significantly between 5 and 11 days after birth: 1.39 ± 0.03 -fold, 1.39 ± 0.12 -fold, and 2.60 ± 0.27 -fold respectively (Table 5, Rows 7-8). The relative quantities of *Usf1* and *Usf2* significantly decreased by 0.55 ± 0.07 and 0.36 ± 0.06 -fold ($p < 0.05$) between 11 and 20 days after birth while the relative quantities of *Trf* increased by 0.61 ± 0.20 -fold ($p < 0.05$; Table 5, Rows 8-9). In contrast to the $\Delta\Delta\text{Ct}$ analysis, relative quantities of *Tcfe2a* (E47) were not significantly elevated in cultured

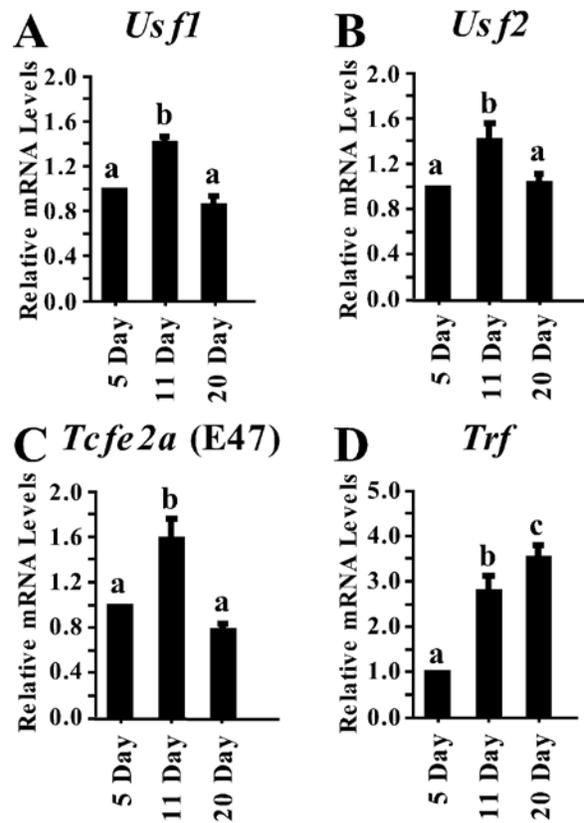


Figure 9. E-box Protein mRNAs Increase 11 Days After Birth in Cultured Sertoli Cells via qPCR.

mRNA isolated from cultured 5, 11, and 20 day-old Sertoli cells was analyzed by qPCR using primers for *Usf1* (A), *Usf2* (B), *Tcfe2a* (E47) (C), and *Trf* (D). Data was analyzed using the $\Delta\Delta\text{Ct}$ method and quantitation of the mean ($\pm\text{SE}$) of three individual experiments for each condition is provided for each primer set. The relative mRNA levels were normalized to *Ppia* levels and made relative to 5 day-old Sertoli cells (=1). Values with different lowercase letters differ significantly ($p < 0.05$).

Sertoli cells isolated from 11 day-old rats (Table 5, Rows 7-8), although the average fold-changes were comparable (Table 5, Row 1-2). Relative quantities of all mRNA transcripts were comparable to *Usf1* at all ages with the exception of *Trf* 20 days after birth, which were significantly increased (Table 5, Rows 10-12). The large average fold changes and standard errors for *Usf2* are due to the smaller Ct values (approximately 19) from one experiment when averaged with two experiments with larger Ct values (approximately 25) (Table 5, Columns 10-12). Additional experiments should be repeated to reduce the variability detected in *Usf2* mRNA levels in cultured Sertoli cells. Based on the qPCR data, the increase in DNA binding of USF1 and USF2 from cultured Sertoli cells corresponds with changes in *Usf1* and *Usf2* mRNA expression.

Table 5. Comparison of mRNA Levels of bHLH Proteins by the $\Delta\Delta$ Ct Method and Efficiency-Corrected Δ Ct Method of qPCR Analysis in Cultured Rat Sertoli Cells.

	Sertoli Cell Age	<i>Usf1</i>	<i>Usf2</i>	<i>Tcfe2a (E47)</i>	<i>Trf</i>	Row Number
$\Delta\Delta$Ct Relative Fold Change Compared to 5 Day-old Sertoli Cells (Figure 9)*	5 Days	1.00 ± 0.0 ^a	1			
	11 Days	1.41 ± 0.03 ^b	1.42 ± 0.13 ^b	1.59 ± 0.15 ^b	2.79 ± 3.52 ^b	2
	20 Days	0.86 ± 0.07 ^a	1.03 ± 0.07 ^a	0.78 ± 0.04 ^a	3.52 ± 0.24 ^c	3
Relative Quantity Using the Efficiency-Corrected ΔCt Method Relative to <i>Ppia</i>	5 Days	0.01609 ± 0.01123	0.30215 ± 0.35748	0.02170 ± 0.00751	0.01882 ± 0.00058	4
	11 Days	0.02275 ± 0.01621	0.38223 ± 0.44987	0.03513 ± 0.01468	0.04905 ± 0.00618	5
	20 Days	0.01272 ± 0.00811	0.30085 ± 0.35523	0.01709 ± 0.00623	0.06061 ± 0.00515	6
Average Fold Change in Relative Quantity Compared to 5 Day-old Sertoli Cells*	5 Days	1.00 ± 0.0 ^a	1.00 ± 0.0 ^a	1.00 ± 0.0	1.00 ± 0.0 ^a	7
	11 Days	1.39 ± 0.03 ^b	1.39 ± 0.12 ^b	1.57 ± 0.15	2.60 ± 0.27 ^b	8
	20 Days	0.84 ± 0.07 ^c	1.03 ± 0.06 ^a	0.78 ± 0.04	3.21 ± 0.20 ^c	9
Average Fold Change in Relative Quantity Over <i>Usf1</i>	5 Days	--	37.44 ± 44.60	1.89 ± 0.70	2.02 ± 0.97	10
	11 Days	--	34.83 ± 41.43	2.12 ± 0.84	3.91 ± 2.14	11
	20 Days	--	45.11 ± 53.74	1.70 ± 0.54	7.12 ± 2.86 ^{**}	12

*Values with different lowercase letters differ significantly within this category (p < 0.05).

**Change between relative quantities when comparing gene to *Usf1* is significantly different (p < 0.05).

4.2.2 USF1 Protein Levels Increase Between 5 and 11 Days After Birth in Whole Cell Extracts from Sertoli Cells Assayed Immediately After Isolation

Western blotting analysis of USF1 and USF2 in whole cell extracts prepared from Sertoli cells immediately after isolation demonstrated that USF1 protein levels increased between 5 and 11 days after birth by 3.3 ± 1.1 -fold ($p < 0.05$; Figure 10 A, C). USF2 protein levels did not

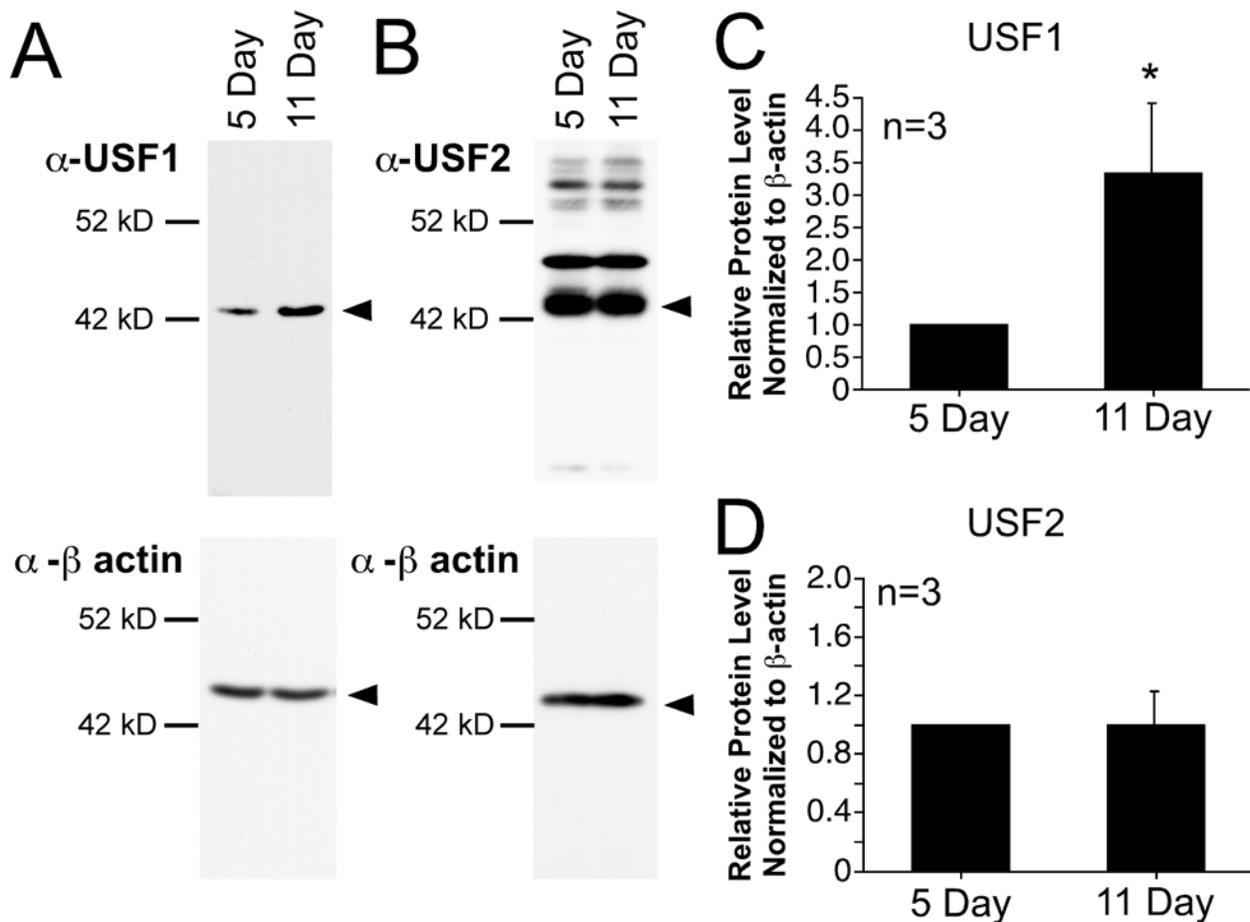


Figure 10. USF1 Protein Levels Increase During Differentiation in Sertoli Cells Assayed Immediately After Isolation.

Western immunoblotting of whole cell extracts from 5 and 11 day-old Sertoli cells was performed sequentially with antisera specifically recognizing USF1 (A) or USF2 (B) followed by β -actin. Arrowheads denote band of interest. The normalized USF1 and USF2 levels for 5 day-old Sertoli cells were arbitrarily set equal to 1. The mean fold induction (\pm SE) of 11 day-old Sertoli cells over 5 day-old Sertoli cells from 3 independent experiments for USF1 and USF2, respectively, is reported (C&D). Values that are significantly different ($p < 0.05$) are indicated with an asterisk (*).

change (Figure 10 B, D). β -actin levels were assayed to ensure equal protein loading. Detection of USF1 required 5-fold more protein extract than that needed for USF2 protein detection. Although the efficiency of the USF1 and USF2 antibodies may vary, the need for less protein to provide a USF2 Western signal is consistent with previous qPCR results demonstrating that *Usf2* mRNA levels are greater than those of *Usf1* (Table 4, Row 5-6). The observed increase in *Usf1* mRNA and USF1 protein levels between 5 and 11 days after birth is likely a major contributor to the increase in DNA binding to USF consensus sites observed 11 days after birth.

4.2.3 USF1 Protein Localizes to the Nuclei of Differentiating Cultured Sertoli Cells

It was hypothesized that an increase in USF1 protein levels within the nuclei of Sertoli cells would contribute to an increase in DNA-binding activity to E-box motifs. To verify the location of E-box proteins within Sertoli cells during differentiation, immunofluorescence analysis was conducted on cultured primary Sertoli cells from 5 and 11 day-old rats (Figure 11). Cells were incubated with antisera against USF1 (top) or USF2 (bottom) and nuclei were stained with Hoechst. Antiserum against vimentin, a Sertoli cell-specific marker, revealed that all USF-positive cells are also Sertoli cells (Appendix A, Figures 34 and 35). USF1 is present in the Sertoli cell cytoplasm, but rarely in the nucleus of 5 day-old cells (Figure 11 top). By 11 and 20 days after birth, USF1 is predominantly nuclear (Figure 11, top). USF2 immunostaining levels were similar in both the cytoplasm and nuclei of Sertoli cells at all stages of differentiation (Figure 11, bottom). These data support the hypothesis that USF1 and USF2 binding increases to E-box binding sites during differentiation due to an increase in USF1 protein within the nucleus of Sertoli cells.

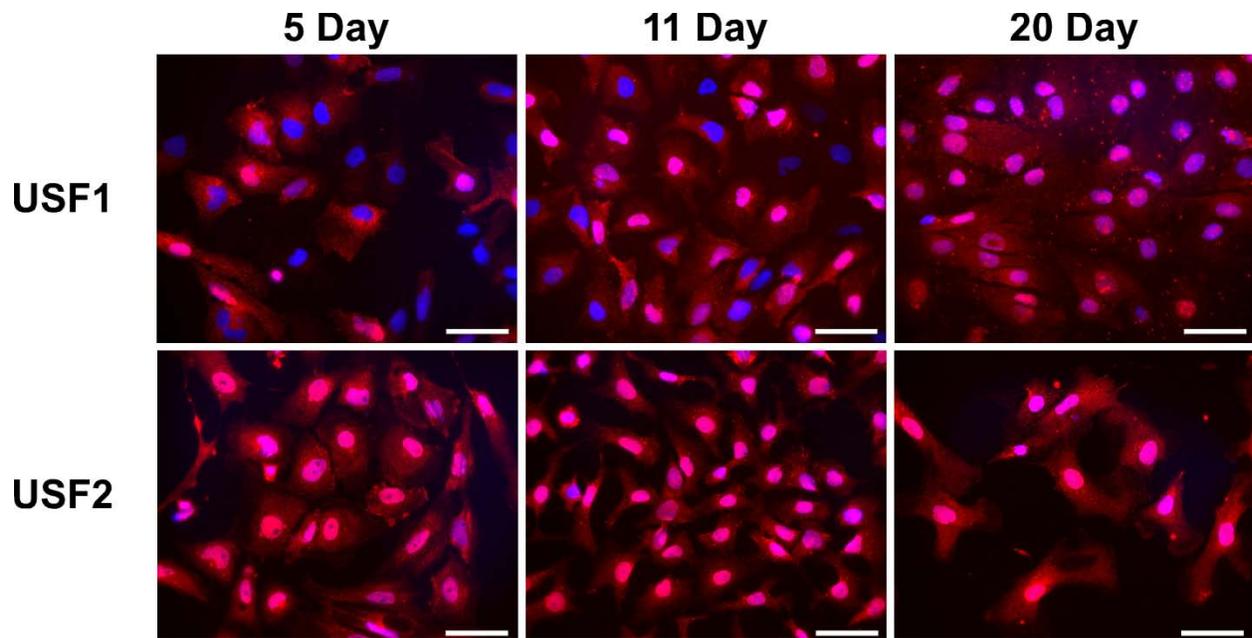


Figure 11. USF1 Protein Localizes to the Nucleus During Differentiation in Cultured Sertoli Cells.

Immunofluorescence of cultured 5, 11 and 20 day-old Sertoli cells was performed with antisera recognizing USF1 (top) or USF2 (bottom) as visualized with Alexa 488 fluorophore (red). Hoechst stains nuclei (blue). Scale bars = 100 μ m. Control immunostaining with the Sertoli cell specific anti-vimentin antiserum is provided in Appendix A, Figures 34 and 35.

4.3 CONCLUSIONS

USF binding to E-box motifs increases in differentiating Sertoli cells (Figures 5 and 6). A hypothesis was proposed that the increase in USF binding to E-box motifs was due to increases in USF mRNA and protein levels during Sertoli cell differentiation. Support for this hypothesis included qPCR studies that demonstrated an increase in *Usf1* mRNA levels between 5 and 11 days after birth (Figures 8 and 9; Tables 4 and 5). Using $\Delta\Delta$ Ct analysis of qPCR results, it was found that *Usf1* mRNA levels were increased 1.73 ± 0.32 -fold ($p < 0.05$) in Sertoli cells immediately after isolation (Figure 8A; Table 4, Row 1) and 1.4 ± 0.03 -fold ($p < 0.05$) in cultured Sertoli cells (Figure 9A; Table 5, Row 2) from 11 day-old rats when compared with mRNA levels in Sertoli cells isolated from 5 day-old rats. *Usf2* and *Tcf2a* (E47) mRNA levels

remained unchanged in immediately assayed cells (Figure 8B,C; Table 4, Row 1) but increased between 5 and 11 days after birth in cultured Sertoli cells (1.42 ± 0.12 -fold and 1.59 ± 0.15 -fold, respectively ($p < 0.05$); Figure 9B,C; Table 5, Row 2). In contrast, mRNA studies of cultured Sertoli cells revealed a significant increase in mRNA levels of not only *Usf1*, but also *Usf2* and *Tcfe2a* (E47) between 5 and 11 days after birth (Figure 9B,C; Table 5, Row 2). The positive control gene, *Trf*, was also found to increase significantly during Sertoli cell differentiation in both cells assayed immediately after isolation and cultured Sertoli cells (Figure 8 and 9; Table 4, Row 1; Table 5, Row 2).

The efficiency-corrected Δ Ct method revealed similar increases in fold-change of RNA from cells assayed immediately after isolation for *Usf1*, *Usf2*, *Tcfe2a* (E47), and *Trf* but only the increased *Trf* levels from 5 to 11 days after birth reached statistical significance (Table 4). The data also revealed that *Usf2* mRNA is expressed 7.31 ± 2.08 -fold ($p < 0.05$) greater than *Usf1* in 5 day-old Sertoli cells (Table 4, Row 5). By 11 days, *Usf2* mRNA levels were still 5.35 ± 2.10 greater than *Usf1* but the difference was no longer statistically significant. In cultured Sertoli cells, all transcripts were expressed at comparable levels to *Usf1*.

mRNA levels were measured in both Sertoli cells assayed immediately after isolation and in cultured Sertoli cells. The results vary between the two populations of cells although similar trends are retained (Table 4 vs. Table 5). For example, *Usf1* mRNA levels increased in cells assayed immediately after isolation 1.73 ± 0.32 -fold ($p < 0.05$; Table 4, Rows 1-2) and in cultured cells by 1.41 ± 0.03 -fold ($p < 0.05$; Table 5, Row 1-2). Both are statistically significant increases although cultured Sertoli cells have a reduced level of *Usf1* induction. In contrast, *Usf2* and *Tcfe2a* (E47) mRNA levels trend upward in both Sertoli cell populations but only in cultured Sertoli cells are the increases significant (Table 4, Rows 1-2 vs. Table 5, Rows 1-2). The

variance in mRNA levels may be explained by the differences in the type of isolation used to study Sertoli cells. Studies of Sertoli cells that are immediately assayed after isolation remain unaltered by culture conditions, whereas cultured Sertoli cells are allowed time to recover from isolation and may be induced by culture conditions to up-regulate expression of *Usf2* and *Tcfe2a* (E47). Regardless of the difference in cell population used, both assays reveal an increase in *Usf1* mRNA levels.

The use of the two methods of qPCR analysis was initially conducted to exploit the strengths of each analysis. The $\Delta\Delta\text{Ct}$ method allows comparisons between mRNA levels of different samples for a single transcript [209]. The efficiency-corrected ΔCt method also allows comparisons between samples but the strength of this method lies in the ability to compare mRNA levels of different transcripts within the same sample [209]. However, both analyses can provide “fold-changes” and differ slightly in the results. The difference seen in the two data sets can be attributed to the $\Delta\Delta\text{Ct}$ method reliance on “perfect” amplification of the primer set. Primer designing for several genes proved difficult; as such the stringency was relaxed from the recommended slope of -3.3 ± 0.1 (efficiency 2.0 ± 0.05). The primers were allowed to have an efficiency of 2.0 ± 0.2 (See Material and Methods, Table 10) which equates to a slope of -3.3 ± 0.5 of the line from a standard serial dilution, created by the plot of the Ct value vs. the log of the input (See Materials and Methods, 10.8). If the primer efficiency was maintained within the recommended range, the values would be expected to be more closely correlated for the two methods of analysis. However, Irregardless of some minor differences in specific mRNA quantities, as a whole, both $\Delta\Delta\text{Ct}$ and efficiency-corrected ΔCt analysis of mRNA from cells assayed immediately after isolation and in culture suggest that there is an increase in *Usf1* or *Usf2* mRNA levels that correlates with an increase in USF DNA binding.

Because mRNA levels do not always correspond to increases in protein levels, USF1 and USF2 protein levels were analyzed. Western blot analysis from Sertoli cells assayed immediately after isolation revealed a 3.3-fold increase in USF1 protein between 5 and 11 days after birth (Figure 10A, C). It is possible that the increases in *Usf* mRNA levels and the subsequent increase in USF1 protein expression could account for the elevation of USF-DNA binding observed in Sertoli cells during differentiation when immediately assayed, a 2-fold increase (Figure 5), or cultured, a 7- to 10-fold increase (Figure 6) between 5 and 11 days after birth. Additionally, USF1 protein accumulates in the nucleus of differentiating Sertoli cells (Figure 11, top). In contrast, the levels of USF2 protein expression remain constant during this stage in postnatal Sertoli cell differentiation and maintain their cellular localization (Figure 11, bottom). Therefore, an increase in USF1 protein expression and an increase of levels of protein in the nucleus during differentiation suggest that increases in the availability of USF1 protein in the nucleus contributes to and correlates with an increase in USF binding to E-box motifs in Sertoli cells. An increase in binding may in turn lead to an increase in gene transcription. It is interesting to note that USF1 levels are up-regulated during differentiation; however, expression of the *Usf2* gene containing two E-box motifs in its promoter [192] is not altered. These data suggest that factors in addition to USF1 are required to activate the *Usf2* promoter in differentiating Sertoli cells.

Data presented thus far support the following model. During proliferation there are low levels of USF1 present within Sertoli cells. With the onset of differentiation, *Usf1* mRNA and USF1 protein production is enhanced. The increased availability of USF1 may lead to the formation of more USF1 homodimers and USF1/USF2 heterodimers. It has been shown that USF1/USF2 heterodimers are the most abundant dimer pair within the testis and ovary of 21

day-old mice, with the second most prevalent dimer pair being USF1 homodimers [210]. The preference for USF1 dimers does not explain why Sertoli cells express 5- to 7-fold more *Usf2* mRNA (Table 4, Rows 7-8) or why detection of USF1 protein by Western blot required 5-fold more protein extract compared to USF2. However, the increase in dimers containing USF1 may lead to more efficient transcriptional activation of USF target genes within Sertoli cells. Therefore, understanding how USF1 is regulated may provide further insights into gene transcription of genes required for Sertoli cell differentiation and differentiated Sertoli cell function.

5.0 ID PROTEINS: INHIBITORS OF SERTOLI CELL DIFFERENTIATION?

Hypothesis: ID proteins inhibit USF1 and USF2 DNA binding during Sertoli cell proliferation.

5.1 INTRODUCTION

5.1.1 Class D bHLH Proteins

Potential regulators of Sertoli cell proliferation and E-box protein actions include the members of the class D bHLH family of transcription factor repressors named inhibitors of cell differentiation or inhibitors of DNA binding (ID) (reviewed in [211-213]). In mammals, ID proteins inhibit class A and class B bHLH proteins. Four ID proteins have been characterized in mammalian cells, ID1, ID2, ID3, and ID4 [155, 214-216]. All four ID proteins contain a conserved helix-loop-helix (HLH) dimerization motif that mediates interactions with other HLH proteins [211, 212, 217]. However, these proteins lack the basic DNA binding domain and function as dominant negative repressors of bHLH transcription factors by forming non-DNA binding, inactive heterodimers [212]. A major consequence of ID protein expression is the inhibition of bHLH protein-mediated activation of genes required for the differentiation of tissues during development [218]. Differentiation of oligodendrocytes [219, 220] and

granulocytes [221] is known to be inhibited by ID proteins. Also, ID proteins promote proliferation through the regulation of cell cycle control proteins [222-225].

5.1.2 ID Protein Localization and Post-translational Regulation

ID proteins require localization to the nucleus to function in the inhibition of DNA binding and thus differentiation. All four ID proteins are small enough to move through nuclear pores by diffusion [226, 227]. ID1 and ID2 have nuclear export signals [226, 227]. No known nuclear export signal has been found for ID3 or ID4 [227]. Although there is not a consensus nuclear localization signal, the presence of the HLH domain is enough to facilitate nuclear localization of the ID proteins [227]. Interaction of ID3 with E47 and ID1 with MyoD allows transport of the ID proteins to the nucleus via the nuclear localization signal of the bHLH proteins [228, 229].

Export to the cytoplasm may allow ID proteins to become sequestered or degraded. ID2 transport from the nucleus requires exportin-1 (XPO1, also known as CRM1) [227]. The blockage of XPO1-mediated export results in ID2 retention to the nucleus [227]. However, when ID2 is present in the cytoplasm, diffusion into the nucleus does not occur suggesting that a regulator may sequester ID2 within the cytoplasm [227]. ID2 associates with three proteins that function to sequester ID2 within the cytoplasm and facilitate its degradation: PDZ and LIM domain 5 [PDLIM5 also known as Enigma homolog (ENH)] [230, 231], interferon inducible protein 204 (IFI204) [232-234], and polycystin-2 (PKD2) [235]. The presence of ID proteins in the cytoplasm facilitates their degradation through the ubiquitin-proteasome system to induce differentiation [236-239]. ID protein function and degradation may be regulated through cell cycle-specific post-translational modifications. ID proteins can be phosphorylated by CDK2

[240], PKA [241], PKC [241], or CDK1 [241]. These data suggest that cellular localization, turnover rate, and phosphorylation state may all alter the ability of ID proteins to perform as inhibitors of differentiation.

5.1.3 ID Proteins in Sertoli Cells

All four ID proteins have been detected in cultured 20 day-old Sertoli cells [178]. *In vivo*, only ID2 and ID3 were expressed in adult Sertoli cells, as detected by immunohistochemistry of tissue sections, representing all stages of the seminiferous epithelium [242]. Stimulation of cultured Sertoli cells for 72 hours with FSH or cAMP inhibited the expression of the *Id1* gene while inducing the expression of the *Id4* gene [178]. *Id3* gene expression was not affected by stimulation of FSH or cAMP [178]. The expression of ID2 is transiently up-regulated by FSH or cAMP within 6 hours of stimulation with FSH or cAMP [243, 244] while 72 hours of stimulation has no effect on *Id2* gene expression [178]. Overexpression of ID1 or ID2 was found to induce and maintain proliferation of cultured post-mitotic Sertoli cells from 20 and 60 day-old rats [166]. In a more recent study, Sertoli cells placed in culture were found to induce ID2 expression and begin proliferating despite being “quiescent” within the testis [130]. ID proteins were found to regulate E-box motifs in the promoters of *Fshr* [172], *Trf* [167], *Fos* [169], and *Shbg* [245] in Sertoli cells. Together, these data support the hypothesis that ID proteins are important inhibitors of differentiation in Sertoli cells and regulate the expression of genes required for differentiated Sertoli cell functions.

5.2 RESULTS

5.2.1 *Id2*, *Id3*, and *Id4* mRNA Levels Increase During Sertoli Cell Differentiation

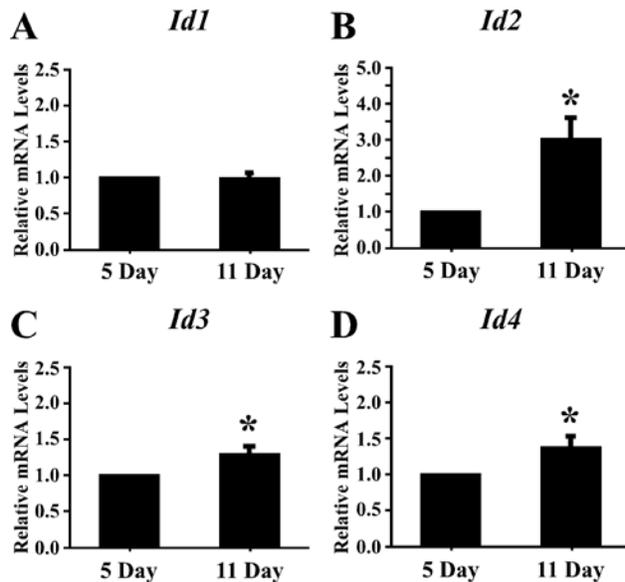


Figure 12. ID Protein mRNAs Increase During Development in Sertoli Cells Assayed Immediately After Isolation via qPCR.

The mRNAs isolated from 5 and 11 day-old Sertoli cells were analyzed by qPCR using primers for *Id1* (A), *Id2* (B), *Id3* (C), and *Id4* (D). Data were analyzed using the $\Delta\Delta C_t$ method, and quantitation of the mean (\pm SE) of three individual experiments for each condition is provided for each primer set. The relative mRNA levels were normalized to *Ppia* levels and were made relative to 5 day-old Sertoli cells (=1). Statistically significant differences ($p < 0.05$) are indicated by an asterisk (*).

To gain understanding of the ability of ID proteins to inhibit USF binding to E-box motifs during Sertoli cell differentiation, the levels of *Id* mRNAs were analyzed by qPCR. Examination of *Id1* mRNA levels from Sertoli cells assayed immediately after isolation revealed no significant differences in the levels of mRNA expression between 5 and 11 days after birth using both $\Delta\Delta C_t$ (Figure 12A; Table 6, Rows 1-2) and efficiency-corrected ΔC_t analyses (Table 6, Rows 5-6). The mRNA levels increased for *Id2* [3.0 ± 0.6 -fold (Figure 12 B)], *Id3* [1.3 ± 0.01

(Figure 12C)], and *Id4* [1.4 ± 0.1 (Figure 12D)] during this period using both qPCR analyses (Table 6, Rows 1-2 and 5-6; $p < 0.05$ for all). The increase in expression of the ID inhibitor mRNA is not consistent with the hypothesis that decreased ID levels cause the up-regulation of USF binding activity during differentiation.

Efficiency-corrected ΔC_t analysis revealed that the relative *Id2* and *Id3* mRNA levels (Table 6, Rows 7-8) in Sertoli cells are similar to that of *Usf1* (Table 4), but the levels of *Id1* and

Table 6. Comparison of *Id* mRNA Levels Using the $\Delta\Delta$ Ct Method and the Efficiency-corrected Δ Ct Method of qPCR Analysis in Isolated Rat Sertoli Cells Assayed Immediately After Isolation.

	Sertoli Cell Age	<i>Id1</i>	<i>Id2</i>	<i>Id3</i>	<i>Id4</i>	Row Number
$\Delta\Delta$Ct Relative Fold Change Compared to 5 Day-old Sertoli Cells (Figure 12)*	5 Days	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1
	11 Days	0.99 ± 0.06	3.03 ± 0.57*	1.30 ± 0.09*	1.37 ± 0.14*	2
Relative Quantity Using the Efficiency-Corrected ΔCt Method Relative to <i>Ppia</i>	5 Days	0.00058 ± 0.00004	0.01232 ± 0.00201	0.00861 ± 0.00200	0.00001 ± 0.000003	3
	11 Days	0.00057 ± 0.00009	0.03675 ± 0.00643	0.01122 ± 0.00288	0.00001 ± 0.00000381	4
Average Fold Change in Relative Quantity Compared to 5 Day-old Sertoli Cells*	5 Days	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	5
	11 Days	0.98 ± 0.09	3.04 ± 0.63*	1.29 ± 0.09*	1.39 ± 0.18*	6
Average Fold Change in Relative Quantity Over <i>Usf1</i>	5 Days	0.07 ± 0.01**	1.57 ± 0.54	1.08 ± 0.41	0.0007 ± 0.0005**	7
	11 Days	0.04 ± 0.01**	2.67 ± 0.78	0.84 ± 0.37	0.0006 ± 0.0004**	8

*Change between 5 and 11 days is significantly different ($p < 0.05$).

**Change between relative quantities when comparing gene to *Usf1* is significantly different ($p < 0.05$).

Id4 mRNAs are 100- and 1,000-fold lower, respectively (Table 6, Rows 7-8). These data suggest that *Id2* and *Id3* are expressed in ratios similar to *Usf1*. Furthermore, if mRNA levels reflect protein levels, ID2 and ID3 may be expressed in levels comparable to USF1 and thus ID2 and ID3 may be present at sufficient levels to inhibit USF binding to DNA. Together, these data suggest that *Id2* is the most abundantly expressed *Id* gene and is greatly induced during the onset of Sertoli cell differentiation (Table 6). Therefore, studies of ID proteins began with ID2.

5.2.2 *Id1* and *Id3* mRNA Levels Decrease with the Termination of Differentiation in Cultured Sertoli Cells

The mRNA levels for ID protein encoding genes were also assayed for cultured Sertoli cells. Analysis of *Id* mRNA levels in cultured Sertoli cells through the use of qPCR revealed a similar increase in *Id3* mRNA levels in cells isolated from 11 day-old rats compared to cells isolated from 5 day-old rats (Figure 13C; Table 7, Rows 1 and 2). In contrast, *Id1* and *Id2* mRNA levels did not change between 5 and 11 days after birth (Figure 13 A,B; Table 7, Rows 1 and 2). *Id2* mRNA levels did significantly increase between 11 and 20 days after birth by 0.29 ± 0.05 ($p < 0.05$; Figure 13B; Table 7, Row 3). Interestingly, *Id1* and *Id3* mRNA levels significantly decreased to less than 20% and 35% of 5 day mRNA levels, respectively (Figure 13A,C; Table 7, Row 3 and 9). *Id4* mRNA levels did not change significantly at any time period (Figure 13D; Table 7, Rows 1-3, 7-9). Efficiency-corrected ΔCt analysis revealed that only

Id2 mRNA was expressed at levels similar to *Usf1* with *Id1*, *Id3*, and *Id4* mRNA levels significantly lower at all ages (Table 7, Rows 10-12). Together, mRNA analysis of the *Id* genes in cultured Sertoli cells suggest that only *Id2* levels increase during differentiation and that *Id2* is

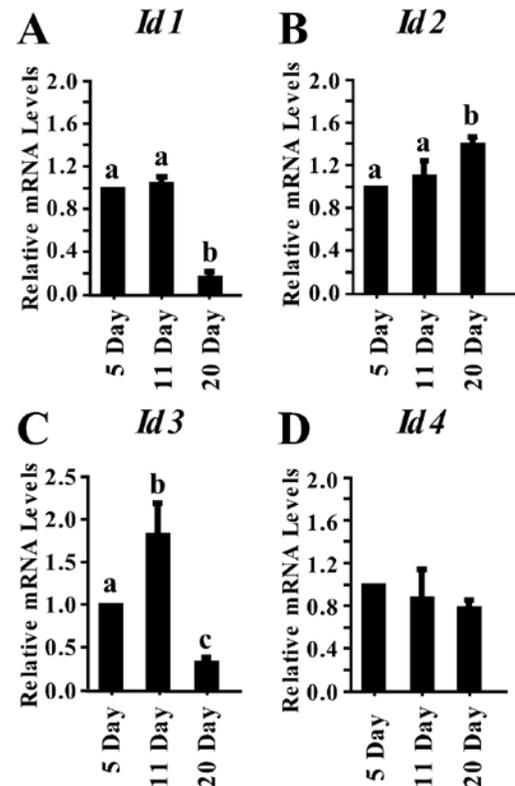


Figure 13. *Id1* and *Id3* mRNA Levels Decrease Whereas *Id2* mRNA Levels Increase 20 Days After Birth in Cultured Sertoli Cells via qPCR.

mRNA isolated from cultured 5, 11, and 20 day-old Sertoli cells was analyzed by qPCR using primers for *Id1* (A), *Id2* (B), *Id3* (C), and *Id4* (D). Data was analyzed using the $\Delta\Delta Ct$ method and quantitation of the mean (\pm SE) of three individual experiments for each condition is provided for each primer set. The relative mRNA levels were normalized to *Ppia* levels and made relative to untreated 5 day-old Sertoli cells (=1). Values with different lowercase letters differ significantly ($p < 0.05$).

expressed at levels comparable to *Usf1*. *Id1* and *Id3* mRNA levels decrease 20 days after birth, leading to a hypothesis that after differentiation the functions of ID1 and ID3 within the cell are less important.

Table 7. Comparison of *Id* mRNA Levels Using the $\Delta\Delta$ Ct Method and Efficiency-Corrected Δ Ct Method of qPCR Analysis in Cultured Rat Sertoli Cells.

	Sertoli Cell Age	<i>Id1</i>	<i>Id2</i>	<i>Id3</i>	<i>Id4</i>	Row Number
$\Delta\Delta$Ct Relative Fold Change Compared to 5 Day-old Sertoli Cells (Figure 13)*	5 Days	1.00 ± 0.0 ^a	1.00 ± 0.0 ^a	1.00 ± 0.0 ^a	1.00 ± 0.0	1
	11 Days	1.05 ± 0.04 ^a	1.10 ± 0.13 ^a	1.83 ± 0.34 ^b	0.87 ± 0.25	2
	20 Days	0.16 ± 0.03 ^b	1.39 ± 0.05 ^b	0.34 ± 0.03 ^c	0.78 ± 0.06	3
Relative Quantity Using the Efficiency-Corrected ΔCt Method Relative to <i>Ppia</i>	5 Days	0.00048 ± 0.00025	0.01968 ± 0.00457	0.00291 ± 0.00114	0.00020 ± 0.00002	4
	11 Days	0.00049 ± 0.00024	0.02203 ± 0.00792	0.00550 ± 0.00249	0.00018 ± 0.00006	5
	20 Days	0.00006 ± 0.00004	0.02748 ± 0.00705	0.00103 ± 0.00045	0.00016 ± 0.00003	6
Average Fold Change in Relative Quantity Compared to 5 Day-old Sertoli Cells*	5 Days	1.00 ± 0.0 ^a	1.00 ± 0.0 ^a	1.00 ± 0.0 ^a	1.00 ± 0.0	7
	11 Days	1.01 ± 0.04 ^a	1.08 ± 0.13 ^a	1.80 ± 0.33 ^b	0.84 ± 0.25	8
	20 Days	0.14 ± 0.03 ^b	1.39 ± 0.05 ^b	0.34 ± 0.03 ^c	0.76 ± 0.06	9
Average Fold Change in Relative Quantity Over <i>Usf1</i>	5 Days	0.07 ± 0.05**	2.15 ± 1.05	0.27 ± 0.15**	0.02 ± 0.01**	10
	11 Days	0.05 ± 0.04**	1.78 ± 1.01	0.32 ± 0.18**	0.01 ± 0.00**	11
	20 Days	0.01 ± 0.01**	3.53 ± 1.79	0.11 ± 0.07**	0.02 ± 0.01**	12

*Values with different lowercase letters differ significantly within this category ($p < 0.05$).

**Change between relative quantities when comparing gene to *Usf1* is significantly different ($p < 0.05$).

5.2.3 Nuclear ID2 Protein Expression Decreases During Differentiation in Sertoli Cells

ID2 protein expression was investigated to determine whether a decrease in ID2 protein levels could contribute to the increase in USF DNA binding in 11 day-old Sertoli cells despite the detected overall increase in *Id2* mRNA levels. To determine ID2 protein expression levels and localization during differentiation, immunofluorescence of cultured Sertoli cells was

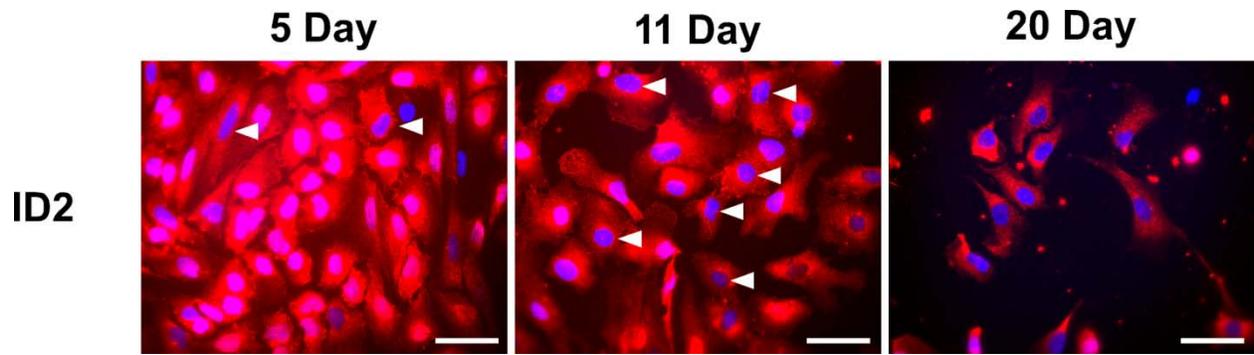


Figure 14. ID2 Protein Decreases Within the Nucleus During Sertoli Cell Differentiation.

Immunofluorescence of cultured 5, 11, and 20 day-old Sertoli cells was performed with antisera recognizing ID2 as visualized with Alexa 488 fluorophore (red). Hoechst stains nuclei (blue). Arrowheads indicate lack of ID2 immunostaining in the nucleus. Scale bars = 100 μm . Control immunostaining with the Sertoli cell specific anti-vimentin antiserum is available in Appendix A, Figure 36.

conducted using antisera against ID2. Nuclei were stained with Hoechst. Cells were also incubated with antiserum against the Sertoli cell-specific vimentin and revealed that all ID2 positive cells were also Sertoli cells (Appendix A, Figure 36). Initially, analysis of immunofluorescence data revealed that ID2 is highly expressed in nearly all of the nuclei of 5 day-old Sertoli cells (Figure 14 left). By 20 days after birth, overall ID2 immunostaining is decreased and primarily localized to the cytoplasm (Figure 14 right). These data raise the possibility that a high level of ID2 protein expression within the nuclei of 5 day-old Sertoli cells is required to maintain Sertoli cells in a proliferative state, whereas the reduction in ID2 protein expression in the nucleus contributes to the termination of differentiation.

To quantify the changes in ID protein expression, analysis was performed using the Metamorph software package. This application allows the measurement of pixel intensity within a given area as specified by the user. Analysis of whole cell and nuclear pixel intensity was measured. Whole cell area was estimated based on immunostaining from vimentin-positive cells. Anti-vimentin antiserum was incubated with the cells as a Sertoli cell-specific marker. Nuclear area was established by the threshold of positive nuclear staining as revealed by Hoechst dye. Analysis was conducted for immunostaining of ID2 in 5, 11, and 20 day-old cultured Sertoli

cells. A decrease in ID2 immunostaining was observed for the total cellular and nuclear intensity during differentiation. A decrease of $23.7 \pm 6.1\%$ and $41.8 \pm 2.5\%$, respectively ($p < 0.05$ for both) was observed when comparing immunostaining in 20 day-old cells when compared with 5 day-old cells (Figure 15A, B). Normalization of cellular and nuclear intensity to measured area revealed a decrease of $27.9 \pm 3.7\%$ and $24.1 \pm 3.6\%$, respectively ($p < 0.05$), for both measurements in 20 day-old cells when compared with 5 day-old cells (Figure 15C, D). As a whole, these results suggest that ID2 levels decrease within Sertoli cell nuclei during differentiation.

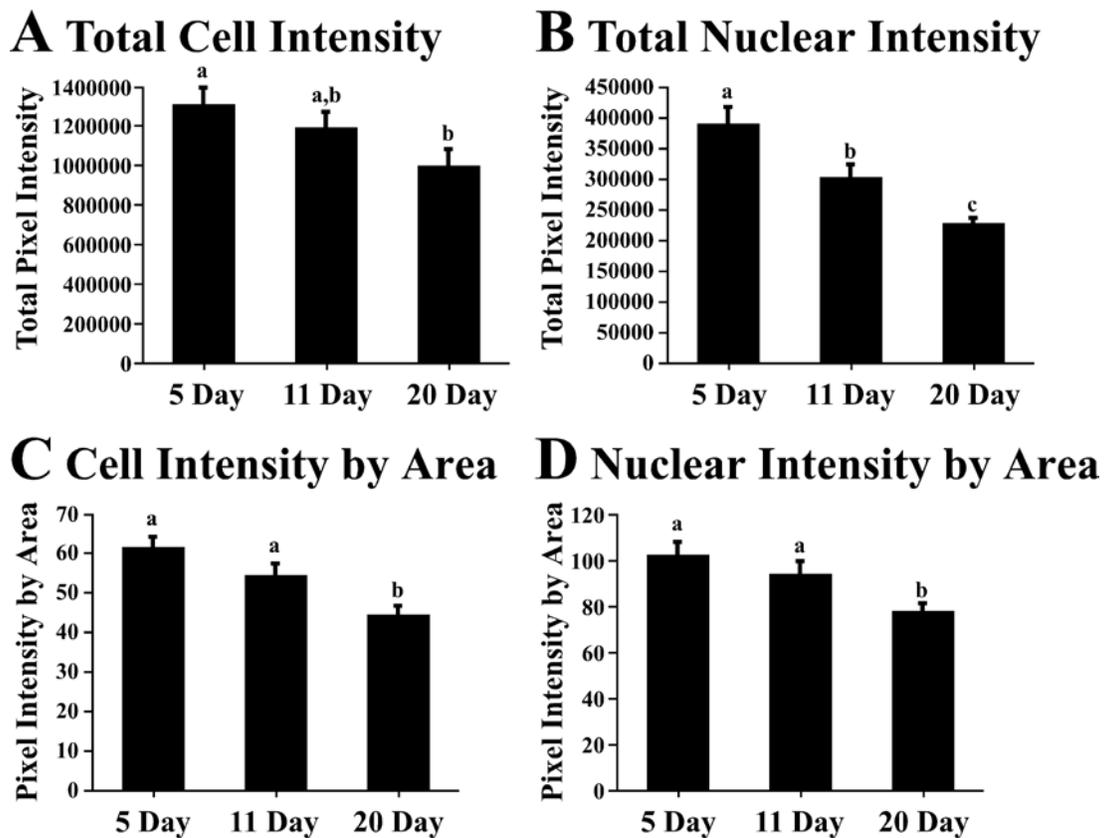


Figure 15. ID2 Immunostaining Decreases by 20 Days After Birth in Cultured Sertoli Cells.

Immunostaining of ID2 in 5, 11, and 20 day-old cultured Sertoli cells was quantified using Metamorph. For each measurement at least 450 cells per age were counted from six independent experiments. (A) The mean cell intensity was determined by measuring the total pixel intensity within the border of the cell. (B) The mean nuclear intensity was determined by measuring the total pixel intensity within the thresholded border of the nucleus, based on Hoechst staining. (C) The mean total cell intensity normalized to the total cell area or (D) the mean total nuclear intensity was normalized to the total nuclear area. Values with different lowercase letters differ significantly ($p < 0.05$).

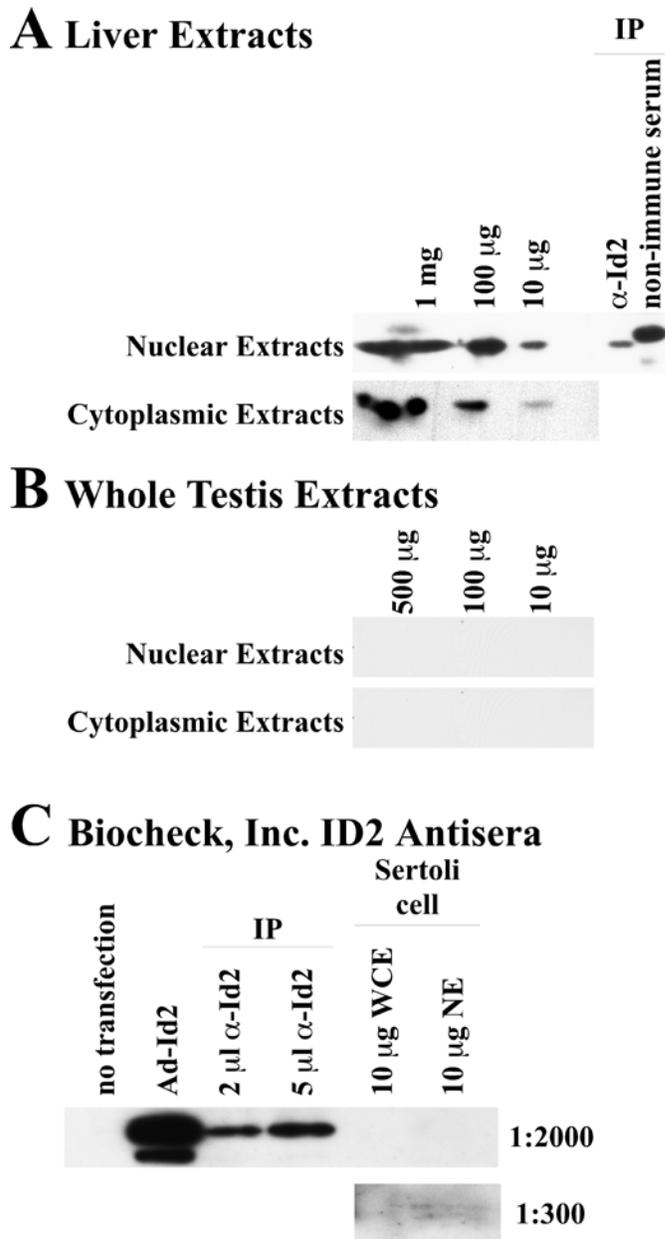


Figure 16. ID Proteins in Whole Testis or Isolated Sertoli Cell Extracts are Undetectable by Western Blotting.

(A) Western immunoblotting of 1 mg, 100 µg, and 10 µg of nuclear (top) and cytoplasmic extracts (bottom) from liver performed with antisera recognizing ID2 (1:500). Antiserum against ID2 but not non-immune serum was able to immunoprecipitate protein from the liver nuclear extracts. (B) ID2 protein was undetectable when Western immunoblotting was conducted of 500 µg, 100 µg, and 10 µg of nuclear (top) and cytoplasmic extracts from whole testis. (C) Western immunoblotting of 10 µg of MSC-1 cell extracts transfected with adenovirus expressing ID2 and 10 µg whole cell extracts or 10 µg nuclear extracts from isolated Sertoli cells with the ID2 antisera from Biocheck, Inc. at a dilution of 1:2000 (top) or 1:300 (bottom).

5.2.4 ID Proteins are Detected in Liver Nuclear Extracts but Not Extracts from Isolated Sertoli Cells or Whole Testis

To compliment the quantitation of ID2 levels observed by immunofluorescence, Western blotting assays were undertaken. Endogenous and radiolabeled ID proteins by Western blot was previously shown to be possible by other groups studying other tissues [246, 247]. However, attempts to detect ID proteins in extracts from cultured Sertoli cells by Western blot were not successful with antisera against ID2 (Santa Cruz, sc-489). Others have also reported an inability to detect endogenous ID proteins in primary Sertoli cells utilizing this antibody [166].

To verify and troubleshoot

Western detection of ID proteins, studies utilizing liver extracts were conducted. Liver extracts express ID2 protein as detected utilizing the Santa Cruz antibody (sc-489) [246]. The protocol of nuclear extraction from Rodriguez, *et al.* (2006), was followed for ID Western analysis of extracts from liver tissue [246]. Liver extracts were homogenized and nuclei were isolated from the cytoplasm of liver homogenate. Varying concentrations of protein were analyzed by Western blotting with antiserum against ID2. ID2 was present in a titratable amount and detectable with as little as 10 μg of protein in both nuclear and cytoplasmic fractions of the liver homogenate (Figure 16A). It should be noted that the excessive amount of protein loaded in the first lane (1 mg) caused the band to spread out within the gel. This was anticipated prior to loading of samples and lanes were skipped between samples. Immunoprecipitation of 1 mg of liver homogenate with antiserum against ID2 resulted in a band of expected size whereas an immunoprecipitation utilizing non-immune serum did not result in a band of the appropriate size (Figure 16A). Whole cell extracts of testis tissue were then prepared according to the same protocol used for the liver extracts to determine if ID2 could be detected. Whole testis extracts were resolved by Western blotting; however, ID2 remained undetectable in both nuclear and cytoplasmic fractions of the testis homogenate after loading as much as 500 μg of extract (Figure 16B).

Because the ID2 antiserum used did not allow detection of ID2 in testis extracts, a second antiserum was obtained from Dr. John Chen of Biocheck, Inc. The efficiency of immunoprecipitation of this new ID2 antiserum was tested using extracts from MSC-1 cells that were infected with an adenovirus expressing ID2. These extracts were immunoprecipitated with decreasing amounts of antibody. Immunoprecipitation of 10 μg of MSC-1 cells extracts containing over-expressed ID2 with 5 μl or 2 μl of ID2 antiserum resulted in 42% or 26%

recovery of protein when compared to a non-immunoprecipitated input by densitometry (Figure 16C). ID2 from over-expressed protein was clearly detectable at a 1:2000 dilution of ID2 antisera. Using the new, more specific ID2 antiserum, ID2 expression within Sertoli cell extracts was still below the level of detection (Figure 16C). Therefore, with the antisera available, it is not possible to determine by Western blotting whether ID2 protein expression levels change during Sertoli cell differentiation via Western blotting.

5.2.5 ENH Protein Levels Increase in Cultured Sertoli Cells During Differentiation

ENH associates with ID1, ID2, and ID3 through their HLH domains [231]. ENH expression increases during neural differentiation and sequesters ID2 in the cytoplasm preventing proliferation and the inhibition of bHLH-mediated transcription via ID2 [231]. As an actin cytoskeleton binding protein, ENH may also perform similar functions in Sertoli cells [230]. Immunofluorescence analysis of ENH in cultured Sertoli cells revealed a dramatic increase in ENH immunoreactivity in the cytoplasm of Sertoli cells between 5 and 20 days after birth (Figure 17). The up-regulation of ENH in the cytoplasm of differentiated Sertoli cells and

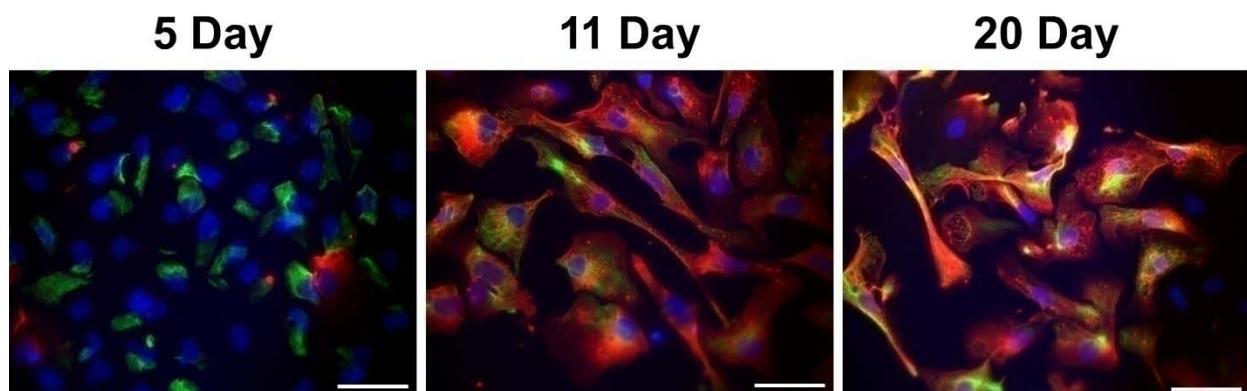


Figure 17. ENH Protein is Up-Regulated During Differentiation in Cultured Sertoli Cells.

Immunofluorescence of cultured 5, 11, and 20 day-old Sertoli cells was performed with antiserum recognizing ENH as visualized with Alexa 488 fluorophore (red). Antisera against vimentin was used as a Sertoli cell-specific marker as visualized with a Cy5 fluorophore (green). Hoechst stains nuclei (blue). Scale bars = 100 μ m.

the reduction of ID expression in the nucleus is consistent with the idea that localization of ID proteins in the cytoplasm of differentiated Sertoli cells relieves ID-mediated inhibition of USF binding to E-box motifs. Possibly, this relief occurs through the sequestration of ID proteins in the cytoplasm of differentiating Sertoli cells by ENH. Further studies are needed to test this hypothesis.

5.3 CONCLUSIONS

The binding of USF E-box proteins increases during Sertoli cell differentiation to E-box motifs. It was hypothesized that during Sertoli cell proliferation, ID proteins, known inhibitors of E-box binding proteins, inhibit USF1 and USF2 binding to E-box motifs. Studies presented here did not directly address this hypothesis but rather attempted to characterize ID protein expression during Sertoli cell differentiation. Direct examination of the hypothesis was not completed due to technical difficulties that will be addressed below.

Analysis of *Id* gene expression within Sertoli cells during differentiation were examined by qPCR analysis. Based on the hypothesis, it was expected that *Id* mRNA levels would decrease during Sertoli cell differentiation. However, instead of observing decreased *Id* mRNA expression during differentiation, the levels of all *Id* mRNAs either remained constant or increased between 5 and 11 days after birth (Figures 12 and 13; Tables 6 and 7). In isolated rat Sertoli cells in culture or assayed immediately after isolation, *Id1* mRNA levels are maintained between 5 and 11 days after birth (Figure 12A, 13A; Table 6, Rows 1-2; Table 7, Rows 1-2), whereas *Id3* mRNA levels increased 11 days after birth (Figure 12C, 13C; Table 6, Rows 1-2; Table 7, Rows 1-2). However, both *Id1* and *Id3* mRNA levels decreased 20 days after birth in

cultured Sertoli cells (Figure 13A,C; Table 7, Row 3). *Id2* and *Id4* mRNA levels increase from 5 to 11 days after birth in immediately assayed Sertoli cells (Figure 12B,D; Table 6, Rows 1-2) but the levels of these mRNAs do not change significantly in cultured cells (Figure 13B,D; Table 7, Rows 1-2). Rather, only *Id2* mRNA levels increase in cultured cells isolated from 20 day-old rats (Figure 13D; Table 7, Row 3). The discrepancy in the timing of *Id2* up-regulation for the two cell models is not clear, but the delayed elevation of *Id2* mRNA levels in cultured cells may be related to short-term loss of contact inhibition and the resumption of proliferation during the initiation of Sertoli cell culture [130]. In contrast, *Id1* and *Id3* mRNA levels decreased dramatically [$84 \pm 3\%$ and $66 \pm 3\%$ ($p < 0.05$ for both), respectively] during the later stages of differentiation between 11 and 20 days after birth (Figure 13A,C; Table 7, Row 3). Efficiency-corrected ΔCt analysis revealed comparable results to the $\Delta\Delta\text{Ct}$ analysis and revealed that only *Id2* mRNA relative quantities were expressed at comparable quantities to *Usf1* in both cell models (Table 6, Row 7-8; Table 7, Rows 10-12). *Id3* mRNA relative quantities were expressed in similar quantities to *Usf1* in cells assayed immediately after isolation only (Table 6, Rows 7-8). These data suggest that lower *Id* mRNA expression and subsequent lower levels of ID1 and ID3 proteins could contribute to increased USF DNA binding in Sertoli cells isolated from 20 day-old rats. Taken in sum, these data suggest that changes in *Id* mRNA expression do not account for the increase in DNA binding of USF1 and USF2 during Sertoli cell development between 5 and 11 days after birth.

ID2 protein levels were examined to determine whether protein levels reflected mRNA levels. In contrast to the qPCR results, immunofluorescence studies revealed that ID2 protein levels decreased within Sertoli cells during differentiation (Figure 14). Although the Sertoli cells from the three developmental time points were isolated, cultured, and fixed concurrently, it is

possible that differing cell morphologies contribute to the changes detected in ID2 immunostaining. Nevertheless, quantitation of immunostaining intensity supports the idea that ID2 levels decrease within the nucleus and the whole cell by 20 days after birth (Figure 15). Interestingly, the decrease in ID2 immunostaining is coincident with the increase in *Id2* mRNA levels (Figure 13D). It is possible that mRNA production is stimulated to compensate for a down-regulation in ID2 protein during differentiation; however, further studies are required to examine this hypothesis. Overall, in contrast to the *Id2* mRNA expression findings, the immunostaining results raise the possibility that decreasing ID2 protein expression contributes to the increase in USF binding to E-box motifs during Sertoli cell differentiation.

Western blot analysis of ID2 protein levels were conducted to reconcile the conflicting results found by mRNA and immunofluorescence analysis in isolated Sertoli cells (Figure 16). A protocol for detection of ID2 proteins by Western blot was established with liver and over-expressed ID2 in MSC-1 cells [246] (Figure 16A,C). Despite having an established Western blot protocol, ID2 protein could not be detected in extracts from isolated Sertoli cells or whole testis (Figure 16B,C). Therefore, Western blotting studies cannot be used to assess ID protein expression during Sertoli cell differentiation with the reagents available for these studies. Further studies addressing ID protein levels in Sertoli cells will require greater sensitivity and specificity. Sensitivity could be increased through concentration of protein from large amounts of tissue or cells. Metabolic labeling of cultured Sertoli cells or whole testis could also be used to increase detection abilities. However, any assay will be limited by the lack of specificity of the antibodies used in this dissertation. The acquisition of the more specific antisera from Biocheck, Inc. provides one avenue for continued studies (Figure 16C). The inability to conduct Western blots hinders studies of USF and ID2 protein-protein interactions which are necessary to definitively

confirm if ID2 can bind to USF1 or USF2. Recent studies in Sertoli cells have shown that ID2 can inhibit USF DNA binding and USF-mediated activation of the *Fshr* promoter in cells from 15 day-old rats [244].

With the possibility raised that ID2 may inhibit USF binding during proliferation, the down-regulation of ID2 protein levels during Sertoli cell differentiation was preliminarily evaluated. ID2 degradation in Sertoli cells has not been examined previously. It was hypothesized that the decrease of ID2 protein levels that was observed may be accounted for by sequestration and degradation within the cytoplasm. ENH could function to sequester ID proteins in the cytoplasm [230, 231]. ENH was found to be up-regulated within the cytoplasm of Sertoli cells during differentiation (Figure 17). This data suggests that increased ENH expression within Sertoli cells is consistent with the idea that ENH could sequester ID2 within the cytoplasm. However, due to the inability to conduct ID2 Westerns, determination of ENH interactions with ID2 in Sertoli cells was not possible. Also, the degradation of ID2 protein cannot be evaluated in the presence of increasing amounts of ENH.

A model has been proposed regarding the onset of Sertoli cell differentiation. During proliferation there are low levels of USF1 present within Sertoli cells and that with the onset of differentiation, USF1 levels are enhanced. The induction of USF1 levels within the cell contributes to increased USF binding to E-box motifs which may lead to transcriptional activation of USF target genes within Sertoli cells. ID proteins were investigated as a potential regulatory mechanism of USF binding. ID proteins were hypothesized to inhibit USF binding during proliferation. Immunofluorescence data raised the possibility that ID2 may inhibit USF binding in proliferating Sertoli cells and that the up-regulation of ENH during differentiation may cause the down-regulation of ID2 to allow USF binding. However, this data is correlative

and requires further studies to confirm the hypothesis that ID proteins inhibit USF1 and USF2 binding in proliferating Sertoli cells.

6.0 THYROID HORMONE: REGULATOR OF SERTOLI CELL NUMBER

Hypothesis: Thyroid hormone induces differentiation through the induction of USF DNA binding.

6.1 INTRODUCTION

A central dogma of male fertility is that the final number of Sertoli cells dictates the total number of sperm that can be produced within the testis [1, 79]. In rodents, all Sertoli cell proliferation occurs during the fetal and neonatal period, predominantly during the first two postnatal weeks ending between 15 and 21 days in rat [78, 85]. How Sertoli cells decide to stop proliferating and begin differentiating is largely unknown. Postnatal Sertoli cells are direct targets of gonadotropins, estrogens, thyroid hormones, and androgens (reviewed in [4, 16, 91, 99, 100, 112, 248-254]). However, thyroid hormone appears to be the most direct regulator of Sertoli cell proliferation and differentiation (reviewed in [252]).

6.1.1 Thyroid Hormone Production in the Rat

Thyroid hormone is secreted under control of the hypothalamo-pituitary-thyroid axis (reviewed in [255]). Thyroid-releasing hormone (TRH) from the hypothalamus is detectable at

birth in the rat and peaks between 16 and 28 days after birth in the rat [256]. TRH stimulates release of thyroid-stimulating hormone (TSH) from the pituitary, which peaks between 7 and 22 days after birth in the rat [256]. TSH then acts on the thyroid to stimulate iodine uptake, thyroglobulin production, and release of iodothyronines from the gland [255]. Iodinated thyroglobulin is degraded intracellularly to release thyroxine (T4) and triiodothyronine (T3) [257]. When stimulated with TSH, stored thyroglobulin from the colloid of the thyroid gland are phagocytosed or endocytosed into the thyrotropes surrounding the colloid stores [255]. Thyroglobulin is degraded through the lysosomal pathway to result in the emergence of T4 which can be further cleaved to T3 by type I or type II 5'-iodothyronine deiodinases within the thyroid or in target tissues [252, 257]. In the rat, T4 levels increase between 4 and 16 days to 6 $\mu\text{g}/100$ ml of serum then decreases to adult levels of 4 $\mu\text{g}/100$ ml of serum [256]. T3 peaks 28 days after birth at 108 ng/100 ml of serum then decreases to adult levels at 70 ng/100 ml of serum [256].

6.1.2 Thyroid Hormone Receptors and Deiodinases in the Testis

The testis is a target tissue of thyroid hormones. Thyroid hormone receptors (TR) are present in high quantities in neonatal Sertoli cells and at lower levels in adult Sertoli cells of the rat (reviewed in [88, 252]). TR α 1 is present in proliferating Sertoli cells and decreases with the onset of differentiation [258, 259]. TR α 2, TR α 3, and TR β 1 are low during the developmental period of Sertoli cells [258]. TR α 1 and TR α 2 co-localize to Sertoli cells in prepubertal human testes but TR α 2 is always expressed at higher levels and the levels of TR α 1 decrease with age [260]. TR β is not detected in human testes [260]. TR α 1 is also in germ cells, from

spermatogonia to mid-cycle pachytene spermatocytes [258]. All three types of deiodinases (D) are expressed at different levels within the testis during postnatal development [252]. D3 levels are expressed highest in the testis during postnatal development and then dramatically decrease in the adult [261]. D1 and D2 are also expressed within the testis however D2 expression is highest during Sertoli cell differentiation, when the levels of TRs are also highly expressed [261]. In adults, D2 expression is limited mostly to elongated spermatids whereas expression in other testicular cells types is almost completely abrogated [262]. Expression levels of the deiodinases have not been studied in extracts specific to Sertoli cells.

6.1.3 Thyroid Hormone Effects on Sertoli Cell Number

Thyroid hormone was implicated in determining Sertoli cell number during studies of hyper- and hypothyroid animals (reviewed in [88, 100, 254, 263]). Hyperthyroidism enhanced Sertoli cell differentiation and shortened the period of Sertoli cell proliferation, resulting in accelerated tubular lumen formation, a smaller testis and a lower daily sperm count [264]. The effects of hypothyroidism were induced neonatally using propylthiouracil (PTU), which blocks the synthesis of thyroid hormones [265-267]. Hypothyroidism induced in neonatal animals results in an overall increase in the number of Sertoli cells in rats despite a delay in testicular growth, germ cell maturation, and tubule lumen formation [268-270]. The increase in Sertoli cell number also resulted in an increase in testicular size and a higher daily sperm count [269-271].

The prolonged period of Sertoli cell proliferation induced by PTU treatment corresponded with prolonged expression of genes associated with Sertoli cell immaturity, including *Amh* and *Thra* (TR α) [272]. Administration of exogenous T3 to these PTU-treated animals caused *Amh* and *Thra* levels to return to control levels [272]. In the presence of PTU

alone, genes representative of Sertoli cell differentiation, *Shbg*, clusterin, and inhibin β B, were delayed in their expression patterns [272]. However, the mRNA levels of Sertoli cell differentiation-associated genes sulfated glycoprotein-1, *Trf*, and inhibin α did not differ in PTU-treated rats when compared with control untreated animals [272].

In sum, the studies of hypo- and hyperthyroid animals suggest that thyroid hormone is able to regulate the period of Sertoli cell proliferation. Thyroid hormone actions normally effect Sertoli cell proliferation between 4 and 8 days after birth [269]. However, artificially induced early onset of thyroid hormone actions inhibits Sertoli cell proliferation prematurely. By contrast, lack of a thyroid hormone signal between 4 and 8 days after birth allows Sertoli cells to continue proliferating beyond the normal 20 days after birth. Studies of the thyroid hormone molecular actions in Sertoli cells are needed to elucidate the molecular mechanisms responsible for altering the timing of the switch between proliferation and differentiation in Sertoli cells during postnatal development.

6.1.4 Molecular Mechanisms of Thyroid Hormone in Sertoli Cells

The regulation of p21^{CIP1} and p27^{KIP1} in the testis are under the control of thyroid hormone and may provide some insights into a potential mechanism involved in the switch between Sertoli cell proliferation and differentiation [273-275]. T3 was found to induce p27^{KIP1} and p21^{CIP1} levels in neonatal Sertoli cells [273-275]. T3 was unable to induce p27^{KIP1} expression in *Thra*^{-/-} animals, suggesting that thyroid hormone is required to induce this cell cycle inhibitor [276]. Administration of PTU to *Cdkn1a*^{-/-} or *Cdkn1b*^{-/-} animals is still able to induce an increase in testis size compared with untreated knockout controls, suggesting that

p21^{CIP1} and p27^{KIP1} are not the only effectors downstream of thyroid hormone to inhibit Sertoli cell proliferation and induce Sertoli cell differentiation [263, 274].

p21^{CIP1} and p27^{KIP1} proteins are degraded through association with the E3 ubiquitin ligase S-phase kinase-associated protein 2 (SKP2) [277, 278]. SKP2-mediated degradation of p21^{CIP1} and p27^{KIP1} may allow Sertoli cell proliferation until the onset of thyroid hormone actions [263]. A lack of SKP2 decreases testis weight by 80% and daily sperm production by 85% suggesting that p21^{CIP1} and p27^{KIP1} may inhibit the cell cycle prematurely within Sertoli cells in the absence of SKP2 [263]. The T3-mediated inhibition of Sertoli cell growth occurs coincidentally with increased expression of connexin 43 [also known as gap junction protein, alpha 1 (GJA1)] in the Sertoli cell plasma membrane [279]. Increasing GJA1 allows an increase in gap junction communication and blockage of gap junction communication inhibits T3-mediated halting of Sertoli cell proliferation [279]. Studies of Sertoli cell-specific *Gjal*^{-/-} mice, display increased Sertoli cell proliferation and delayed Sertoli cell maturation in adulthood resulting in a loss of germ cells and reduced fertility [252, 279]. Further studies of thyroid hormone contributions to the onset of Sertoli cell differentiation need to be conducted. Studies presented in this dissertation explore the ability of T4 to increase USF DNA binding during Sertoli cell differentiation.

6.2 RESULTS

6.2.1 Thyroid Hormone Induces USF-DNA binding in Proliferating Sertoli Cells

To determine whether thyroid hormone, a known Sertoli cell differentiating agent, could increase the DNA binding of USF proteins, cultured Sertoli cells were incubated with vehicle or T4 (10^{-6} M). T4 was used because T4 levels are 100-fold greater than T3 in the testis during the period of Sertoli cell differentiation [256]. Furthermore, Sertoli cells express iodothyronine deiodinase type 2 (D2) that converts T4 to T3, the form able to interact with the thyroid hormone receptor [262, 280]. Finally, by using T4, the effects of both T3 and T4 can be assayed. The dose of T4 that was used is 12.5-fold greater than the peak T4 serum level (8×10^{-8} M) reached in rats during differentiation [256].

A 24 hour stimulation of cultured Sertoli cells from 5 day-old rats with T4 resulted in induced protein binding to the USF consensus and *Fshr* promoter E-box probes (Figure 18A,B). T4 stimulation for 24 hours was unable to further induce E-box protein binding in Sertoli cells from 11 and 20 day-old rats. At 11 days, T4 caused a significant reduction in protein binding to the USF consensus and *Fshr* promoter E-box probes (Figure 18A,B). Supershift analysis of 5 day-old Sertoli cells treated with T4 confirmed that USF1 and USF2 were the proteins whose DNA binding was augmented (Figure 18C). T4 stimulation of Sertoli cells induced the formation of a second, larger DNA protein complex to the USF consensus binding sites (Figure 18C). The components of the second, upper band are not known and supershift results do not identify a component of the complex (Figure 18C). These studies suggest that T4 is able to promote USF DNA binding in Sertoli cells prior to the commitment to differentiation.

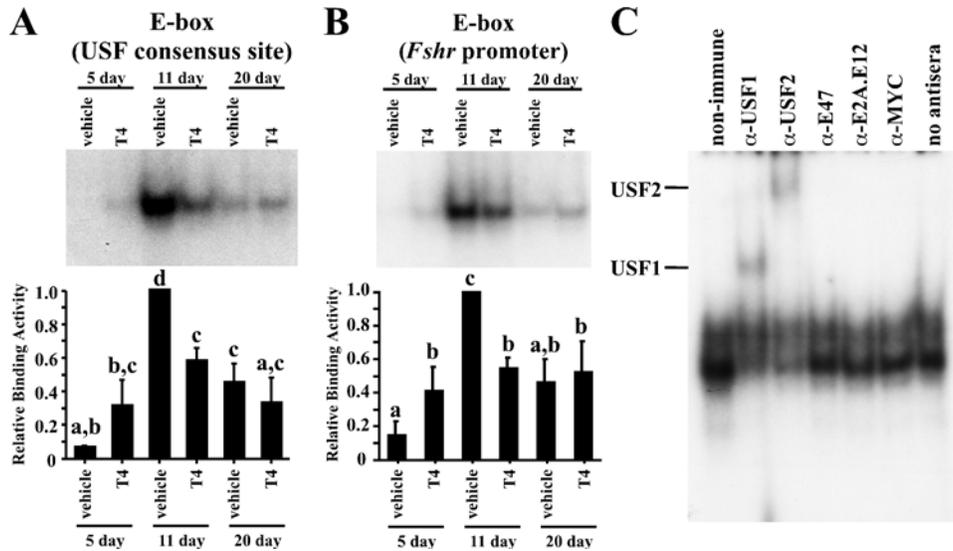


Figure 18. Thyroid Hormone Induces USF DNA Binding in 5 Day-old Cultured Sertoli Cells.

In EMSAs, radiolabeled probes containing a consensus E-box that is selective for USF binding (A) or a region of the *Fshr* promoter containing an E-box known to bind USF proteins (B) were incubated with nuclear extracts isolated from cultured 5, 11, and 20 day-old Sertoli cells that were treated with vehicle or T4 (10^{-6} M) for 24 hours. The results of vehicle treated cells were previously shown in Figure 6. Representative images of the resulting DNA protein complexes are shown, and quantitation of the mean (\pm SE) of three independent experiments is provided. Free probe has been run off the gel. The relative binding activity for each condition is normalized to that of vehicle-treated 11 day-old Sertoli cells (=1). Values with different lowercase letters differ significantly ($p < 0.05$). C) USF1 and USF2 are responsible for the increase in DNA-binding activity during Sertoli cell differentiation. DNA-protein complexes from nuclear extracts isolated from cultured 11 day-old Sertoli cells were incubated with non-immune sera or antisera for USF1, USF2, E47, E2A, or MYC. Supershifted DNA protein complexes containing USF1 or USF2 are indicated. Exposure times for all complexes are less than 48 h.

6.2.2 Thyroid Hormone Does Not Greatly Alter the Expression of mRNAs Encoding E-box or ID Proteins

The ability of thyroid hormone to regulate E-box and ID protein gene expression was tested using cultured Sertoli cells. Using qPCR with $\Delta\Delta$ Ct analysis, T4 stimulation (10^{-6} M) did not significantly alter the expression of *Usf2*, *E47*, or *Trf* compared with basal levels (Figure 19B-D). However, T4 stimulation for 24 hours increased *Usf1* mRNA levels by approximately $19.8 \pm 3.8\%$ ($p < 0.05$) in cells isolated from 11 day-old rats (Figure 19A). This increase in *Usf1* mRNA does not correlate to the T4-induced decrease in USF binding to E-box consensus sites (Figure 18A,B). However, basal *Usf1* and *Usf2* mRNA levels are induced 11 days after birth

(Figure 19A,B) coincident with increased DNA binding 11 days after birth when compared with extracts from 5 day-old rats (Figure 18A,B).

Id2 and *Id3* mRNAs in are induced in isolated Sertoli cells from 6 day-old rats that were cultured for 4 days in the presence of T3 [275]. Stimulation of the cultured Sertoli cells with T4 for 24 hours resulted in a slight further stimulation of *Id2* mRNA levels ($21 \pm 12\%$, $p < 0.05$) in cells isolated 11 days after birth but not from cells isolated 5 or 20 days after birth (Figure 20B). It is possible that an increase in ID2 levels

could contribute to the decrease in USF binding 11 days after birth in the presence of thyroid hormone. T4 stimulation did not result in any significant changes to *Id1*, *Id3*, or *Id4* basal mRNA expression at any time point (Figure 20A,C-D). The mRNA levels of the housekeeping gene, *Ppia*, did not change upon stimulation with T4 (data not shown).

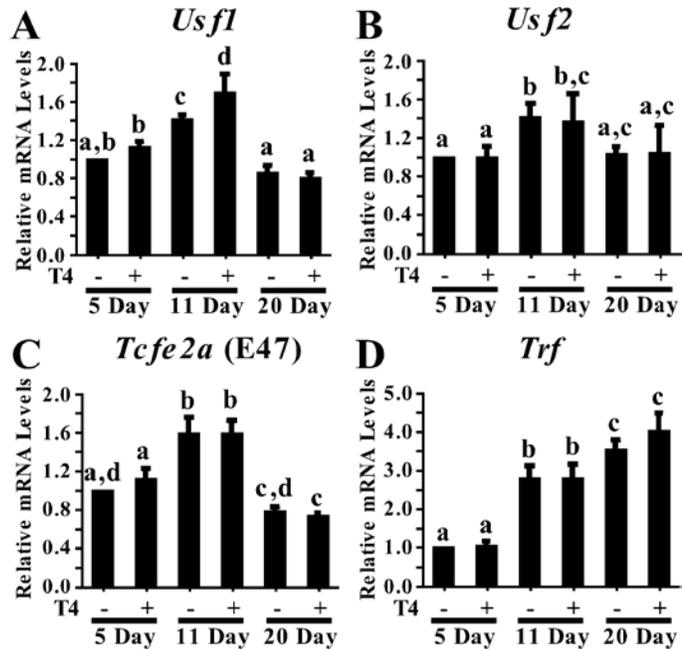


Figure 19. Thyroid Hormone Induces *Usf1* mRNA levels 11 Days After Birth in Cultured Sertoli Cells.

mRNA isolated from cultured 5, 11, and 20 day-old Sertoli cells treated with vehicle (-) or T4 (10^{-6} M) for 24 hours were analyzed by qPCR using primers for *Usf1* (A), *Usf2* (B), *Tcfe2a (E47)* (C), and *Trf* (D). Vehicle treated values were previously shown in Figure 9. Data was analyzed using the $\Delta\Delta C_t$ method and quantitation of the mean (\pm SE) of three individual experiments for each condition is provided for each primer set. The relative mRNA levels were normalized to *Ppia* levels and made relative to untreated 5 day-old Sertoli cells (=1). Values with different lowercase letters differ significantly ($p < 0.05$).

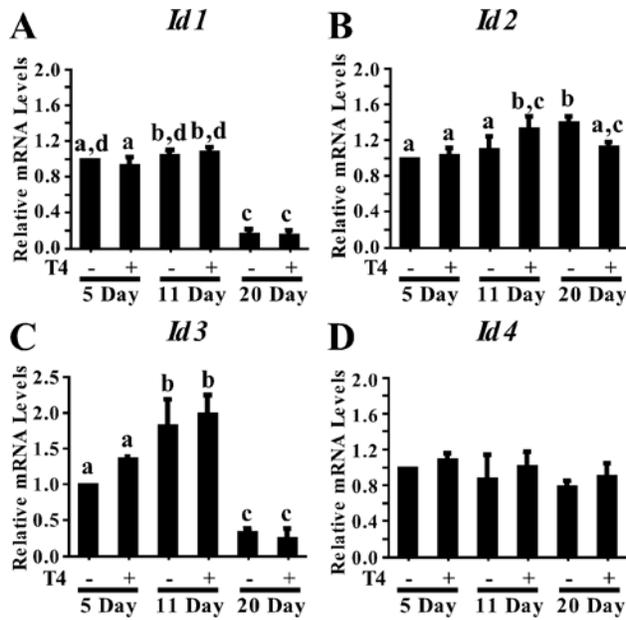


Figure 20. Thyroid Hormone Does Not Alter ID Protein mRNA Levels in Cultured Sertoli Cells.

mRNA isolated from cultured 5, 11, and 20 day-old Sertoli cells treated with vehicle (-) or T4 (10^{-6} M) for 24 hours were analyzed by qPCR using primers for *Id1* (A), *Id2* (B), *Id3* (C), and *Id4* (D). Vehicle-treated values were previously shown in Figure 13. Data was analyzed using the $\Delta\Delta C_t$ method and quantitation of the mean (\pm SE) of three individual experiments for each condition is provided for each primer set. The relative mRNA levels were normalized to *Ppia* levels and made relative to untreated 5 day-old Sertoli cells (=1). Values with different lowercase letters differ significantly ($p < 0.05$).

T4 stimulation of Sertoli cells from 5 day-old rats for 24 hours resulted in increased USF1 expression in the nucleus and a staining pattern similar to that observed in cells cultured from 20 day-old animals (Figure 21). USF2 immunostaining intensity was similar in both the cytoplasm and nuclei of Sertoli cells in the presence and absence of thyroid hormone (Figure 21, left vs. right panels). These data support the hypothesis that thyroid hormone is able to induce USF1 binding to E-box binding sites due to an increase in USF1 protein within the nucleus of proliferating Sertoli cells.

6.2.3 Thyroid Hormone can Induce Premature Nuclear Localization of USF1 in Cultured Sertoli Cells from 5 Day-old Rats

To determine whether T4 could induce USF protein expression in cultured Sertoli cells, immunofluorescence analysis was conducted on cultured primary Sertoli cells from 5 day-old rats. Cells were stained with antisera against USF1 (Figure 21, top) or USF2 (Figure 21, bottom). Nuclei were stained with Hoechst. Antiserum against vimentin was used as a Sertoli cell-specific marker (Appendix A, Figures 34 and 35).

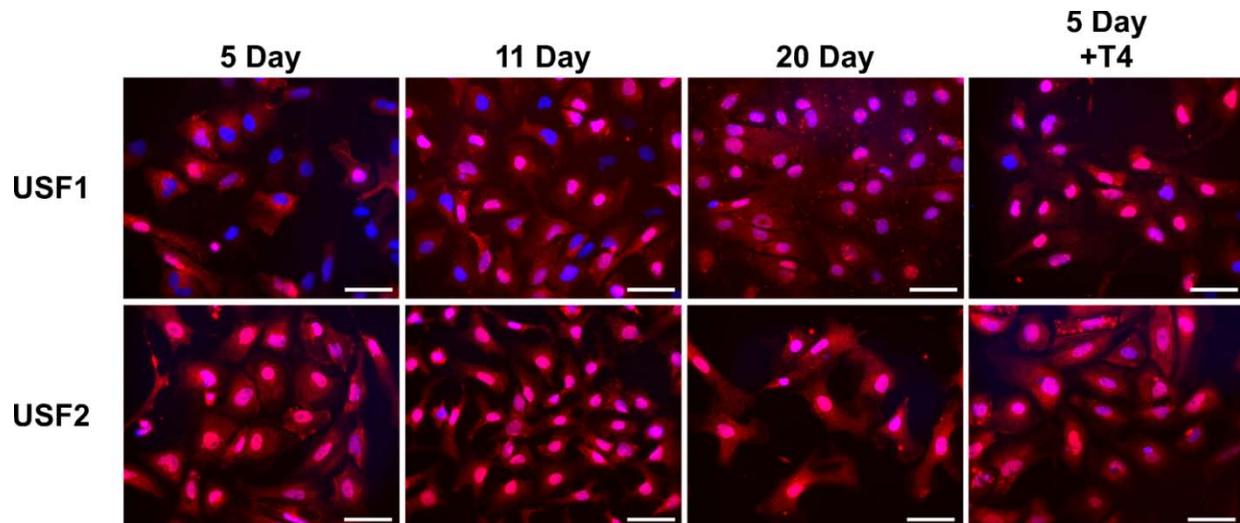


Figure 21. USF1 Protein is Up-regulated in the Nucleus of 5 Day-old Sertoli Cells in the Presence of Thyroid Hormone.

Immunofluorescence of cultured 5, 11, and 20 day-old Sertoli cells was performed with antisera recognizing USF1 (top row) or USF2 (bottom row) as visualized with Alexa 488 fluorophore (red). Hoechst stains nuclei (blue). Cultured 5 day-old Sertoli cells were incubated with or without T4 (10^{-6} M) for 24 hours prior to fixation and staining as above. Panels of vehicle treated cells were previously shown in Figure 11 and are provided here again for reference. Scale bars = 100 μ m. Control immunostaining with the Sertoli cell specific anti-vimentin antiserum is provided in Appendix A, Figures 34 and 35.

6.2.4 Thyroid Hormone Induces ENH Expression in 5 Day-old Sertoli Cells

ENH protein levels were up-regulated in cultured Sertoli cells during differentiation (Figure 17). To determine whether thyroid hormone was able to induce the up-regulation of ENH protein within cultured proliferating Sertoli cells, immunofluorescence analysis of ENH levels in 5 day-old Sertoli cells was evaluated in the presence and absence of thyroid hormone for 24 hours (Figure 22). Stimulation of cultured Sertoli cells from 5 day-old rats with thyroid hormone increased ENH expression to levels similar to 20 day-old cultured Sertoli cells predominantly within the cytoplasm. These data suggest that thyroid hormone can up-regulate ENH during Sertoli cell differentiation.

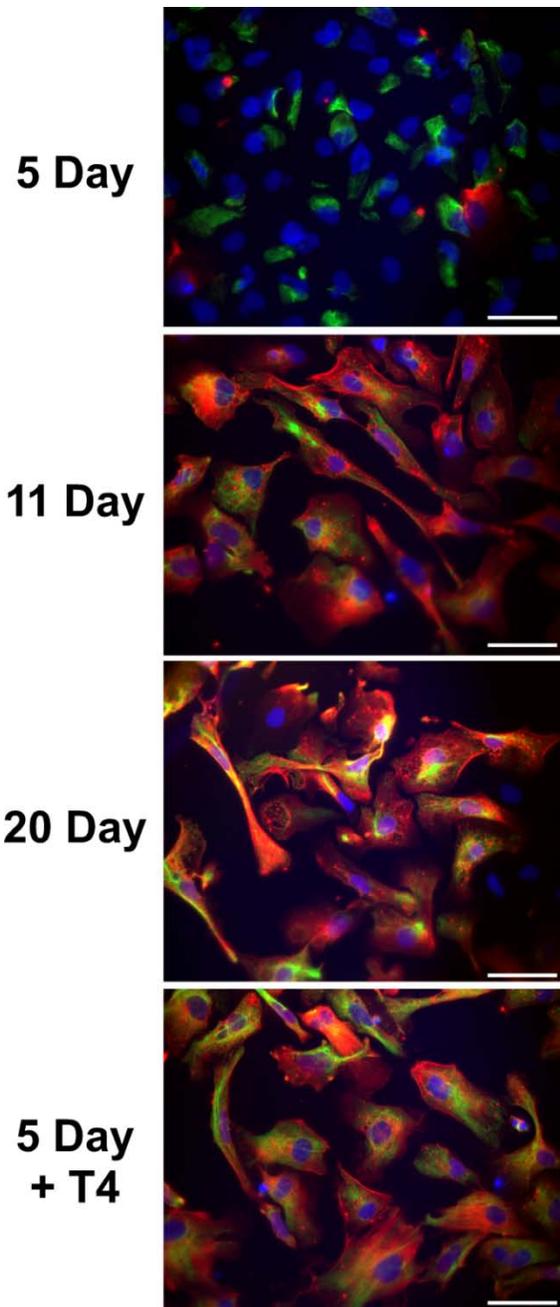


Figure 22. ENH is Up-regulated in the Presence of Thyroid Hormone in 5 Day-old Cultured Sertoli Cells.

Immunofluorescence of cultured 5, 11, and 20 day-old Sertoli cells was performed with antiserum recognizing ENH as visualized with Alexa 488 fluorophore (red). Antiserum against vimentin was used as a Sertoli cell-specific marker as visualized with a Cy5 fluorophore (green). DAPI stains nuclei (blue). Cells were cultured in the presence or absence of 10^{-6} M T4 for 24 hours. Vehicle treated controls were previously shown in Figure 17.

6.3 CONCLUSIONS

Thyroid hormone, a known inducer of Sertoli cell differentiation, was proposed to induce USF binding to E-box motifs. T4 stimulation of cultured Sertoli cells induced USF DNA binding in undifferentiated Sertoli cells from 5 day-old rats (Figure 18A,B). T4 also induced USF1 protein expression within the nucleus of proliferating Sertoli cells to the levels of cells that have already differentiated, 20 days after birth (Figure 21). Although T4 did not alter *Id* gene mRNA levels (Figure 20), T4 induced the up-regulation of an inhibitor of ID proteins, ENH, in 5 day-old Sertoli cells (Figure 22). Together, these data support the hypothesis that T4 can promote Sertoli cell differentiation through the induction of USF DNA binding due to the enhanced expression of ENH to sequester the ID2 inhibitor. Further evaluation of protein levels by Western blotting are required to support the immunofluorescence and mRNA data.

At later time points, in cells that were

differentiating (11 days after birth) or differentiated (20 days after birth), T4 had alternative effects. T4 stimulation of cultured 11 day-old Sertoli cells decreased USF binding to a consensus E-box probe and the *Fshr* probe (Figure 18A,B). The decrease in binding activity occurred in correlation with an induction of *Id2* mRNA levels (Figure 20B). In contrast, *Usf1* mRNA levels were induced by T4 in cells isolated 11 days after birth (Figure 19A). These results are difficult to interpret because protein levels were not assayed in cells from 11 day-old rats treated with T4. It is possible that if USF1 levels are increasing, ID2 levels may be sufficient to inhibit USF DNA binding or that other mechanisms, such as post-translational modifications, may contribute to decreased USF binding [281-284]. T4 did not appear to have any effects on E-box or ID protein mRNA levels or USF binding in Sertoli cells from 20 day-old rats.

The timing of the T4-mediated increases in USF DNA binding is consistent with previous data showing that thyroid hormone promotes Sertoli cell differentiation between 4 and 8 days after birth in the rat [264, 269]. Returning to the model of USF-mediated Sertoli cell differentiation, thyroid hormone can now be added as an inducer of USF binding in proliferating Sertoli cells. During proliferation, low levels of USF1 are present within Sertoli cells and ID proteins may be elevated to inhibit USF binding. Signals, such as thyroid hormone, then activate Sertoli cells to begin differentiating. ID protein actions may be inhibited through the up-regulation of ENH. USF binding is up-regulated at E-box motifs, which is expected to activate transcription of genes required for differentiated Sertoli cell functions. Thyroid hormone is one of many signals that could induce Sertoli cell differentiation. The effects of other hormones known to contribute to Sertoli cell differentiation, including T and FSH (reviewed in [100, 112, 253, 254]), need to be evaluated for their ability to induce USF DNA binding in undifferentiated Sertoli cells.

7.0 UP-REGULATION OF USF TARGET GENES, *Nr5a1* AND *Shbg*, CORRELATES WITH THE ONSET OF DIFFERENTIATION IN RAT SERTOLI CELLS

Hypothesis: USF1 and USF2 up-regulate target genes during Sertoli cell differentiation by binding to E-box consensus motifs within target gene promoter regions.

7.1 INTRODUCTION

7.1.1 Known USF Target Genes in Sertoli Cells

USF1 and USF2 have been identified as potential mediators of Sertoli cell differentiation signals (Chapter 3 and 4). E-box motifs having consensus USF binding sites are present in the promoters of a number of genes that are markers for differentiated Sertoli cell status including *Fshr*, nuclear receptor subfamily 5, group A, member 1 [*Nr5a1*, also known as steroidogenic factor 1 (SF1)] and GATA binding protein 4 (*Gata4*) [168, 171-173]. USF1 and USF2 binding to the *Fshr* promoter has already been discussed in previous chapters (Figures 5 and 6). USF1 and USF2 contribute to an increase in DNA binding to the E-box within the *Fshr* promoter during the postnatal differentiation of Sertoli cells (Figure 5). The transcription of *Nr5a1* requires USF1 and

USF2 within Sertoli cells and Leydig cells [171]. *Nr5a1* expression within the rat testis is maintained through 21 days after birth and then decreases in adulthood when NR5A1 is detected primarily in Leydig cells [285]. *Gata4* contains an E-box through which USF1 and USF2 induce transcription [286]. However, the expression pattern of *Gata4* within the testes remains controversial. Studies investigating the levels of *Gata4* mRNA in whole testis revealed that *Gata4* mRNA expression is maintained through days 7 to 14 after birth and then decreases [287, 288]. Immunohistochemical studies suggest that GATA4 is present through 25 days after birth albeit at a reduced level after 14 days [287, 288]. However, studies conducted in germ cell-deficient mice suggest that observed reductions in GATA4 expression were due to dilution by developing germ cells and found that GATA4 levels remained constant in Sertoli cells despite postnatal age or stage of spermatogenesis [289]. Both *Nr5a1* and *Gata4* encode transcription factors that can lead to downstream regulation of other key genes during the onset of differentiation in Sertoli cells.

7.1.2 E-box Regulated Genes that are Up-regulated During Differentiation in Sertoli Cells

Trf and *Shbg* have E-box motifs within their promoters and are expressed within the testis. Transferrin, secreted by Sertoli cells, facilitates iron transport to cells beyond the blood-testis barrier [28]. It has been shown that E47 regulates this promoter in fully-differentiated Sertoli cells [168]. However, increased binding of E47 to the E-box region of the *Trf* promoter does not occur during the switch between Sertoli cell proliferation and differentiation (Figure 4). *Shbg* expression is a marker of Sertoli cell differentiation and its product within the testis, androgen binding protein, functions to carry testosterone through the lumen of the seminiferous tubules and throughout the male reproductive tract [101, 103]. Three E-box motifs have been

detected within the *Shbg* promoter [245]. Studies in fully-differentiated Sertoli cells revealed that the proximal E-box (-36 to -31 bp) and the distal E-box (-268 to -263 bp) within the promoter are necessary for *Shbg* expression [245]. The third E-box overlaps with a CREB binding site and is capable of binding the bHLH protein, E47 [245]. ID1, an inhibitor of bHLH proteins, inhibits the responsiveness of the *Shbg* promoter to FSH and cAMP [245]. The identities of the bHLH proteins that are capable of binding to the distal and proximal E-boxes are not known. The USF-mediated regulation of *Fshr*, *Nr5a1*, *Gata4*, and *Shbg* during Sertoli cell differentiation is not known.

7.2 RESULTS

7.2.1 The DNA Binding of USF Proteins Increase During Sertoli Cell Differentiation on Target Gene Promoters

Because USF1 and USF2 contribute to increased binding at the *Fshr* promoter E-box (Figures 5 and 6), it is hypothesized that USF binding to the promoters of other USF target genes would also increase in differentiating Sertoli cells. The *Gata4* promoter was found to contain an E-box within -118 bp upstream of the transcription start site that is also regulated by USF1 and USF2 [286]. The *Nr5a1* promoter contains an E-box at -82 to -77 bp from the transcription start site and binds USF1 and USF2 in rat Sertoli cells and whole testis [171, 173]. Nuclear extracts prepared from Sertoli cells immediately after isolation from 5 and 11 day-old rats were incubated with radiolabeled, double-stranded oligonucleotide probes containing E-box motifs from the *Nr5a1* and *Gata4* promoters. For probes containing the USF

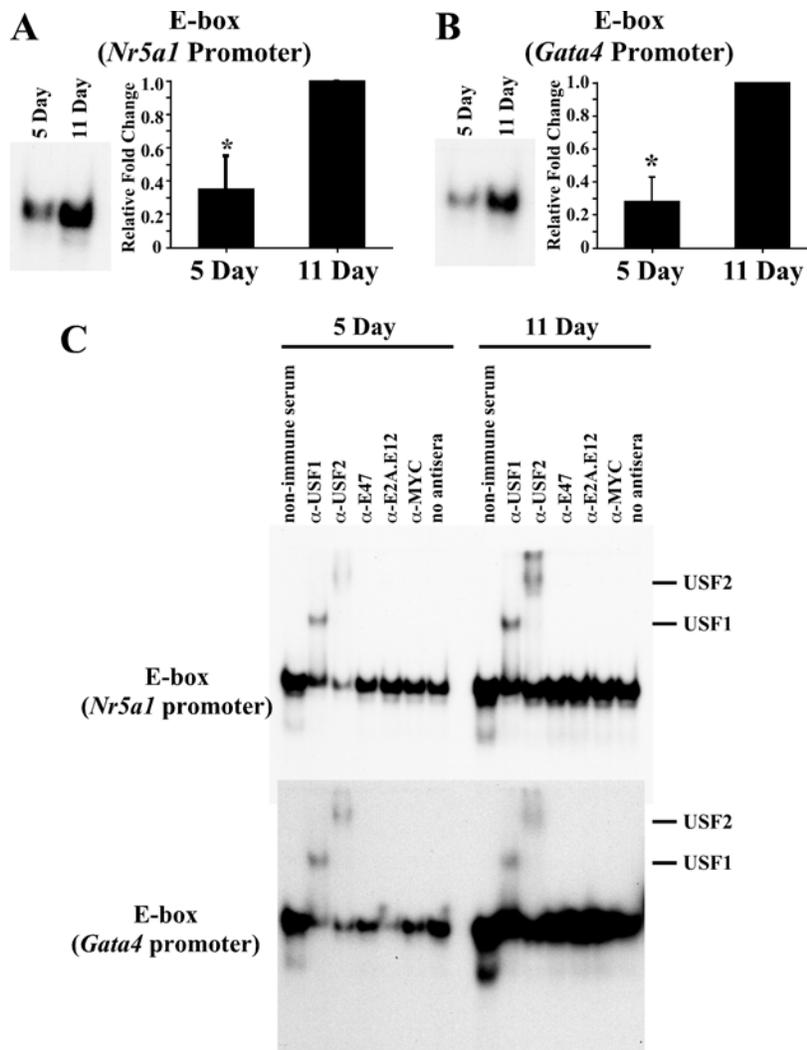


Figure 23. USF1 and USF2 DNA Binding Increases at E-box Motifs in the *Nr5a1* and *Gata4* Promoters in Sertoli Cells Assayed Immediately After Isolation.

In EMSAs, radiolabeled probes containing E-box regions of the *Nr5a1* promoter (A) or the *Gata4* promoter (B) were incubated with nuclear extracts of Sertoli cells immediately assayed after isolation from 5 and 11 day-old testis. Representative images of the resulting DNA-protein complexes are shown, and quantitation of the mean (\pm SE) of three independent experiments is provided. The relative binding for each condition is normalized to that of 11 day-old Sertoli cells (=1). Statistically significant differences ($p < 0.05$) are indicated by an asterisk (*). C) DNA-protein complexes from nuclear extracts of Sertoli cells isolated from 5 and 11 day-old Sertoli cells were incubated with non-immune sera or antisera for USF1, USF2, E47, E2A, or MYC immediately after isolation. Supershifted DNA-protein complexes containing USF1 or USF2 are indicated. Exposure times for all complexes are less than 48 h. Free probe was run off the gel.

binding site from the *Nr5a1* promoter or the *Gata4* promoter, protein binding was low in extracts from 5 day-old Sertoli cells but binding activity increased approximately 3-fold at both promoter regions at 11 days (Figure 23A, B). To confirm that USF proteins are responsible for forming the DNA-protein complexes, supershift studies were performed. Sertoli cell nuclear extracts from 5 and 11 day-old testes were preincubated with non-immune serum or antisera against USF1, USF2, E47, E2A, or MYC E-box binding transcription factors before incubation with probes containing the E-box regions

of the *Nr5a1* or *Gata4* promoters (Figure 23C). The supershift assay demonstrated that both USF antisera supershifted DNA-protein complexes, whereas the E47, E2A, and MYC antisera had little effect. These data confirm that USF1 and USF2 binding to E-box motifs within target promoters increases during Sertoli cell differentiation.

7.2.2 Expression of the USF Target Gene *Nr5a1* Increases During Sertoli Cell Differentiation

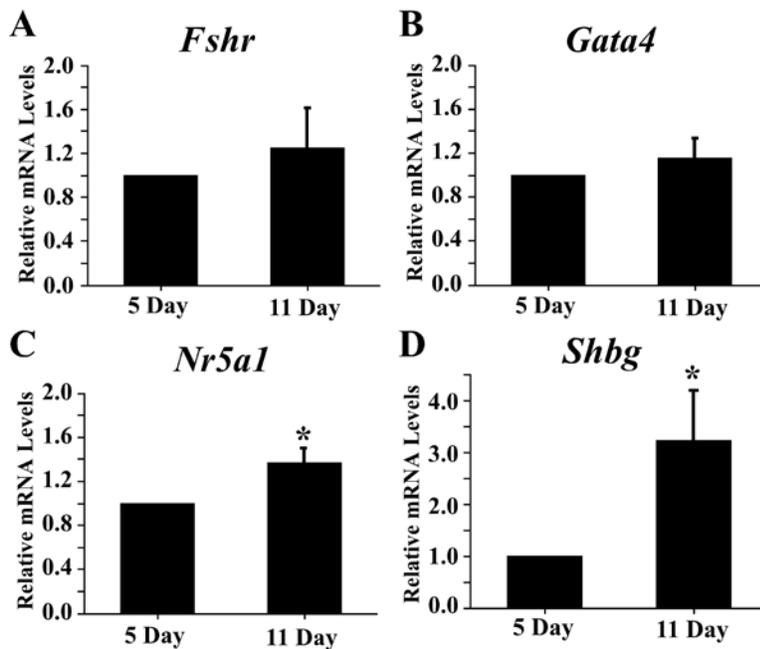


Figure 24. *Nr5a1* and *Shbg* mRNA Levels Significantly Increase During Differentiation in Sertoli Cells Assayed Immediately After Isolation.

The mRNAs isolated from 5 and 11 day-old Sertoli cells were analyzed by qPCR using primers for *Fshr* (A), *Gata4* (B), *Nr5a1* (C), and *Shbg* (D). Data were analyzed using the $\Delta\Delta C_t$ method, and quantitation of the mean (\pm SE) of three individual experiments for each condition is provided for each primer set. The relative mRNA levels were normalized to *Ppia* levels and were made relative to 5 day-old Sertoli cells (=1). Statistically significant differences ($p < 0.05$) are indicated by an asterisk (*).

maintained throughout Sertoli cell postnatal differentiation [18, 289]. *Nr5a1* is expressed in Sertoli cells and Leydig cells within the testis. The expression of NR5A1 peaks 7 days after

To determine whether increased USF DNA binding during Sertoli cell differentiation corresponded with increased USF target gene expression for Sertoli cell- or testis-specific genes, the expression of mRNAs encoding *Fshr*, *Gata4*, and *Nr5a1* were assayed during Sertoli development using qPCR with $\Delta\Delta C_t$ analysis. *Fshr* and *Gata4* are Sertoli cell-specific genes within the testes whose levels are known to increase or are

birth [285]. Assays of mRNA preparations from Sertoli cells immediately after isolation revealed that the mRNA levels of *Fshr* and *Gata4* do not increase between 5 and 11 days after birth in the rat (Figure 24A,B). In contrast, *Nr5a1* mRNA levels increased 1.37 ± 0.13 -fold ($p < 0.05$) from 5 to 11 days after birth (Figure 24C). For all studies, the mRNA levels of the housekeeping gene *Ppia* did not change over the course of differentiation (data not shown). Based on these data, the increase in DNA binding of USF1 and USF2 from Sertoli cells assayed immediately after isolation to the *Nr5a1* promoter E-box (Figure 23A) correlates to an increase in mRNA expression of *Nr5a1* (Figure 24A). These studies identify *Nr5a1* as a USF target gene during postnatal Sertoli cell differentiation between 5 and 11 days after birth.

7.2.3 The *Shbg* Promoter is a Novel Target of USF1 and USF2 Action Through Binding to the Proximal E-box

Shbg mRNA levels are known to increase during Sertoli cell differentiation. *Shbg* levels are expressed at low levels from 0 to 15 days after birth with increasing levels between 15 and 60 days after birth [272]. mRNA levels of *Shbg* were confirmed to increase 3.2 ± 1.0 -fold ($p < 0.05$) between 5 and 11 days after birth in Sertoli cells assayed immediately after isolation (Figure 24D). Previously, the only evidence for E-box involvement in the regulation of the *Shbg* gene suggested that E47 bound to the E-box between -129 and -124 bp upstream of the transcription start site within the promoter region in fully-differentiated rat Sertoli cells [245]. Therefore, to determine whether USF1 and USF2 were able to act on one of the two other E-box regions within the *Shbg* promoter, EMSA analysis of nuclear extracts from Sertoli cells immediately assayed after isolation from 5 and 11 day-old rats was conducted using probes containing E-box motifs for each of the three E-box regions within the *Shbg* promoter (Figure 25). For the

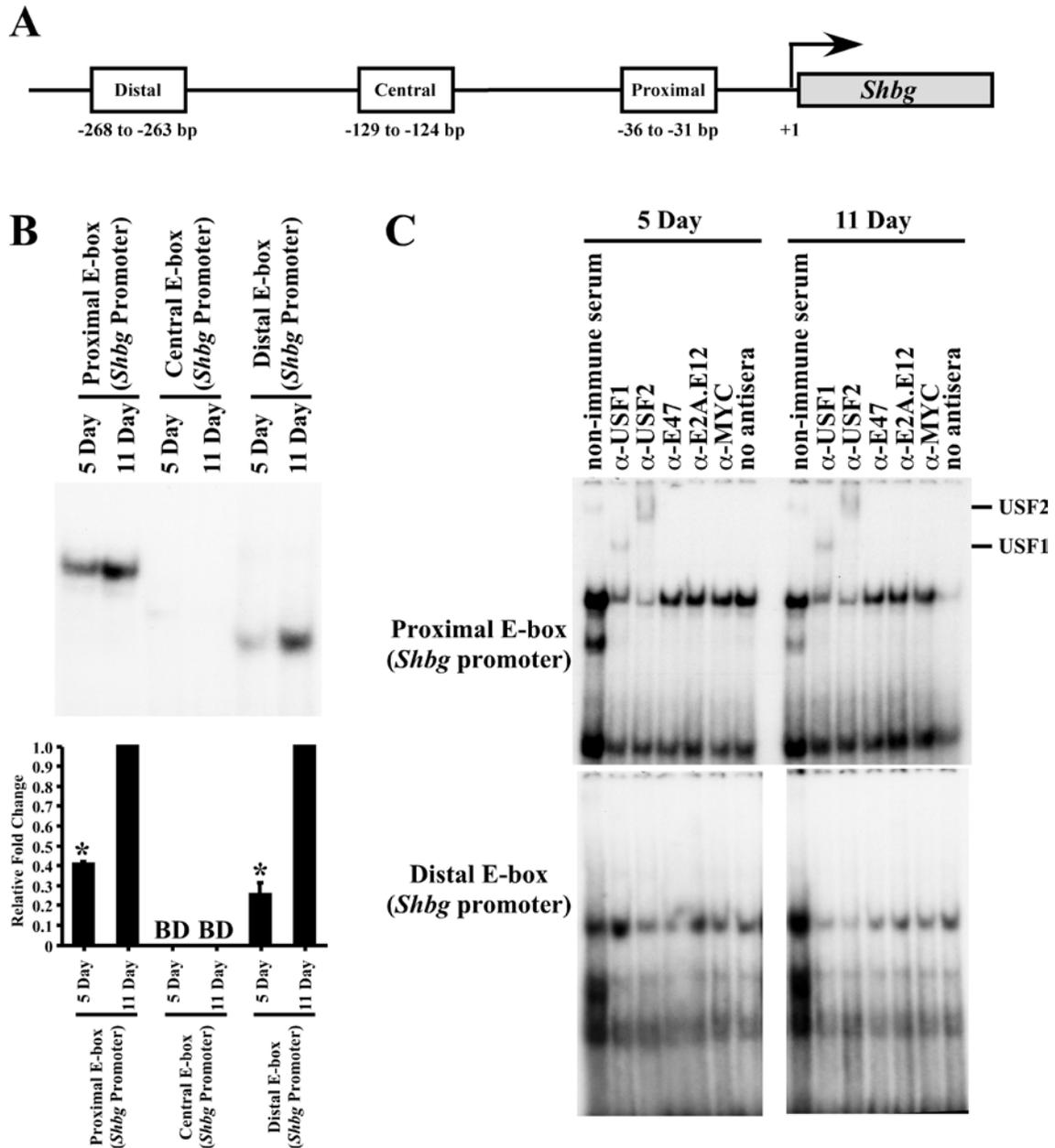


Figure 25. USF1 and USF2 Exhibit Increasing DNA Binding at the Proximal E-box of the *Shbg* Promoter in Differentiating Sertoli Cells Assayed Immediately After Isolation.

A) A schematic of the *Shbg* promoter showing the presence of three E-box motifs: the proximal E-box motif from -36 to -31 bp, the central E-box motif from -129 to -124 bp, and the distal E-box from -268 to -163 bp [245]. Promoter region is not drawn to scale. B) In EMSAs, radiolabeled probes containing a region of the *Shbg* promoter containing the proximal E-box, the central E-box, or the distal E-box (B, top) were incubated with nuclear extracts from 5 and 11 day-old Sertoli cells immediately after isolation. Representative images of the resulting DNA-protein complexes are shown, and quantitation of the mean (\pm SE) of three independent experiments is provided. The relative binding for each condition is normalized to that of 11 day-old Sertoli cells (=1). Statistically significant differences ($p < 0.05$) are indicated by an asterisk (*) (B, Bottom). BD = below detection limit of the assay. C) DNA-protein complexes from nuclear extracts isolated from cultured 11 day-old Sertoli cells were incubated with non-immune sera or antisera for USF1, USF2, E47, E2A, or MYC. Supershifted DNA-protein complexes containing USF1 or USF2 are indicated. Exposure times for all complexes are less than 48 h. Free probe was run off of the gel.

probes containing the proximal (-36 to -31 bp) and distal (-268 to -263 bp) E-box motifs, protein binding was low in extracts from 5 day-old Sertoli cells but DNA binding increased 2.5 ± 0.004 -fold and 3.3 ± 0.05 -fold, respectively ($p < 0.05$ for both), in extracts from 11 day-old Sertoli cells (Figure 25A). DNA binding was not detected at the central E-box from -129 to -124 bp at either 5 or 11 days after birth where E47 binds in fully-differentiated Sertoli cells [245]. To determine whether USF1 or USF2 contributed to the DNA-protein complexes at either the proximal or distal E-box motifs, supershift studies were performed (Figure 25B). Sertoli cell nuclear extracts from assayed immediately after isolation from 5 and 11 day-old rats were preincubated with non-immune serum or antisera against USF1, USF2, E47, E2A, or MYC before incubation with probes containing the proximal or distal E-box regions of the *Shbg* promoter (Figure 25B). The supershift assay demonstrated that both USF antisera supershifted DNA-protein complexes only at the proximal E-box motif, whereas the E47, E2A, and MYC antisera had little effect. None of the antibodies shifted the lower band whose protein components are unknown. In contrast, none of the antibodies tested were able to shift or disrupt the DNA-protein complexes detected for the distal E-box. Additionally, the upper-most DNA-protein complex formed at the distal E-box motif migrated faster than that usually observed for USF bound probes suggesting that an alternative protein or protein complex is bound at the site. These data suggest that USF1 and USF2 may be involved in the transcriptional activation of *Shbg* through the proximal E-box region and that this increase in DNA binding correlates with an increase in *Shbg* mRNA levels.

7.2.4 Chromatin Immunoprecipitation Studies Confirm the Presence of USF1 and USF2 at the *Nr5a1* Promoter Region 5 and 11 Days After Birth in Whole Testes

To confirm that USF1 and USF2 occupy the promoters of USF target genes during Sertoli cell differentiation *in vivo*, chromatin immunoprecipitation (ChIP) analysis was employed to analyze USF1 and USF2 binding to the *Nr5a1* promoter E-box. Formaldehyde cross-linked chromatin from whole testes of 5 and 11 day-old rats was used for immunoprecipitation with antibodies to several members of the bHLH transcription factor family, CREB, and non-immune serum (Figure 26). Whole testes were used to ensure that enough material could be obtained for the assay.

Eluates from USF1 and USF2 immunoprecipitates of chromatin prepared from 5 and 11 day-old testes were significantly enriched for DNA containing the E-box region of the *Nr5a1* promoter in three independent trials (Figure 26, solid bars). In contrast, no such enrichment was observed for DNA containing a region downstream of the E-box within the *Nr5a1* coding sequence (Figure 26, hatched bars). Immunoprecipitation with antisera against E47, E2A.E12, MYC, and CREB did not enrich eluates with DNA from either the E-box region of the *Nr5a1* promoter or a region downstream of the E-box within the *Nr5a1* coding sequence demonstrating the specificity of the E-box interaction with USF1 and USF2. In two out of three experiments, the binding of USF1 and USF2 to the E-box region of the *Nr5a1* promoter appeared to decrease in testes from 11 day-old rats (Figure 26A, B). However, only in one trial was USF1 binding greater than USF2 binding (Figure 26C). These data confirm that USF1 and USF2 bind to the *Nr5a1* promoter between -130 bp and -10 bp upstream of the transcription start site in which the E-box is located at both 5 and 11 days after birth in the testis *in vivo* [171, 173].

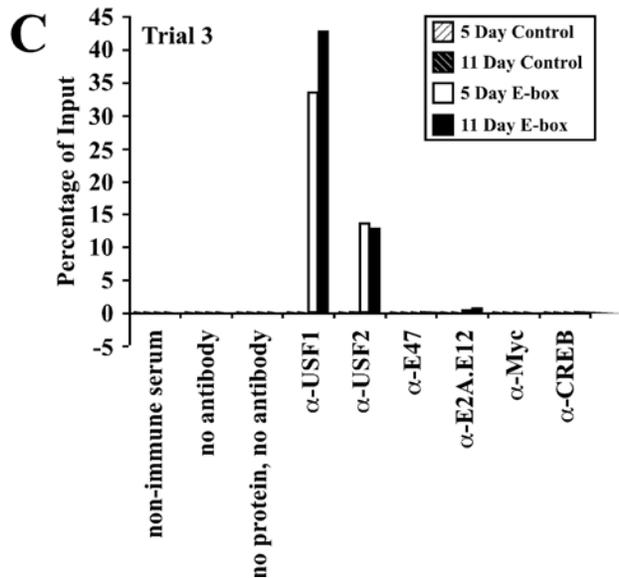
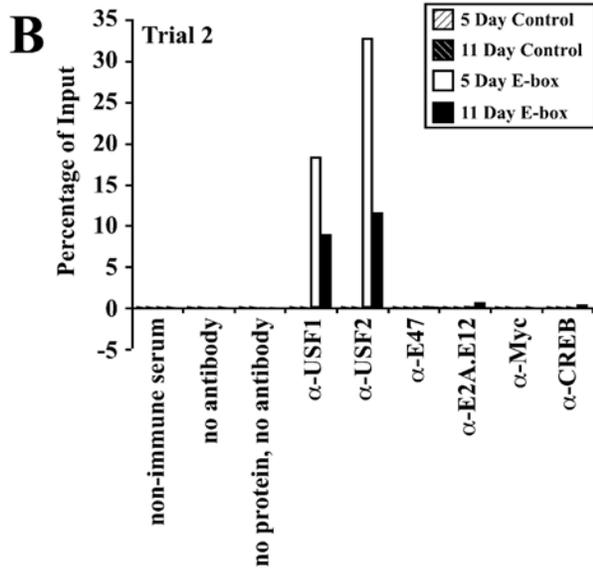
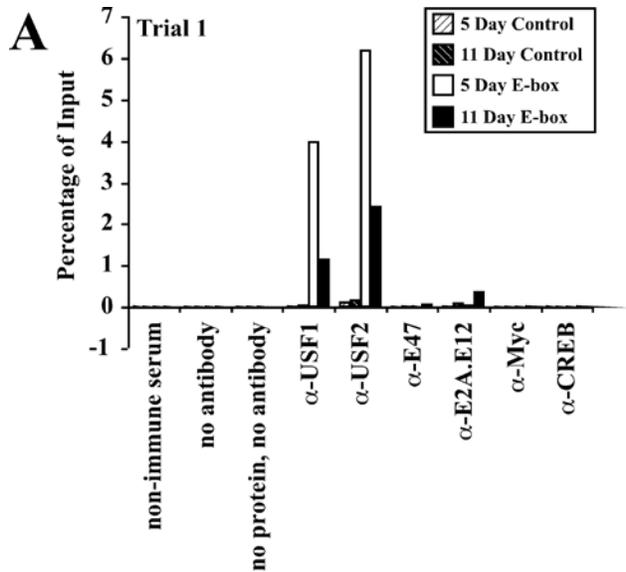


Figure 26. USF1 and USF2 are Enriched at the E-box Within the *Nr5a1* Promoter at Both 5 and 11 Days After Birth *In Vivo*.

Interactions between the USF proteins and *Nr5a1* promoter, *in vivo*, were evaluated using ChIP with cross-linked chromatin from whole testes at 5 and 11 days after birth. In DNA isolated from each immunoprecipitated chromatin sample, the promoter region (solid bars) and a negative control region located 5000 bp downstream of the E-box (hatched bars) were amplified by qPCR. Immunoprecipitations for USF1 or USF2 were compared with immunoprecipitations with antisera against E47, E2A.E12, and MYC. Antisera against CREB, which does not bind the *Nr5a1* E-box, and normal rabbit IgG were used as controls. Three independent trials are shown.

7.3 CONCLUSIONS

USF1 and USF2 are hypothesized to up-regulate target genes during Sertoli cell differentiation by binding to E-box motifs within target gene promoter regions. *Fshr*, *Nr5a1* and *Gata4* were identified as potential USF target genes during Sertoli cell differentiation, based on previous studies [168, 171-173]. EMSA analyses revealed that USF1 and USF2 bind to E-box regions within the *Fshr* (Figures 5 and 6), *Nr5a1* and *Gata4* promoters (Figure 23A,B). Only *Nr5a1* mRNA levels were up-regulated in rat Sertoli cells isolated between 5 and 11 days after birth (Figure 24C), thus identifying *Nr5a1* as a potential USF target gene during the onset of differentiation in rat Sertoli cells. Studies of the *Nr5a1* promoter by ChIP revealed that USF1 and USF2 bind to the *Nr5a1* promoter in 5 and 11 day-old testes, *in vivo* (Figure 26). However, it is not possible to confirm that the DNA binding by USF increased *in vivo* during this stage of Sertoli cell differentiation.

Studies of USF DNA binding through the use of EMSA and ChIP would be expected to reveal similar results. However, it is possible that these two methods could provide different outcomes. Cell extracts analyzed by EMSA are incubated with a buffer that can be altered to favor the binding of different transcription factors. This *in vitro* system also provides binding sites in excess without chromatin structure and in the absence of *in vivo* regulators. ChIP assays allow evaluation of proteins bound to native chromatin, in the context of the intrinsic cell environment. Therefore, results obtained from ChIP assays reveal the *in vivo* ability of a given protein to recognize and bind a consensus motif with the added dimension of chromatin structure. Whereas the results of an EMSA are quantitative [290], the quantitative capability of ChIP assays [291] are limited to studies involving homologous cell populations. When comparing DNA binding of a given protein to the same region of chromatin in samples arising

from a homologous or similar cell population (i.e. a cell line or isolated cell type), ChIP may allow relative quantitative comparisons. However, using cells derived from heterogeneous cell populations of whole testis at different developmental stages may introduce unknown variables and may not truly reveal quantitative differences. Whole testes are a heterogeneous population of cells with varying contributions from germ cells during Sertoli cell development. Testes in 5 day-old mice are composed of approximately 85% Sertoli cells whereas testes from 11 day-old mice contain approximately 45% Sertoli cells [292]. Similar studies of testis cellular composition in rat do not provide the total percentage of Sertoli cells in the testis but would be expected to yield similar results [8, 81, 293]. Therefore, if the amount of total chromatin isolated from 11 day-old cells was diluted in half by chromatin from developing germ cells in rat testes and if the DNA binding was normalized to the percentage of Sertoli cell DNA, then the ChIP assays would reveal approximately equal DNA binding for USF1 and USF2. Future studies of isolated Sertoli cells are required to better test the hypothesis that USF protein binding increase *in vivo* during differentiation. Nevertheless, the data obtained indicate that USF proteins bind to the *Nr5a1* promoter E-box and *Nr5a1* mRNA expression increases during the onset of Sertoli cell differentiation (Figure 26).

Shbg was identified as a potential target of USF proteins during differentiation based on the presence of three E-box motifs within the promoter and the knowledge that *Shbg* expression increases during Sertoli cell differentiation [245, 272]. *Shbg* mRNA levels were confirmed to increase between 5 and 11 days after birth in isolated Sertoli cells (Figure 24D). In EMSA studies of the *Shbg* promoter, DNA-binding activity to both the proximal and distal E-box motif increased between 5 and 11 days after birth (Figure 25A). USF1 and USF2 were only found to bind to the proximal E-box (Figure 25B). The data provide a hypothesis that the binding of two

complexes, a USF containing complex at the proximal E-box and an unknown complex at the distal E-box, could synergize to induce *Shbg* mRNA expression during Sertoli cell differentiation. Future studies should investigate the components of the protein-DNA complex bound at the distal E-box and its involvement in *Shbg* regulation. Additionally, it is interesting to note that no binding was found at the central E-box at -129 and -124 bp upstream of the transcription start site (Figure 25A). This E-box motif was previously shown to bind E47 and may suggest an alternative mechanism of regulation for *Shbg* which occurs in fully-differentiated Sertoli cells [245].

A model has been proposed to describe the actions of USF-mediated transcription during the onset of Sertoli cell differentiation. During proliferation, USF1 levels are low and ID protein levels may be elevated to inhibit USF binding to E-box motifs within the promoters of genes required for differentiated Sertoli cell functions. A differentiating promoting signal, such as T4, is received by the Sertoli cell and USF1 levels are up-regulated while ID levels are down-regulated. These changes in USF1 and ID proteins allows an increase in USF binding to E-box motifs during the onset of Sertoli cell differentiation in the promoter regions of USF target genes, including *Nr5a1* and *Shbg*. The ensuing transcription of *Nr5a1* and *Shbg* genes would allow genes to be expressed that are required for Sertoli cell differentiated functions. Future studies are required to determine if USF proteins directly mediate *Nr5a1* and *Shbg* promoter activity. Also, the ability of T4 and ID proteins to induce or inhibit promoter activity, respectively, needs to be investigated. Finally, USF-mediated NR5A1 and SHBG protein production should be assessed to determine if protein levels are elevated to reflect changes in mRNA levels to contribute to the onset of Sertoli cell differentiation.

8.0 FSHR: REGULATION OF A USF TARGET GENE IN DIFFERENTIATED SERTOLI CELLS

Hypothesis: FSH-induced up-regulation of ID2 inhibits USF-mediated transcription of Fshr in Sertoli cells.

8.1 INTRODUCTION

To this point USF has been identified as a potential mediator of gene transcription that may facilitate the onset of differentiation in Sertoli cells. This chapter will explore the regulation of *Fshr* in fully-differentiated Sertoli cells. Previous studies showed that FSH down-regulates its receptor within four hours of induction and that *Id* mRNA levels are induced by FSH [243, 294]. Therefore, this chapter discusses a possible regulatory mechanism of USF-mediated *Fshr* transcription as regulated by FSH and ID proteins. Although these studies were conducted in differentiated Sertoli cells, this chapter will bring together the ideas of USF binding, hormonal regulation, and ID protein inhibitors at a USF target gene. Specifically, a mechanism is identified to explain the cyclic regulation of *Fshr* during spermatogenesis in fully-differentiated Sertoli cells.

8.1.1 Molecular Mechanisms of FSHR Actions

FSHR is a G-protein coupled receptor (GPCR) that can mediate multiple signaling cascades within the Sertoli cell [21]. FSH stimulation can lead to the regulation of multiple genes required by the Sertoli cell, including: *Fshr*, *Shbg*, *Trf*, and *Ar* [245, 295-297]. The most well studied pathway of FSHR downstream signaling is the ligand-induced activation of adenylyl cyclase by the GPCR to induce cAMP and PKA (reviewed in [21]). FSH binds to FSHR, which leads to the dissociation of the α subunit of receptor-associated G_s protein [21] and the activation of adenylyl cyclase to produce cAMP [298]. cAMP production is highest between stages XIII and IV and is lowest between stages VII and IX [299]. Accumulation of cAMP leads to the release of the catalytic subunit of PKA. PKA then goes on to phosphorylate CREB, a transcription factor [21]. Phosphorylation of CREB can lead to the activation of downstream target genes within Sertoli cells.

8.1.2 *Fshr* Gene Regulation by cAMP in Differentiated Sertoli Cells

The exact mechanisms of *Fshr* gene regulation are not yet understood. *Fshr* gene expression is decreased upon FSH stimulation for 4 hours by 90% through a cAMP-dependent mechanism [294]. FSH stimulation for 16 hours allowed *Fshr* mRNA levels to return to control levels [294]. Stimulation with cAMP or forskolin also produce similar results and treatments in the presence of the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX), can prolong the actions of FSH [294]. A region of the *Fshr* promoter between -100 and +123 relative to the first transcriptional start site was identified as the minimal promoter region needed for transcription [163, 172, 300]. Block mutations of the minimal promoter identified an E-box, an E2F binding

site, and an inverted GATA binding site that may be involved in promoter activation [172, 300]. However, only the E-box motif has significant effects in Sertoli cells and is conserved in the promoter of all species [163].

USF1 and USF2 bind to the E-box within the *Fshr* promoter (Figures 5 and 6). In cultured Sertoli cells, FSH stimulation transiently represses binding to a consensus E-box, decreasing 85% after 12 hours and returning to 45% of basal binding levels within 24 hours [243]. FSH-mediated transient up-regulation of *Id2* gene expression within 2 hours of stimulation coincided with the inhibition of E-box binding [243]. *Id2* mRNA levels then peaked between 6 and 12 hours and decreased by 24 hours after stimulation with FSH [243]. In transient transfections of *Id2* promoter driven reporters, forskolin, FSH, and PKA induced *Id2* promoter activity in primary Sertoli cells [243]. In contrast, after prolonged stimulation (72 hours) of 20 day-old Sertoli cells with FSH or cAMP, *Id1* mRNA decreased and *Id4* mRNA increased while no change was observed in the mRNA levels of *Id2* or *Id3* [169]. Together, these data suggest that FSH activation of FSHR may lead to transient down-regulation of the *Fshr* gene promoter through the up-regulation of ID proteins within Sertoli cells to negatively regulate USF-mediated gene transcription.

8.2 RESULTS

8.2.1 FSH Transiently Decreases *Fshr* mRNA Levels

To confirm the transient down-regulation of *Fshr* mRNA levels upon stimulation with FSH, Sertoli cells from 15 day-old rats were stimulated with FSH+IBMX for 0, 2, 6, 12, 24, or

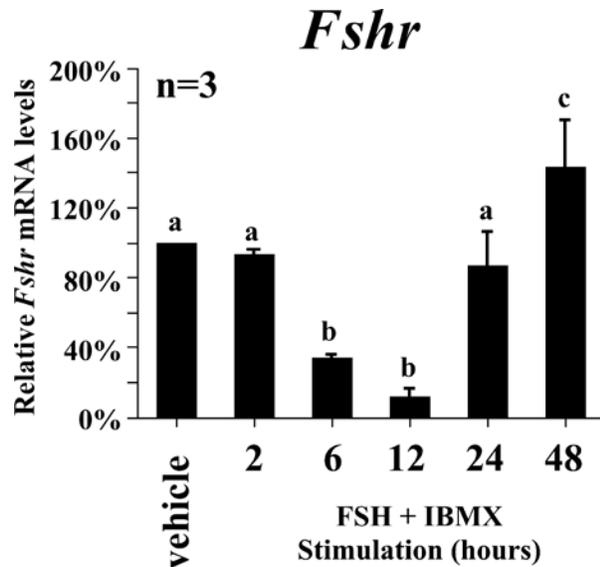


Figure 27. Transient Down-regulation of *Fshr* mRNA with FSH Stimulation in Cultured 15 Day-old Sertoli Cells.

mRNA isolated from cultured 15 day-old Sertoli cells treated vehicle or FSH (100 ng/ml) + IBMX (0.5 mM) for 2 to 24 h were analyzed by qPCR using primers for *Fshr*. Data was analyzed using the $\Delta\Delta C_t$ method and quantitation of the mean (\pm SE) of three experiments is presented. The relative mRNA levels were normalized to *Ppia* levels and made relative to vehicle-treated Sertoli cells (=100%). Values with different lowercase letters differ significantly ($p < 0.05$). Figure modified from [244] with permission from the Endocrine Society (Copyright 2009).

mRNA levels were normalized to that of *Ppia*. These data suggest that FSH-mediate the transient down-regulation of *Fshr* mRNA.

8.2.2 FSH Stimulation Transiently Decreases *Usf1* Gene Expression

USF1 and USF2 are known to bind to the E-box within the *Fshr* promoter (Figures 5 and 6). Therefore, it was hypothesized that FSH+IBMX would transiently down-regulate *Usf* gene expression to contribute to the transient decrease in *Fshr* gene expression (Figure 27). Cultured Sertoli cells from 15 day-old rats were stimulated with FSH + IBMX for 0, 2, 6, 12, 24, and 48

48 hours (Figure 27). Previous studies revealed decreased *Fshr* expression after 4 hours and increased *Fshr* expression after 16 hours of stimulation with FSH [294]. Studies of *Fshr* mRNA levels through the use of qPCR with $\Delta\Delta C_t$ analysis revealed a significant decrease in *Fshr* mRNA levels 6 hours after stimulation ($-66 \pm 1.4\%$, $p < 0.05$), reaching a nadir at 12 hours ($-88 \pm 4.1\%$, $p < 0.05$), and recovering to control levels within 48 hours (Figure 27). The mRNA levels of *Ppia* were studied as a control gene and mRNA levels were not found to change upon stimulation with FSH + IBMX (data not shown). All

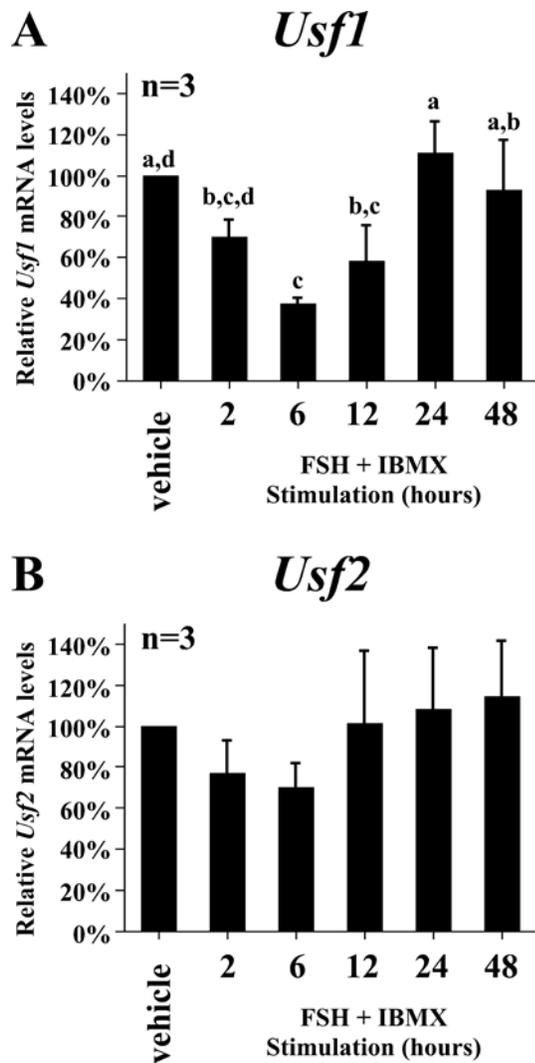


Figure 28. FSH Stimulation Transiently Decreases *Usf1* mRNA Levels in Cultured 15 Day-old Sertoli Cells.

mRNA isolated from cultured 15 day-old Sertoli cells treated with vehicle or FSH (100 ng/ml) + IBMX (0.5 mM) for 2 to 24 h were analyzed by qPCR using primers for *Usf1* (A) or *Usf2* (B). Data was analyzed using the $\Delta\Delta C_t$ method and quantitation of the mean (\pm SE) of three experiments is presented. The relative mRNA levels were normalized to *Ppia* levels and made relative to vehicle-treated Sertoli cells (=100%). Values with different lowercase letters differ significantly ($p < 0.05$). Figure modified from [244] with permission from the Endocrine Society (Copyright 2009).

by FSH [243]. To confirm this result, mRNA levels from cultured rat Sertoli cells isolated from 15 day-old rats stimulated with FSH+IBMX for 0, 2, 6, 12, 24, and 48 hours were evaluated by

hours (Figure 28). qPCR with $\Delta\Delta C_t$ analysis revealed that *Usf1* mRNA levels decreased significantly after 6 hours of stimulation ($-63 \pm 3.1\%$, $p < 0.05$), remained decreased through 12 hours ($-42 \pm 17.5\%$, $p < 0.05$), and returned to control levels within 24 hours (Figure 28A). In contrast, *Usf2* mRNA levels were not found to change significantly in Sertoli cells stimulated with FSH + IBMX (Figure 28B). The mRNA levels of *Ppia* were studied as a control gene and mRNA levels were not found to change upon stimulation with FSH+IBMX (data not shown). All mRNA levels were normalized to that of *Ppia*. These data suggest that FSH induced a decrease in *Usf1* mRNA and may contribute to the transient decrease in *Fshr* expression.

8.2.3 FSH Transiently Up-regulates *Id2* mRNA Levels

Id2 gene expression is transiently up-regulated in cultured Sertoli cells when stimulated

qPCR with $\Delta\Delta C_t$ analysis (Figure 29). *Id2* mRNA levels were transiently up-regulated after 2 hours of stimulation ($+279 \pm 101\%$, $p < 0.05$) and returned to basal levels within 12 hours (Figure 29). This data reveals a more immediate response to FSH compared to previous studies which found that *Id2* mRNA expression in Sertoli cells peaked between 6 and 12 hours [243], although both increases in *Id2* mRNA were transient. Together, these data suggest that if ID2 protein levels reflect *Id2* mRNA levels, then increases in ID2 protein could inhibit USF-mediated DNA binding at the *Fshr* promoter E-box to result in a transient decrease of *Fshr* expression.

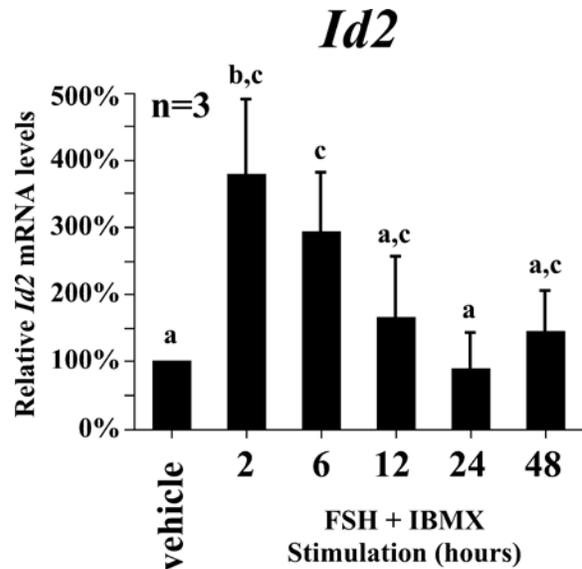


Figure 29. FSH Stimulation Transiently Increases *Id2* mRNA Levels in 15 Day-old Cultured Sertoli Cells.

mRNA isolated from cultured 15 day-old Sertoli cells treated vehicle or FSH (100 ng/ml) + IBMX (0.5 mM) for 2 to 24 h were analyzed by qPCR using primers for *Id2*. Data was analyzed using the $\Delta\Delta C_t$ method and quantitation of the mean (\pm SE) of three experiments is presented. The relative mRNA levels were normalized to *Ppia* levels and made relative to vehicle treated Sertoli cells (=100%). Values with different lowercase letters differ significantly ($p < 0.05$).

Efficiency-corrected ΔC_t analysis of the qPCR data was used to evaluate the relative quantities of *Usf1* to *Id2* within the Sertoli cells after FSH stimulation (Table 8). At all time points, *Id2* mRNA was expressed at equal or greater levels compared to *Usf1* (Table 8, Column 5). From 2 to 12 hours, *Id2* mRNA levels were 5 to 15-fold greater than *Usf1*, only reaching significance 6 hours after FSH stimulation, a 14.69 ± 7.22 -fold increase ($p < 0.05$) (Table 8, Column 5). This increased ratio of *Id2:Usf1* within the Sertoli cell corresponds to the time period when *Id2* mRNA levels are up-regulated (Figure 29) and when *Usf1* mRNA levels are at their lowest (Figure 28A). Together, this data suggests that, if protein levels follow mRNA

expression patterns, ID2 is present in sufficient quantities to inhibit USF binding to an E-box motif.

Table 8. Comparison of *Usf1* and *Id2* mRNA Levels in 15 Day-old Cultured Sertoli Cells Stimulated with FSH and IBMX.

Duration of FSH+IBMX Stimulation	Gene	$\Delta\Delta Ct$ Relative Fold Change (Figure 24A)*	Relative Quantity Using Efficiency Corrected ΔCt Method Compared to <i>Ppia</i>	Gene	$\Delta\Delta Ct$ Relative Fold Change (Figure 24)*	Relative Quantity Using Efficiency Corrected ΔCt Method Compared to <i>Ppia</i>	Average Fold Change in Relative Quantity of <i>Id2</i> Over <i>Usf1</i>
No treatment	<i>Usf1</i>	1.00 ± 0 ^{a,d}	0.124 ± 0.026	<i>Id2</i>	1.00 ± 0 ^a	0.188 ± 0.080 ^a	1.67 ± 0.75
2 hours		0.70 ± 0.08 ^{b,c,d}	0.083 ± 0.010		3.79 ± 1.10 ^{b,c}	0.670 ± 0.231 ^{b,c}	8.62 ± 3.50
6 hours		0.37 ± 0.03 ^c	0.044 ± 0.006		2.93 ± 0.88 ^c	0.598 ± 0.286 ^{a,b}	14.69 ± 7.22**
12 hours		0.58 ± 0.18 ^{b,c}	0.067 ± 0.008		1.66 ± 0.89 ^{a,c}	0.342 ± 0.184 ^{a,c,d}	5.34 ± 3.03
24 hours		1.11 ± 0.15 ^a	0.138 ± 0.030		0.90 ± 0.52 ^a	0.163 ± 0.088 ^{a,d}	1.38 ± 0.79
48 hours		0.93 ± 0.24 ^{a,b}	0.107 ± 0.007		1.45 ± 0.59 ^{a,c}	0.280 ± 0.130 ^{a,c}	2.63 ± 1.23
Column Number		1	2		3	4	5

*Values with different lower case letters are significantly different from other values in the same column ($p < 0.05$).

**Change between relative quantities when comparing gene to *Usf1* is significantly different ($p < 0.05$).

8.3 CONCLUSIONS

FSH was hypothesized to induce the up-regulation of ID2 to inhibit USF-mediated transcription of *Fshr* in differentiated Sertoli cells. This hypothesis was proposed to investigate a potential regulatory mechanism involving hormonal signals and inhibitor ID proteins that may regulate USF binding to E-box motifs at multiple target genes. Stimulation of cultured Sertoli cells for 0, 2, 6, 12, 24, or 48 hours with FSH+IBMX revealed a transient decrease in *Fshr* mRNA expression at 6 and 12 hours after stimulation (Figure 27). This is consistent with

previous studies that showed decreased *Fshr* expression after 4 hours and increased expression of *Fshr* after 16 hours of stimulation with FSH [243]. This data also correlates with a decrease in USF binding to the *Fshr* promoter E-box 6 hours after stimulation with FSH+IBMX and an increase in USF binding 48 hours after stimulation with FSH+IBMX [244].

USF levels were investigated as a cause for the FSH-mediated transient down-regulation of USF binding to the E-box within the *Fshr* promoter and a subsequent decrease in *Fshr* gene expression. *Usf1* mRNA levels were found to decrease upon stimulation of Sertoli cells with FSH+IBMX with similar kinetics as the decrease in the expression of *Fshr* (Figure 28A). In contrast, *Usf2* mRNA levels were not found to decrease upon stimulation with FSH+IBMX (Figure 28B). This data is supported by a decrease in USF1 protein between 2 and 12 hours after stimulation with FSH+IBMX [244]. Together, these data support the hypothesis that FSH induces an inhibition of USF-mediated transcription of *Fshr* in Sertoli cells.

ID2 was hypothesized to be an inhibitor that is responsible for the down-regulation of USF-mediated *Fshr* expression based on previous studies showing that *Id2* mRNA is transiently up-regulated upon stimulation with FSH+IBMX [243]. *Id2* mRNA levels were confirmed to be transiently up-regulated within 2 hours of stimulation with FSH+IBMX and returned to basal levels within 12 hours (Figure 29). ID2 protein levels also increase transiently within the nuclei of Sertoli cells after 6 hours of stimulation with FSH+IBMX and ID2 levels begin to decrease within the nuclei after 24 hours [244]. Additionally, *Id2* mRNA levels are expressed at quantities similar to or greater than *Usf1* (Table 8). If mRNA levels reflect protein expression levels, then ID2 would be expected to be expressed at comparable levels to USF1 and could inhibit USF binding. This data supports the hypothesis that ID2 is up-regulated with FSH+IBMX and may inhibit USF binding to the E-box within the *Fshr* promoter.

The ability of ID2 to inhibit USF-mediated *Fshr* transcription was not directly addressed in this dissertation; however, recent studies further support this hypothesis. *In vitro*, increasing levels of ID2 are able to inhibit USF binding to the *Fshr* promoter E-box in Sertoli cells [244]. In addition, ID2 is able to inhibit USF-mediated *Fshr* promoter activity in a dose-dependent manner in Sertoli cells [244]. As a whole, these data support the hypothesis that FSH-induced up-regulation of ID2 inhibits USF-mediated transcription of *Fshr* in Sertoli cells. FSH binding to the FSHR allows internalization of the receptor for either recycling or degradation [21, 294, 297, 301]. Thus, decreased *Fshr* transcription may lead to the down-regulation of the FSHR at the cell surface. ID2-mediated mechanisms of FSHR down-regulation may be involved in the cyclical control of FSHR levels during the stages of spermatogenesis in differentiated Sertoli cells [19]. Further studies need to be conducted in Sertoli cells isolated from staged tubules in order to confirm these ideas.

This data can also provide a model for the regulation of other USF target genes. In this model, USF binds to the E-box motif of the target gene, allowing transcription. FSH signaling acts on the Sertoli cell to transiently down-regulate USF levels and up-regulate ID protein levels. These changes inhibit USF binding to E-box motifs within the promoters of target genes. Upon prolonged signaling or loss of the signal that caused the down-regulation, USF and ID protein expression returns to basal levels. The return to basal levels again allows USF binding to the E-box within the target gene promoter and activates gene transcription. Future studies of other USF target genes are required to determine if this proposed model is maintained across USF-regulated gene promoters.

9.0 A PROPOSAL OF HOW USF REGULATES THE ONSET OF SERTOLI CELL DIFFERENTIATION

Sertoli cells divide until the onset of puberty after which time they never divide again [1, 79]. Sertoli cells are required for the maintenance of male fertility. Each Sertoli cell can only support a given number of germ cells [79]. Therefore, the number of Sertoli cells establishes the upper level of male fertility [1]. The molecular mechanisms that regulate the terminal differentiation of Sertoli cells are only beginning to be investigated. The studies presented in this dissertation identify one potential mechanism that contributes to the onset of Sertoli cell differentiation and the initiation of transcription of genes required for Sertoli cell differentiated functions.

The data presented in this dissertation support the following model. In undifferentiated Sertoli cells, USF1 levels are low and ID protein levels may be elevated to prevent USF binding to E-box motifs. The Sertoli cell receives signals, including those from thyroid hormone, to initiate differentiation. This signal up-regulates USF expression and down-regulates ID2 expression levels to induce USF binding to E-box motifs. USF binding to E-box motifs in the promoters of USF target genes can activate transcription of genes required for Sertoli cell differentiation and differentiated Sertoli cell functions.

9.1 ISOLATION OF SERTOLI CELLS FOR BIOCHEMICAL ANALYSIS

To study Sertoli cells, the cells were isolated from the testis. A protocol was adapted in order to assay cells immediately after isolation from testis tissue allowing Sertoli cells to be studied as close to the *in vivo* conditions as possible (Figure 3). The purity of the Sertoli cells was 85 ± 0.03 % (Figure 3). The studies were also conducted in cultured Sertoli cells, which are routinely 95% pure [302]. Studies conducted in cultured Sertoli cells allow manipulation of the cells for studies of hormonal actions and allows the use of immunodetection to determine protein localization and levels within the cell. A caveat of Sertoli cell isolation is that the cell populations are not pure and the other cell types present may affect the results of biochemical assays. Additionally, the effects of the enzymatic digestion of the testis and removal of Sertoli cells from the surrounding germ cells and interstitial cells are unknown. These actions may activate signaling cascades that are not part of the Sertoli cells' endogenous biochemical make-up. However, better techniques have not been developed and isolated Sertoli cells are routinely used for biochemical assays. Sertoli cells used for these studies were isolated from 5, 11, or 20 day-old rats representative of proliferating, differentiating, or differentiated Sertoli cells, respectively. These time points allowed biochemical analysis of the molecular changes that are occurring during the onset of Sertoli cell differentiation. Studies utilizing both Sertoli cells assayed immediately after isolation and those that were cultured revealed overall similar results. The results suggest that cultured Sertoli cells are effective tools for biochemical analysis of Sertoli cells.

9.2 THE DNA BINDING OF BHLH PROTEINS INCREASES DURING SERTOLI CELL DIFFERENTIATION

bHLH proteins are known contributors of cell differentiation (reviewed in [131, 141]). The presence of Class A and B bHLH proteins were previously shown to be important in the regulation of the differentiation associated genes: *Trf* and *Fshr* [168, 177, 179, 296]. Class A and B bHLH proteins were investigated to determine if their actions could influence the onset of Sertoli cell differentiation. The Class A proteins [TCFE2A (E47), etc.] investigated were not found to bind to E-box consensus sites in differentiating Sertoli cells (Figure 4) in contrast to what had previously been found in differentiated Sertoli cells [168, 169, 174]. The binding of Class B bHLH proteins, specifically USF1 and USF2, was found to increase during Sertoli cell differentiation between 5 and 11 days after birth to E-box consensus motifs *in vitro* (Figures 5 and 6). Class D bHLH proteins were also investigated (see 9.3.2 below). Other classes of bHLH proteins were not evaluated in these studies as prior evidence was not available indicating their involvement in the regulation of genes associated with differentiation in Sertoli cells. These data support the hypothesis that DNA binding of bHLH proteins increases during Sertoli cell differentiation. The increase in USF1 and USF2 binding to E-box motifs during the onset of Sertoli cell differentiation may activate transcription of genes required for Sertoli cell differentiation and differentiated Sertoli cell functions.

9.3 MECHANISMS OF USF REGULATION

USF1 and USF2 binding increased at E-box motifs during the onset of Sertoli cell differentiation (Figures 5 and 6). Therefore, three potential regulatory mechanisms of USF binding were investigated. First, the mRNA and protein levels of the USF proteins were evaluated (Chapter 4). Second, the ability of ID proteins to prevent USF binding in proliferating Sertoli cells was evaluated as an inhibitor of bHLH proteins (Chapter 5). Finally, the ability of thyroid hormone to induce USF binding in proliferating Sertoli cells was evaluated as a known inducer of Sertoli cell differentiation (Chapter 6).

9.3.1 *Usf1* mRNA and Protein Expression Levels Increase During Differentiation

The mRNA and protein levels of USF1 and USF2 were evaluated. *Usf1* mRNA and USF1 protein levels were found to increase during the onset of Sertoli cell differentiation (Figures 8-10; Tables 4-5). This increase also corresponded to an up-regulation of USF1 protein in the nuclei of Sertoli cells (Figure 11). In contrast, the mRNA and protein levels of USF2 were not altered between 5 and 11 days after birth during Sertoli cell differentiation. USF1 and USF2 are capable of activating transcription as homodimers or heterodimers. USF1 containing dimers are thought to be the preferred dimer pair for the initiation of *Fshr* gene expression [179]. Studies presented here did not evaluate the relative quantity of dimer pairs present in Sertoli cells during Sertoli cell differentiation or their relative binding affinities. However, the increase in USF1 protein availability within the Sertoli cell may allow the formation of more USF1 containing dimers to initiate transcription of genes required for Sertoli cell differentiation or

differentiated Sertoli cell functions. Thus, USF1 homodimers or USF1:USF2 heterodimers may be required to promote Sertoli cell differentiation.

9.3.2 ID2 Proteins May Inhibit USF1 and USF2 Binding During Sertoli Cell Proliferation.

The second regulatory mechanism investigated was the ability of USF binding to be inhibited by Class D bHLH proteins, specifically the ID proteins. The ID proteins are present in Sertoli cells and induce terminally differentiated Sertoli cells to begin proliferating [166, 178]. The results of studies involving ID proteins are difficult to interpret. *Id* mRNA levels were found to remain constant or increase during Sertoli cell differentiation (Figures 12, 13 and Tables 6, 7). In contrast, ID2 protein levels were found to decrease within the nucleus of the Sertoli cell during differentiation by immunofluorescence (Figures 14, 15). Western blots were not able to be conducted with the available reagents to confirm either result (Figure 16). Although these data cannot definitely determine the expression patterns of ID proteins in Sertoli cells, this data raises the possibility that ID protein levels decrease during differentiation. Recent studies provide evidence that ID2 is able to inhibit USF binding in differentiated Sertoli cells under hormonal control [244]. Studies presented here do not investigate the ability of ID proteins to inhibit USF binding to E-box motifs or USF-mediated transcription. If ID proteins are inhibitors of USF binding in proliferating Sertoli cells, the decrease in ID protein levels would allow an increase in USF binding during Sertoli cell differentiation resulting in the transcription of genes required for Sertoli cell differentiation or differentiated Sertoli cell functions.

9.3.3 Thyroid Hormone Induces USF Binding in Proliferating Sertoli Cells.

The final regulatory mechanism investigated was the ability of thyroid hormone, a known inducer of Sertoli cell differentiation, to induce USF binding to E-box motifs in proliferating Sertoli cells. Stimulation of cultured Sertoli cells with thyroid hormone for 24 hours induced USF binding in 5 day-old Sertoli cells (Figure 18). In contrast, thyroid hormone was unable to induce USF binding, but rather inhibited or did not alter binding, in Sertoli cells isolated from 11 and 20 day-old rats, respectively (Figure 18). Thyroid hormone also induced USF1 within the nuclei of 5 day-old Sertoli cells to levels comparable with differentiated Sertoli cells (Figure 21). In contrast, *Usf1* mRNA levels were not altered by thyroid hormone 5 days after birth (Figure 19) and suggest that thyroid hormone regulation of USF binding is through a post-transcriptional mechanism. These data are consistent with previous data in which thyroid hormone is thought to contribute to Sertoli cell differentiation between 4 and 8 days after birth in the rat [264, 269]. Thyroid hormone induces USF binding during the onset of differentiation and may promote gene transcription of genes required for Sertoli cell differentiation or differentiated Sertoli cell functions.

9.3.4 Future Studies Into the Regulation of USF Binding During Sertoli Cell Differentiation

The data investigating the regulation of USF binding during differentiation supports the proposed model. However, the data presented is largely correlative. Additional studies, will contribute to the understanding of USF regulation during differentiation. To determine whether USF1 is induced in Sertoli cell nuclei *in vivo* during differentiation, immunohistochemistry

should be conducted in whole tissue sections. The ability of the different USF1 containing dimers to regulate gene expression through E-box motifs in proliferating and differentiating Sertoli cells could determine the relevance of the increase in USF1 protein levels. If USF1 containing dimers preferentially bind to E-box motifs during differentiation, then the increase in USF1 protein would be a legitimate regulatory mechanism of USF binding. If this was found to be true, additional studies into the regulation of the *Usf1* gene transcription should be conducted.

Similar immunohistochemical studies of testis tissue could also be performed to evaluate the levels of ID proteins present within Sertoli cells during the onset of differentiation. Additionally, the acquisition of other ID protein antisera or the concentration of more protein should facilitate Western blotting to support either the mRNA or immunofluorescence data. The ability of ID proteins to inhibit USF binding or USF-mediated gene transcription need to be evaluated in Sertoli cells isolated from 5 and 11 day-old rats. If ID proteins are unable to inhibit USF binding to E-box motifs or USF-mediated gene transcription during differentiation, then the changes in ID protein levels are irrelevant to the regulation of USF binding during differentiation.

The ability of thyroid hormone to induce USF binding needs to be verified *in vivo*. Additionally, the ability of other hormones present during the onset of Sertoli cell differentiation to induce USF binding needs to be tested. How thyroid hormone induces USF binding is unknown. It is not likely that the regulation is transcriptionally mediated because *Usf1* mRNA levels were not affected by the addition of thyroid hormone in proliferating 5 day-old Sertoli cells. It is possible that thyroid hormone causes post-translational modifications on USF to facilitate USF binding. The type of modification and the signaling mechanism that occurs will need to be investigated. Phosphorylation of USF can regulate USF binding to E-box motifs,

although no studies have been conducted in Sertoli cells [281, 282, 303, 304]. Thyroid hormone could induce changes in the chromatin structure of the E-box region or may negatively regulate ID proteins. Additionally, studies will need to evaluate the ability of thyroid hormone to induce USF-mediated gene transcription, perhaps through the use of reporter assays. The mechanisms involved in the regulation of USF binding during Sertoli cell differentiation remain to be completely understood and provides an area of much larger research that could be conducted. Understanding the regulation of USF binding and the downstream effects could provide important insights into the function of USF1 and USF2 transcription factors in the onset of Sertoli cell differentiation.

9.4 USF BINDING TO TARGET GENE PROMOTER E-BOX MOTIFS

9.4.1 *Nr5a1* is a USF Target Gene During the Onset of Sertoli Cell Differentiation

USF1 or USF2 are able to regulate the promoters of *Fshr*, *Nr5a1*, and *Gata4*, in Sertoli cells. Therefore, these genes were investigated as potential target genes regulated during differentiation by USF1 or USF2. USF binding was found to increase *in vitro* at all three promoter E-box regions during Sertoli cell differentiation (Figures 5-6 and 23). However, only the mRNA levels of *Nr5a1* were found to increase during differentiation between 5 and 11 days after birth (Figure 24C). This identified *Nr5a1* as a USF target gene during the onset of Sertoli cell differentiation. As a result, USF binding to the *Nr5a1* promoter E-box was evaluated *in vivo*. ChIP assays confirmed that USF1 and USF2 bound to the *Nr5a1* promoter E-box region but were unable to verify the increase between 5 and 11 days after birth (Figure 26). These

studies established that USF binding increased the *Nr5a1* promoter E-box *in vitro* during the onset of Sertoli cell differentiation. The induction of *Nr5a1*, if correlated with protein levels, could result in the activation of the SF1 transcription factor, which could regulate genes required for the onset of Sertoli cell differentiation and differentiated Sertoli cell functions.

9.4.2 *Shbg* is Identified as a Novel USF Target Gene During the Onset of Sertoli Cell Differentiation

Shbg was investigated as a potential USF target gene. *Shbg* contains three E-box motifs within its promoter [245]. The central E-box was previously found to bind E47 in differentiated Sertoli cells [245]. *Shbg* levels increase during Sertoli cell differentiation (Figure 24D) [268]. Protein binding was found to increase at the proximal and distal E-box site during the onset of Sertoli cell differentiation but only the proximal E-box was found to bind USF1 and USF2 (Figure 25). Interestingly, the lack of binding at the central E-box suggests differential regulation of the promoter in differentiating cells compared to fully differentiated cells. It is also possible that during differentiation two protein complexes, the USF containing complex at the proximal E-box and an unknown complex at the distal E-box, synergize to induce *Shbg* gene expression. These data identify *Shbg* as a novel USF target gene in differentiating Sertoli cells and confirm that USF binding increases to the proximal E-box motif within the promoter region during the onset of Sertoli cell differentiation. If USF binding to the proximal E-box contributes to *Shbg* gene expression, the induction of *Shbg* mRNA could correlate with an increase in ABP secretion from Sertoli cells to help facilitate androgen transport throughout the remainder of the male reproductive tract.

9.4.3 Future Studies of USF-mediated Gene Transcription During the Onset of Sertoli Cell Differentiation

USF binding to E-box motifs within the promoters of *Nr5a1* and *Shbg* was found to increase by *in vitro* methods (EMSA; Figures 23, 25), but not *in vivo* methods (ChIP; Figure 26). Isolated Sertoli cells will need to be utilized to conduct additional ChIP assays to determine if USF1 and USF2 bind to the E-box motifs within the *Nr5a1* and *Shbg* promoters and if USF binding increases between 5 and 11 days after birth. The induction of USF binding *in vitro* correlated with an increase in mRNA levels between 5 and 11 days after birth for both genes (Figure 24C,D). However, these data do not directly show that the induction of USF binding mediates an induction in mRNA expression. Additional studies will need to evaluate the ability of USF to drive the transcription of *Nr5a1* and *Shbg* in Sertoli cells. Since two target gene promoters have been identified as USF targets during the onset of Sertoli cell differentiation, further studies are required to determine if thyroid hormone and ID proteins are able to regulate USF binding to these promoter regions and regulate gene transcription. High-throughput methods can be used to identify more USF target genes during the onset of Sertoli cell differentiation. The use of microarray analysis or ChIP-sequencing of samples of Sertoli cells from 5 and 11 day-old rats will provide a large list of other USF regulated genes that may be essential during the onset of Sertoli cells differentiation. The overall goal is to identify the role that USF has in regulating genes necessary for Sertoli cell differentiation and differentiated Sertoli cell functions.

9.5 FSHR: A MODEL FOR USF-MEDIATED GENE TRANSCRIPTION IN DIFFERENTIATED SERTOLI CELLS

USF1 and USF2 are known to regulate the *Fshr* gene through the E-box motif within the minimal promoter [177, 179]. Additionally, it is known that during the cycle of the seminiferous epithelium, *Fshr* gene expression is cyclically regulated [19]. Studies presented in this dissertation provide a potential mechanism of *Fshr* gene regulation through transient inhibition of transcription as induced by FSH and ID proteins. FSH stimulation of differentiated Sertoli cells over the course of 48 hours revealed a transient decrease in *Fshr* mRNA, *Usf1* mRNA, and USF1 protein levels within 6 to 12 hours after stimulation (Figures 27, 28) [244]. Additionally, FSH transiently up-regulates *Id2* mRNA (Figure 29) [243] and ID2 protein with similar kinetics [244]. Recent studies also confirmed that ID2 proteins can inhibit USF binding to the *Fshr* promoter E-box and USF-mediated *Fshr* promoter activity in isolated differentiated Sertoli cells [244]. Additional studies need to be conducted using immunostaining of testis tissue sections to determine whether USF and FSHR expression levels are similar over the prolonged 13 day cycle in rat Sertoli cells. The transient hormonal induction of the inhibitor ID2 and the transient inhibition of USF binding to the *Fshr* promoter E-box and USF expression provide a potential explanation for *Fshr* regulation during the cycle of the seminiferous epithelium.

9.6 CONCLUSIONS

Although numerous factors regulating Sertoli cell development have been identified, the molecular mechanisms that contribute to the onset of differentiation are complex and are only

beginning to be understood. The importance of USF1 and USF2 transcription factors in Sertoli cell differentiation is not known. The studies described here advance the understanding of one developmentally regulated molecular mechanism for Sertoli cells in which USF proteins regulate the onset of differentiation in rat Sertoli cells. The model proposes that during proliferation USF1 levels are low within the cell and ID protein levels are elevated to inhibit USF binding to E-box motifs. When a signal such as thyroid hormone is received to initiate differentiation, USF1 levels are up-regulated and ID proteins are down-regulated to increase USF binding to E-box motifs. The increase in USF binding can lead to the transcription of USF target genes within the cell. Future studies of the downstream targets of USF target genes may provide key insights into the importance of the genetic program initiated by USF1 and USF2 during the onset of Sertoli cell differentiation. These studies identified the *Nr5a1* gene as a USF target gene during this developmental time period. NR5A1 is also a transcription factor which can further activate other genes to perpetuate the differentiation signal. It is possible that additional USF target genes also encode transcription factors. Together, the actions of USF1, USF2, NR5A1, and yet unknown USF-mediated transcription factors, when appropriately up-regulated, will promote the onset of Sertoli cell differentiation. Proper timing of differentiation in Sertoli cells sets the final number of Sertoli cells and thus establishes the upper level of male fertility.

10.0 MATERIALS AND METHODS

10.1 ANIMAL CARE AND USE

Male Sprague Dawley rat pups were obtained from Charles River Laboratories (Boston, MA). Animals used in these studies were maintained and euthanized according to the principles and procedures described in the NIH Guide for the Care and Use of Laboratory Animals. These studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

10.2 ISOLATION OF SERTOLI CELLS FOR DIRECT ASSAY

Rats were euthanized 5 and 11 days after birth and Sertoli cells were isolated as described by Anway, *et al.* [127] with slight modifications. Briefly, decapsulated testes were digested with collagenase (0.5 mg/ml) in enriched Krebs-Ringer bicarbonate medium (EKRB; 118.5 mM NaCl, 4.7 mM KCl, 2.1 mM CaCl₂-H₂O, 1.0 mM KH₂PO₄, 1.2 mM MgSO₄-7H₂O, 25 mM NaHCO₃, 10 mM HEPES, and 0.1% BSA) at 34°C for 10-15 minutes in shaking water bath (80 oscillations/minute), followed by three settlings in EKRB to isolate seminiferous tubules. Tubules were dispersed through digestion with trypsin (0.5 mg/ml) in the presence of DNase (1 µg/ml) for 5-10 minutes at 37°C without shaking. Following the digestion, tubule fragments were

washed with soybean trypsin inhibitor (0.3 mg/ml) followed by two washes with EKRB. Sertoli cells were separated from germ cells by incubation with 0.1% collagenase, 0.2% hyaluronidase, 0.04% DNase I, and 0.03% trypsin inhibitor in shaking water bath for 40 minutes at 34°C (80 oscillations/minute). Following digestion, cells were collected by centrifugation and washed three times with EKRB. To remove contaminating germ cells, the suspension of single cells and clusters of 5-10 cells were subjected to hypotonic shock by resuspending the pellet in EKRB diluted with water (final concentration 0.2x EKRB), gently inverting 3 times to disperse cells followed by immediate centrifugation at 700 rpm (63 x g) for 10 minutes. Cells were then washed three times with serum-free media containing 50% Dulbecco modified Eagle medium (DMEM), 50% Ham's F-12, 5 mg/ml insulin, 5 mg/ml transferrin, 10 ng/ml epidermal growth factor, 3 µg/ml cytosine β-D-arabino-furanoside, 1 mM sodium pyruvate, 200 units/ml penicillin, and 200 µg/ml streptomycin. The resuspended cells were counted and directly aliquoted for preparation of protein extracts, RNA, or plated for analysis of purity.

10.3 ISOLATION OF SERTOLI CELLS FOR CELL CULTURE

Rats were euthanized 5, 11, 15, and 20 days after birth, as described previously [123]. Decapsulated testes were digested with collagenase (0.5 mg/ml, 33°C, 12 minutes) in EKRB, followed by three settlings in EKRB to isolate seminiferous tubules. The tubules were digested with trypsin (0.5 mg/ml, 32°C, 12 minutes) in the presence of DNase (2 µg/ml), and cell aggregates were passed repeatedly through a Pasteur pipette. An equal volume of DMEM containing 10% fetal bovine serum was added to the Sertoli cells, which were then pelleted (500 x g, 5 minutes) and resuspended in supplemented serum-free media used above. Sertoli cells

were cultured on Matrigel (Collaborative Research, Bedford, MA) coated dishes (33°C, 5% CO₂). Sertoli cells were routinely 95% pure as determined by phase microscopy and alkaline phosphatase staining [305]. Cells were cultured three days prior to treatment. Sertoli cells were stimulated with vehicle (EtOH) or thyroxine (10⁻⁶ M) for 24 hours.

10.4 DETERMINING PURITY OF SERTOLI CELLS FOR IMMEDIATE BIOCHEMICAL ANALYSIS

In a 24-well plate, 12 mm² glass coverslips were coated with poly-L-lysine and allowed to dry after excess liquid was removed. Coverslips were washed with PBS and 2.5 x 10⁴ cells were plated per well. Cells were allowed to attach to coverslips for approximately 2 hours after which Sertoli cells and peritubular cells were identified by immunofluorescence. Leydig cells were identified by lipid-specific staining. Briefly, coverslips were washed with PBS and fixed with 4% formalin for 15 minutes at room temperature and stored in 70% ethanol overnight. The coverslips were incubated with antiserum against Sertoli cell-specific vimentin (1:200 dilution; V6630, Sigma-Aldrich, St. Louis, MO) or antiserum against peritubular cell-specific alpha smooth muscle actin (1:2000 dilution; A2547, Sigma-Aldrich, St. Louis, MO). Primary antisera were detected by secondary antibodies tagged with Alexa 488 or Cy5. Nuclei were visualized using Hoechst 33258 (861405, Sigma-Aldrich, St. Louis, MO). Leydig cells were detected by staining with the lipid-specific stain Oil Red O (O0625, Sigma-Aldrich, St. Louis, MO). The cells were fixed in 2% paraformaldehyde for 5 minutes, washed three times with distilled water, incubated in propylene glycol for 5 minutes and stained with 0.5% Oil Red O in propylene glycol for 8 minutes at 60°C. Cells were rinsed in 85% propylene glycol for 5 minutes and

washed with distilled water prior to staining with hematoxylin. Cells were washed repeatedly with water over the course of 3 minutes followed by two changes of distilled water prior to mounting. All images were taken on an Olympus Provis AX70 Microscope with a 60x objective and quantification was performed using Northern Eclipse v. 7.0 Software (MVIA Inc., Monaca, PA). Ten random fields from multiple coverslips were analyzed to determine the total number of nuclei and the number of cells expressing each marker. The mean percentage of cells expressing the cell-specific marker (\pm SE) was calculated. Greater than 500 cells for each detection method were counted.

10.5 CELL CULTURE FOR CELL LINES, TRANSFECTION, AND WHOLE CELL LYSATES

The mouse-derived Sertoli cell line, MSC-1, was cultured at 33°C and COS7 cells were maintained at 37°C in culture. Both cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. For studies involving over-expressed ID2, cells were transfected with 5 µl of an adenovirus expressing ID2 (1.9×10^{11} pfu) for 48 hours. COS7 or MSC-1 Sertoli cells were transfected with 0.5 µg of expression vectors expressing USF1, USF2, or E47 (pSV-USF1, pSV-USF2, or pcDNA E47) using FUGENE reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Two days after transfection, cells were lysed in extract lysis buffer (ELB; 250 mM NaCl, 0.1% NP-40, 50 mM HEPES, pH 7.0, 5 mM EDTA, 0.5 mM DTT, and a cocktail of protease and phosphatase inhibitors) and then sonicated and subjected to centrifugation (12,000 x g, 10 min). Supernatants containing whole cell lysates were stored at -

80°C. Protein concentrations were determined by the Bradford method (Bio-Rad protein assay, Bio-Rad Laboratories, Inc., Hercules, CA).

10.6 NUCLEAR PROTEIN EXTRACTS

Sertoli cells for immediate assay (1.5×10^6 cells/sample) or three-100 mm² plates of cultured Sertoli cells were collected in a total of 1 ml of 1x PBS and used for the preparation of nuclear extracts. Nuclear and cytoplasmic extracts were prepared by detergent lysis [306]. Briefly, after pelleting, the cells were incubated in buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail) for 15 minutes on ice followed by the addition of 0.06% Nonidet P40. Cells were vortexed for 10 seconds and nuclei were collected by centrifugation (12,000 x g, 30 seconds). The nuclei pellet was then washed once with buffer A and resuspended in buffer C (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail). Nuclei were incubated for 15 minutes on shaking vortex platform at 50% power at 4°C. The cell debris was pelleted (12,000 x g, 5 minutes) and the supernatant containing nuclear proteins was stored at -80°C. Protein concentrations were determined by the Bradford method (Bio-Rad protein assay, Bio-Rad Laboratories, Inc., Hercules, CA).

10.7 DNA-PROTEIN BINDING STUDIES

³²P-radiolabeled DNA probes were generated by annealing 24-42 nucleotide templates containing protein binding motifs plus flanking promoter sequences to 10-nucleotide primers that are complimentary to the 5' end of the templates (Table 9). The overhangs were filled in with Klenow in the presence of [α -³²P]-dATP and 5 mM each of dCTP, dGTP, dTTP and 5-bromo-2'-deoxyuridine-5'-triphosphate (BrdU) at 16°C overnight. Electrophoretic mobility shift assays (EMSA) were performed as described [307]. Briefly, ³²P-labeled probes were incubated with nuclear extracts (0.2-1.0 μ g). Binding reactions were incubated 15 minutes at room temperature in the presence of 1 μ g of poly (deoxyinosine-deoxycytosine), 0.25 mg/ml bovine serum albumin, 5 mM dithiothreitol, 50 to 100 mM KCl, 20 mM HEPES, pH 7.9, 20% glycerol, and 0.2 mM EDTA. For supershift assays, 1 μ l antiserum was added to the mixture for 15 minutes at room temperature prior to the addition of the probe. All antisera were obtained from Santa-Cruz Biotechnologies (Santa Cruz, CA): α -USF1 (sc-229), α -USF2 (sc-862), α -E47 (sc-763), α -

Table 9. Electrophoretic Mobility Shift Assay Probes

DNA Motif	Template	Reference
USF Consensus E-box	5' – CACCCGGT <u>CACGTG</u> GCCTACA – 3'	
<i>Fshr</i> Promoter E-box	5' – GATCGGTGGGT <u>CACGTG</u> ACTTTGC – 3'	
E47 Consensus E-box	5' – AGCTTCC <u>CAGCTG</u> AGGAATTCCACAGCTGGGC – 3'	
<i>Trf</i> Promoter E-box	5' – GATCGCCCAAG <u>CAGCTG</u> TACCATGC – 3'	
<i>Id2</i> Promoter E-box	5' – CGGGGCGCGAGCC <u>CAGCTG</u> GGGTGGTAAATA- 3'	
CREB Consensus CRE	5' – GATCCGGCTGACGTCATCAAGCTAGAA – 3'	
<i>Nr5a1</i> Promoter E-box	5' - GTGCAGAGT <u>CACGTG</u> GGGGCAGAG - 3'	[173]
<i>Gata4</i> Promoter E-box	5' - CGGGATCCCTT <u>GACGTG</u> ACTCCC - 3'	[286]
<i>Shbg</i> Promoter E-box (Proximal)	5' – CCTTTAACCCCTCCACCACC <u>CATGTG</u> GAGAGGCTACCTACC CCC - 3'	[245]
<i>Shbg</i> Promoter E-box (Distal)	5' – GAGAATTGTTTCCAGATCT <u>CAAGTG</u> TAACCTAGATAGCC TCC - 3'	[245]
<i>Shbg</i> Promoter E-box (Putative)	5' – GGACTCTGTCCCTCATCT <u>CATCTG</u> CCCTTCAGAGGGGCCGC ATGG - 3'	[245]

E-box motifs are underlined.

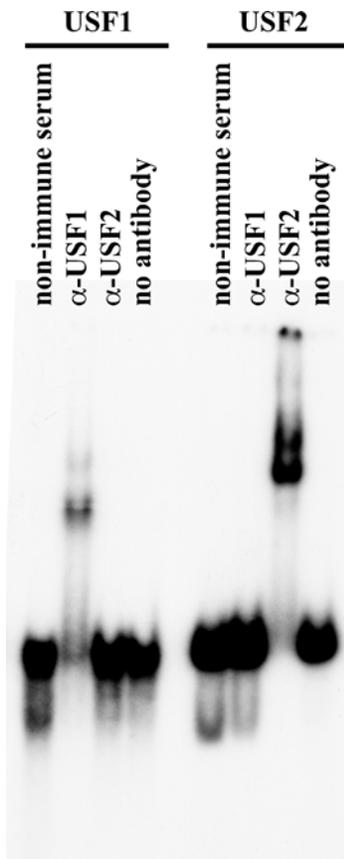


Figure 30. USF1 and USF2 Antisera are Not Cross-reactive in Supershift Assays.

Extracts from COS7 cells transfected with plasmids encoding USF1 (left) or USF2 (right) were incubated with radiolabeled oligos containing the E-box region of the *Fshr* promoter. Supershift analysis determined that the antisera against USF1 or USF2 did not cross-react with the other USF protein. Free probe was run off of the gel.

and purified by phenol/chloroform extraction. The RNA was subjected to reverse transcription using random hexamers [308]. For reverse transcription, 250 ng of RNA was incubated with 100 μ l of reaction mix containing 7.5 mM MgCl₂, 400 μ M deoxynucleotide triphosphates (Promega, Madison, WI), 10x PCR II Buffer (Applied Biosystems Foster City, CA), 40 U RNasin RNase inhibitor (Promega, Madison, WI), 2.25 μ M random hexamers (Integrated DNA Technologies, Coralville, IA), 250 U Superscript reverse transcriptase II (Invitrogen), and nuclease-free water

E2A.E12 (sc-349), α -MYC (sc-40). USF1 and USF2 antisera were specific for their respective proteins in preliminary experiments (Figure 30). DNA loading dye was added to the protein-DNA complexes, which were resolved via 5% polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions in a Tris borate/EDTA buffer. The gels were dried at 80°C for 1 hour and exposed to Classic X-Ray Film, Blue Sensitive (Laboratory Products Sales, Rochester, NY) at -80°C for 1 hour to 1 week.

10.8 QUANTITATIVE PCR ANALYSIS OF GENE

EXPRESSION

RNA was isolated from Sertoli cells using the RNeasy Mini Kit (Quiagen, Inc., Valencia, CA) according to manufacturer's instructions. RNA was treated with RNase-free RQ1 DNase (Promega, Madison, WI) for 2 hours at 37°C

Table 10. Primers for Quantitative PCR

Gene	Accession Number	Sequence	Efficiency	Region Amplified	Reference (*)
<i>Usf1</i>	NM_031777	Forward 5' – AAGTCAGAGGCTCCCAGGA – 3' Reverse 5' – CGGCGTCCACTTCGTTAT – 3'	2.05	693-761	[309]
<i>Usf2</i>	NM_031139	Forward 5' – AGACCAACCAGCGTATGCAG – 3' Reverse 5' – GCTCCTCGGATCTGCTGCCT – 3'	1.90	1386-1473	[309]
<i>Tcfe2a (E47)</i>	NM_01107865	Forward 5' – CACCTTCGGGAGAGACACCA – 3' Reverse 5' – AGGAAGCCAGCCTGACTCAG – 3'	1.99	304-354	
<i>Id1</i>	NM_012797	Forward 5' – CGACTACATCAGGGACCTGCA – 3' Reverse 5' – GAACACATGCCGCCTCGG – 3'	2.12	336-479	[310]
<i>Id2</i>	NM_013060	Forward 5' – ATCCCCAGAACAAGAAGGT – 3' Reverse 5' – CTGTCCAGGTCTCTGGTGGT – 3'	2.01	220-349	[311]
<i>Id3</i>	NM_013058	Forward 5' – GCATCTCCCAGTCCAGACAG – 3' Reverse 5' – TTGGAGATCACAAGTCCCGGA – 3'	2.00	299-349	
<i>Id4</i>	NM_175582	Forward 5' – CGATAACGTGCTGCAGGATCT – 3' Reverse 5' – AGGCTCGTGCCACCAT – 3'	2.09	229-295	[312]
<i>Fshr</i>	NM_199237.1	Forward 5' - ACGCCATTTTCACCAAGAAC - 3' Reverse 5' - TGGGCTTGCAATTCATAACA - 3'	1.86	1944-2022	
<i>Nr5a1</i>	XM_001072904.1	Forward 5' - ATTCCTGAACAACCACAGCC - 3' Reverse 5' - ACCTCCACCAGGCACAATAG - 3'	1.94	1350-1481	
<i>Gata4</i>	NM_144730	Forward 5' - CCCTCTGTGTGGGAACAAC - 3' Reverse 5' - GTGCTCCACCTGGAAAGGTA - 3'	2.00	1970-2081	
<i>Dmrt1</i>	NM_053706.1	Forward 5' - GAGTCTGCGTCCTCTGAACC - 3' Reverse 5' - GCTCACTCGTCCTCATCCTC - 3'	1.85	1159-1229	
<i>Amh</i>	NM_012902.1	Forward 5' - GGAGACCTACCAAGCCAACA - 3' Reverse 5' - CATTTTTAGCAGCAGCACCA - 3'	2.00	1419-1518	
<i>Shbg</i>	NM_012650.1	Forward 5' - GACGGACCCTGAGACACATT - 3' Reverse 5' - AGGGTTTGCTGATTTTGGTG - 3'	2.01	116-244	
<i>Trf</i>	NM_001013110	Forward 5' – CTGTGACCTGTGTATTGGCCC – 3' Reverse 5' – CGTCTCCCTTCTCAACGAGG – 3'	1.90	1575-1675	
<i>Ppia</i>	M19533.1	Forward 5' – ATGGTCAACCCACCGTGT – 3' Reverse 5' – TCTGCTGTCTTTGGAACCTTGTCT – 3'	1.93	43-143	

*Primers without a reference were designed for use in this study with the aid of Primer Express Software v 3.0 (Applied Biosystems, Foster City, CA).

(Ambion). Parallel reactions were performed without reverse transcriptase to control for the presence of contaminant DNA. The samples were incubated at 25°C for 10 min, at 48°C for 30 min, and at 95°C for 5 min followed by 4°C for 5 min.

Real-time PCR (qPCR) amplifications were performed in a 96-well plate in the ABI Prism 7900HT Sequence Detection System v 2.3 (Applied Biosystems, Foster City, CA). For studies of bHLH protein studies, reactions were conducted in a total volume of 25 µl, which included 2 µl of cDNA, 12.5 µl of ABsolute SYBR Green ROX Mix (ThermoFisher Scientific, ABgene House, Surrey, UK) and 30 µM of each primer. For studies of USF target genes,

reactions were conducted in a total volume of 20 μ l, which included 2 μ l of cDNA, 10 μ l of PerfeCTa SYBR Green Fast Mix ROX (Quanta Biosciences, Inc., Gaithersburg, MD) and 30 μ M of each primer. Amplification reactions were also performed on samples containing no cDNA, either from reverse transcription reactions lacking reverse transcriptase or no template, were analyzed as negative controls. Primers used for each gene are listed in Table 10 and primer pairs were independently validated for use in the $\Delta\Delta$ Ct method of gene expression analysis [209] through the use of a standard curve derived from serial dilutions of the cDNA obtained from the reverse transcription reactions. The resulting Ct values for each sample were plotted vs. the log of the mRNA concentration present in each cDNA dilution. The slope of the line was used to calculate the efficiency of amplification [efficiency = $10^{(1/\text{slope})}$]. Primers with an efficiency of 2 ± 0.2 were considered acceptable. *Ppia* (peptidylprolyl isomerase A, commonly known as cyclophilin) was used as an endogenous control. The qPCR analysis initiated with melting of cDNA at 95°C for 15 min, followed by 40 amplification cycles (15 sec at 95°C and 1 min at 60°C). A dissociation curve was performed immediately after amplification to ensure there was only one (gene-specific) amplification peak.

Ct values were recorded and analyzed via the $\Delta\Delta$ Ct method and also via the efficiency-corrected Δ Ct method [209]. The $\Delta\Delta$ Ct method was used to characterize relative fold changes in mRNA expression between treatment groups. The following terms were defined prior to calculation: GOI = gene of interest; Reference = Ct value for GOI of 5 day-old untreated sample; Unknown = Ct value for GOI of samples from any time point or treatment; Control = Ct value of *Ppia* for a given treatment. The Δ Ct was calculated for each sample according to the following formula: Δ Ct = Unknown - Control. The $\Delta\Delta$ Ct was calculated by comparing each sample to the Reference according to the following equation: $\Delta\Delta$ Ct = Δ Ct_{Unknown} - Δ Ct_{Reference}. The Fold Change

was then calculated relative to the Reference according to the formula: Fold Change = $2^{(-\Delta\Delta Ct)}$. The means (\pm SE) of 3 individual experiments were determined for each GOI in each treatment group. The relative quantity of mRNA for each GOI was determined through the efficiency-corrected ΔCt method. The relative quantity is derived from the equation: Quantity = (Efficiency)^{-Ct}. For each sample, the calculated Quantity is then normalized to the quantity found for *Ppia*.

10.9 WESTERN BLOTTING FOR USF PROTEINS

Sertoli cells were prepared for immediate assay as above in ELB. Protein was concentrated when necessary using a Microcon Ultracel YM-3 Centrifugal Filter Unit (Millipore, Billerica, MA). For detection of USF1 and USF2, 50 μ g and 10 μ g of protein, respectively, were added to boiling 2x Laemmli sample buffer for 2 minutes. Lysates were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and incubated with primary antisera against USF1 (1:1000 in TBS with 0.1% Tween-20; Santa Cruz Biotechnologies, Santa Cruz, CA, sc-229), USF2 (1:1000 in TBS with 0.1% Tween-20; Santa Cruz Biotechnologies, Santa Cruz, CA, sc-862), or β -actin (1:100,000 in TBS with 0.1% Tween-20; Sigma-Aldrich, St. Louis, MO, A1978), followed by either goat anti-rabbit antisera (1:20,000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, 11-035-003) or goat anti-mouse antisera (1:50,000; Bio-Rad Laboratories, Inc., Hercules, CA, 170-6516). The antigen-antibody complex was visualized with Millipore Immobilon Western Chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA). USF1 and USF2 antisera were specific and non-cross reactive in preliminary

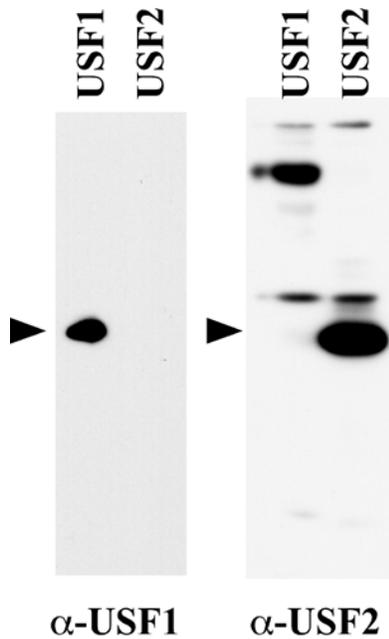


Figure 31. Antisera for USF1 and USF2 Do Not Cross-react with Each Other via Western Blotting. Extracts from COS7 cells transfected with plasmids encoding USF1 or USF2 were analyzed by Western blotting to determine if the antisera against USF1 (left) or USF2 (right) cross-reacted with the other USF protein. Arrows at the left indicate the desired USF band of interest.

experiments (Figure 31). The levels of USF1 and USF2 were normalized to β -actin expression levels.

10.10 WESTERN BLOTTING FOR ID2 PROTEIN

10.10.1 Whole Tissue Nuclear and Cytoplasmic Extracts

Whole liver or testis tissues (approximately 1.5 g each) were washed twice with 10 ml of cold PBS and immersed in 8 ml of PBS supplemented with protease inhibitor and phosphatase inhibitors. Tissues were disrupted with a Dounce homogenizer and centrifuged at 1500 x g for 5 min. The pellets were washed once with PBS and repelleted. The pellets were resuspended in 3 ml of cell lysis buffer (5 mM HEPES, pH 8.0, 85 mM KCl and 0.5% Nonidet P40 supplemented with protease and phosphatase inhibitors), incubated at 4°C for 15 min with rocking and nuclei were pelleted by centrifugation at 3500 x g for 5 min. The supernatant was saved as the cytoplasmic fraction. The nuclei were resuspended in 3 ml of nuclear lysis buffer (1x PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, and 0.1% SDS supplemented with the protease and phosphatase inhibitors). The samples were incubated at 4°C for 15 min with rocking. Nuclei were examined under microscope to ensure lysis. Nuclear debris was collected by centrifugation at full speed. The supernatant was saved as the nuclear fraction. Lysates were stored at -80°C.

Protein concentrations were determined by the Bradford method (Bio-Rad protein assay, Bio-Rad Laboratories, Inc., Hercules, CA). Lysates were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membranes and incubated with primary antiserum against ID2 (1:500, Santa Cruz Biotechnologies, Santa Cruz, CA, sc-489) followed by goat anti-rabbit antiserum as above. Immunodetection was carried out as above.

10.10.2 Assays MSC-1 Cell Extracts with ID2 Antisera from Biocheck, Inc.

MSC-1 whole cell lysates (10 µg) were immunoprecipitated with 2 or 5 µl of ID2 antiserum (Biocheck, Inc.) overnight. Lysates were fractionated by Western blotting as above. Membranes were incubated with primary antiserum against ID2 (Biocheck, Inc.) diluted in 0.015M NaPO₄, 0.85% NaCl, 0.25% BSA, and 0.1% sodium azide, pH 7.4. Membrane washes were conducted in 0.015 M NaPO₄, 0.85% NaCl, and 0.05% Tween-20, pH 7.4. Detection with goat anti-rabbit secondary antiserum was carried out as above.

10.11 IMMUNOFLUORESCENCE

Primary Sertoli cells were cultured in 35-mm² plates containing coverslips three days prior to treatment as described. Sertoli cells were stimulated with vehicle (EtOH) or thyroxine (10⁻⁶ M) for 24 hours. The cells were then washed twice in ice-cold PBS and fixed with 2% paraformaldehyde in saline for 20 min, washed three times with PBS, blocked with goat serum (150 µl per 10 ml of 1x PBS/0.5% BSA/0.15% Glycine), and then incubated with a rabbit antiserum from Santa Cruz Biotechnologies, CA for USF1 (1:1000, sc-229), USF2 (1:1000, sc-

862), ID1 (1:500, sc-488), or ID2 (1:500, sc-489). Sertoli cells were identified by co-staining with mouse antiserum against the vimentin (1:200; Sigma-Aldrich, St. Louis, MO, V6630). The cells were washed a total of six times for 3 minutes each with 1x PBS/0.5% BSA/0.15% Glycine and then incubated with fluorescent secondary goat anti-rabbit Alexa488 alone or also with goat anti-mouse Cy5. Following six washes for 3 minutes each of 1x PBS/0.5% BSA/0.15% Glycine, nuclei were visualized by staining with Hoechst 33258. Hoechst was applied for 10 seconds and immediately washed two times with 1x PBS. Coverslips were then mounted onto glass slides with gelvatol. Images were obtained on an Olympus Provis AX70 Microscope with a 60x objective. All image files were digitally processed for presentation in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

Initial studies were conducted in 5 day-old Sertoli cells that were probed with ID2

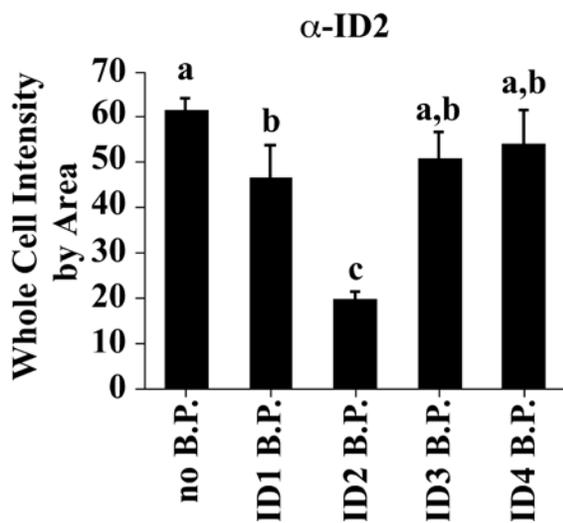


Figure 32. ID2 Antiserum is Specific in Cultured 5 Day-old Sertoli Cells.

Immunostaining of ID2 in 5 day-old cultured Sertoli cells were quantified using Metamorph. Antiserum was preincubated with or without blocking peptides (B.P.) for ID1, ID2, ID3, or ID4. The mean total cell intensity normalized to the total cell area was plotted on the graph (\pm SE). Values with different lowercase letters differ significantly ($p < 0.05$).

antisera pre-incubated with blocking peptides for ID1, ID2, ID3, and ID4 antisera. Control studies for ID2 immunostaining utilized blocking peptides at a ratio of 1 unit of antiserum to 5 unit of blocking peptide that were supplied by the manufacturer (Figure 32). When blocking peptides were incubated with ID2 antiserum, ID1 blocking peptide was able to inhibit the immunofluorescence signal by approximately $23.9 \pm 7.0\%$ ($p < 0.05$), whereas ID2 blocking peptide was able to reduce the immunofluorescence signal by $67.8 \pm 1.7\%$ ($p <$

0.05; Figure 32). ID3 and ID4 blocking peptides were not able to diminish the immunofluorescence signal for ID2 antisera significantly.

To quantify immunofluorescent signals, images were taken in a blind and random manner. Images were quantified utilizing Metamorph Software (Molecular Devices, Downingtown, PA). Within each field, the number of nuclei present and the number of vimentin-positive cells were recorded. Five vimentin-positive cells in each field were then analyzed by recording the nuclear area, nuclear intensity, cell area, and cell intensity. Nuclear area was determined by thresholding the nuclei based on Hoechst staining using the blue channel. The integrated value of staining for antisera against the protein of interest was established within the thresholded area on the red channel. The integrated value indicates the sum of the intensity of all pixels within the designated area. Approximate cell area was estimated as best guess using present staining but was not exact as all cell borders were not clearly visible. The integrated value of the approximate cell area was also recorded. Calculations could then be conducted to determine the average pixel intensity for each cell and nuclei counted. Cells were categorized by age and density. Pixel intensity was analyzed from greater than 450 vimentin-positive cells for each age from six independent experiments.

10.12 CHROMATIN IMMUNOPRECIPITATION

Whole testes were removed from rats euthanized 5 and 11 days after birth. Testes were decapsulated before exposure to 1% formaldehyde for 10 min at room temperature. Glycine was added to a final concentration of 0.125 M, and, after 5 min, the samples were washed two times and harvested in cold PBS with protease inhibitors. Tubules were collected by centrifugation for

5 min at 1000 x g. Tubules were suspended in 1 ml swelling buffer (10 mM Hepes, 1.5 mM MgCl₂, 100 mM KCl, 200 μM sodium orthovanadate, 150 μM spermine, 500 μM spermidine, 1 mM DTT, 0.2% igepal, 1 mM EDTA, pH 8.0, 5% sucrose) per 100 μl of pellet. The sample was then homogenized ten times using a Dounce homogenizer and an A Pestle followed by ten strokes with a B Pestle. The sample was then layered onto 0.5 ml cushion buffer (10 mM Tris, pH 7.5, 15 mM NaCl, 60 mM KCl, 150 μM spermine, 500 μM spermidine, 1 mM EDTA, pH 8.0, 10% Sucrose) into thick-walled centrifuge tubes and nuclei were pelleted by centrifugation for 1 hour at 30,000 x g in a Sorvall RC M120GX. The pellet was transferred to eppendorf tubes and was washed two times in ice cold PBS with protease inhibitors. Nuclei were recollected by centrifugation at 13,000 x g for one minute between washes. The pellet was then suspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0).

Chromatin immunoprecipitation (ChIP) assays were performed following the Upstate Biotechnology ChIP assay kit protocol, with modifications. Briefly, the 0.5 ml/tube of cell lysate was sonicated to yield 200–1,000 bp genomic DNA fragments utilizing the Bioruptor (Diagenode, Inc., Sparta, NJ) at 4°C in ice water on high for 10 minutes of cycles: 30 seconds on, 30 seconds off. Sonication parameters were determined in preliminary experiments (Figure 33). Each 0.5 ml sample was mixed with 2.5 ml of dilution buffer (16.7 mM Tris, pH 8.1, 167 mM NaCl, 0.01% SDS, 1.2 mM EDTA, pH 8.0, 1.1% Triton-X 100) and stored at -80°C in 0.5 ml aliquots. Protein concentrations were determined using the BCA Protein Assay (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL). Fragmentation was verified by taking 10 μl of sample, reversing the cross-link (see below) and resolving the purified chromatin in 0.05% glycerol on a 5% polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions in a Tris borate/EDTA buffer (Figure 33).

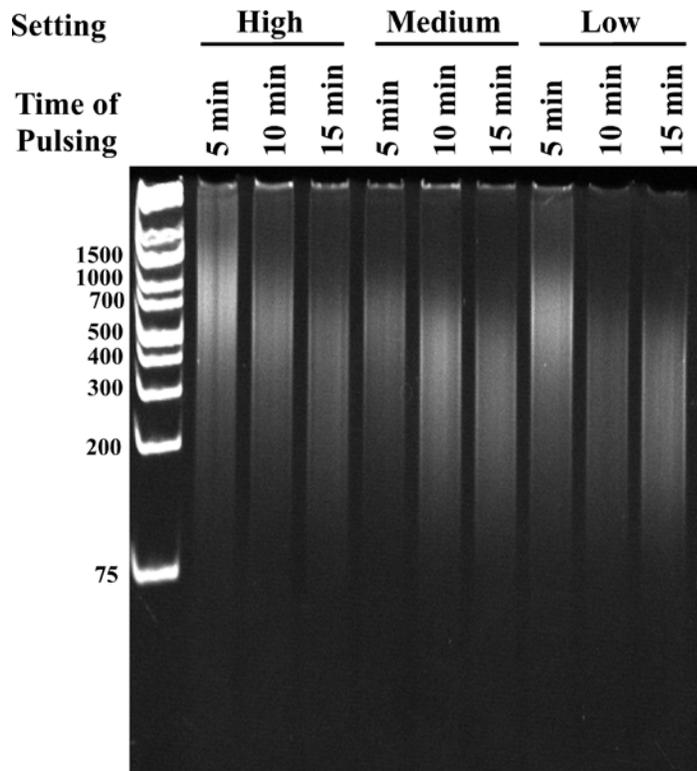


Figure 33. Sonication of Chromatin for Chromatin Immunoprecipitation Assays.

Chromatin isolated from whole testis (0.5 ml of cell lysate/tube) was sonicated to yield 200–1,000 bp genomic DNA fragments utilizing the Bioruptor on high, medium or low for 5, 10 or 15 minutes of cycles: 30 seconds on, 30 seconds off. The optimal fragmentation was achieved with 10 minute cycles on the high setting.

763), E2A.E12 (Santa Cruz Biotechnologies, Santa Cruz, CA, sc-349), MYC (Santa Cruz Biotechnologies, Santa Cruz, CA, sc-40), or CREB (Upstate, Lake Placid, NY, 06-863).

Controls were also incubated with non-immune serum or without protein. Samples were incubated overnight with rocking at 4°C. Immunocomplexes were recovered by incubation with 50 µl of 50% slurry of protein A sepharose for 1 h at 4°C. The beads were washed 5 min each with low-salt (20 mM Tris pH8.1, 150 mM NaCl, 2 mM EDTA, pH 8.0, 1% Triton-X 100, 0.1% SDS), high-salt (20 mM Tris, pH 8.1, 500 mM NaCl, 2 mM EDTA, pH 8.0, 1% Triton-X 100, 0.1% SDS), and LiCl buffer (250 mM LiCl, 1% Igepal, 1% sodium deoxycholate, 10 mM Tris, pH 8.1, 1 mM EDTA, pH 8.0). The beads were then washed two times in 10 mM Tris, pH 8.0, 1

The lysate (200 µg) in a total of 1000 µl of dilution buffer was precleared two times with 40 µl of a 50% slurry of protein A sepharose in the presence of 20 µg of salmon sperm DNA. After centrifugation at 1,000 x g for 1 min, aliquots of the supernatant were incubated with 8 µg of antiserum directed against USF1 (Santa Cruz Biotechnologies, Santa Cruz, CA, sc-229 Trans-X), USF2 (Santa Cruz Biotechnologies, Santa Cruz, CA, sc-862 Trans-X), E47 (Santa Cruz Biotechnologies, Santa Cruz, CA, sc-

mM EDTA buffer. The chromatin complexes were eluted by incubation with 250 μ l freshly prepared elution buffer (1% SDS, 0.1 M NaHCO₃) with rotation at room temperature for 15 min. The supernatant was saved to a new tube and the elution was repeated. The supernatant from the second elution was pooled with the first elution. The cross-linking was reversed by adding NaCl to a final concentration of 200 mM and heating at 65°C overnight. After incubation with 40 μ g proteinase K for 1 h at 45°C, the DNA was purified using phenol/chloroform extraction.

Immunoprecipitation of promoter regions were quantified by qPCR with PerfeCTa SYBR Green Fast Mix ROX (Quanta Biosciences, Inc., Gaithersburg, MD) using 2 μ l of the purified DNA as template (see above). Primers are listed in Table 10. Standard curves for each primer set sample were constructed using two-fold serial dilutions of the unbound chromatin fragments for each sonicated sample as a reference input. The log of the percentage of input was plotted vs. Ct value to obtain a standard curve. Ct values for each samples were plotted on the line to determine the logarithmically transformed value of the percentage of input. The resulting value from the line was reverse transformed to obtain the percentage of input. The percentage obtained for the IgG control sample was subtracted from all other samples to account for non-specific binding. Average percentages of inputs were calculated for each antibody used.

Table 11. Chromatin Immunoprecipitation Primers

Gene	Efficiency (*)	Region Amplified	Sequence
<i>Nr5a1</i> CDS	2.03	+1350 to +1481	Forward 5' – AAT CCT GAA CAA CCA CAG CC – 3' Reverse 5' – ACC TCC ACC AGG CAC AAT AG – 3'
<i>Nr5a1</i> E-box	2.18	-130 to -10	Forward 5' – CCC AAA TGA AGA GAA ACA CCA – 3' Reverse 5' – CGA TCG TCC TCT CCT CCT C – 3'

*Efficiency determined by a standard curve of input cDNA.

10.13 STATISTICAL ANALYSIS

DNA-protein complex formation and autoradiograms were quantified from digitized images of the X-ray films using ImageJ v1.37 software [313, 314]. Amplicons were quantified using Image J v 1.37 software from digital images of agarose gels visualized with AlphaEase®FC software version 4.1 (Alpha Innotech Corporation, San Leandro, CA). For all studies, the mean (\pm SE) value or intensity of three individual experiments were determined for each sample group unless otherwise noted. Results were analyzed by ANOVA with Fisher's PLSD at a 5% significance level utilizing Statview 4.5 (Abacus Concepts, Inc., Berkeley, CA).

APPENDIX A

SERTOLI CELL POSITIVE IMMUNOSTAINING CONTROLS

Immunofluorescent images presented throughout this dissertation have been shown without the presence of immunostaining with a Sertoli cell-specific marker. The images provided in this appendix include immunostaining with antiserum against the Sertoli cell-specific vimentin cytoskeletal protein. Due to overexposure of the vimentin immunostaining, vimentin immunostaining was excluded in earlier figures to facilitate visualization of the proteins of interest.

A.1 USF1 PROTEIN IMMUNOSTAINING

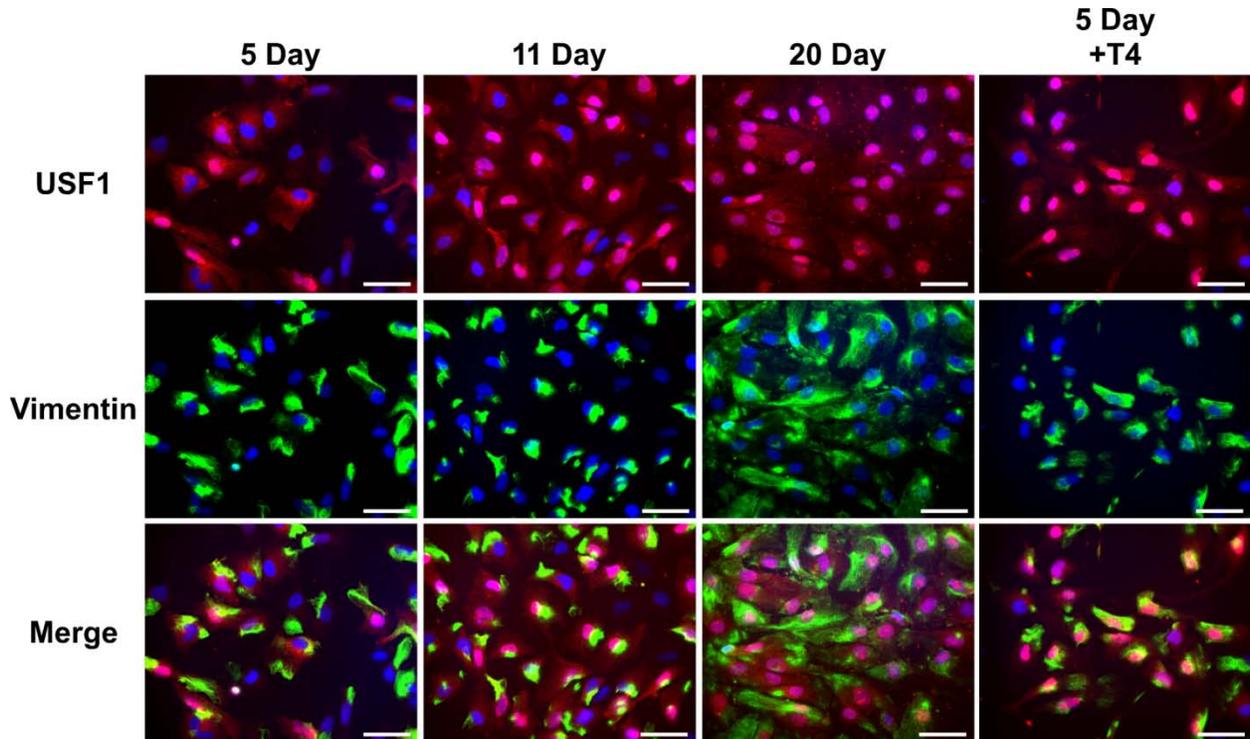


Figure 34. Immunostaining for USF1 Protein with Vimentin Co-localization in Cultured Sertoli Cells – Control for Figures 11 and 21.

Immunofluorescence of cultured 5, 11 and 20 day-old Sertoli cells was performed with antisera recognizing USF1 (top) as visualized with Alexa 488 fluorophore (red). Sertoli cells were identified by immunostaining with antisera against vimentin (middle) as visualized with the Cy5 fluorophore (green). Hoechst stains nuclei (blue). The bottom row of images merges the top two panels to show co-localization. The column on the right depicts 5 day-old Sertoli cells that were stimulated with T4 for 24 hours. Scale bars = 100 μ m.

A.2 USF2 PROTEIN IMMUNOSTAINING

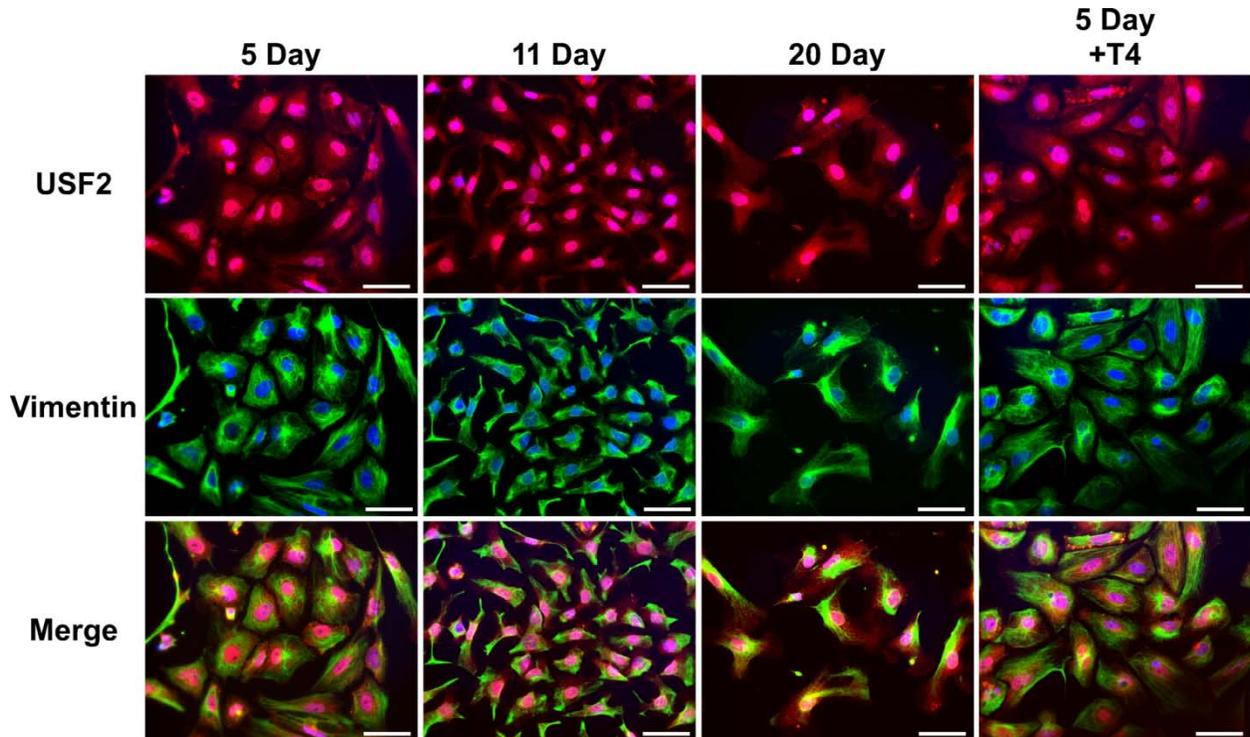


Figure 35. Immunostaining for USF2 Protein with Vimentin Co-localization in Cultured Sertoli Cells – Control for Figures 11 and 21.

Immunofluorescence of cultured 5, 11 and 20 day-old Sertoli cells was performed with antisera recognizing USF2 (top) as visualized with Alexa 488 fluorophore (red). Sertoli cells were identified by immunostaining with antisera against vimentin (middle) as visualized with the Cy5 fluorophore (green). Hoechst stains nuclei (blue). The bottom row of images merges the top two panels to show co-localization. The column on the right depicts 5 day-old Sertoli cells that were stimulated with T4 for 24 hours. Scale bars = 100 μm .

A.3 ID2 PROTEIN IMMUNOSTAINING

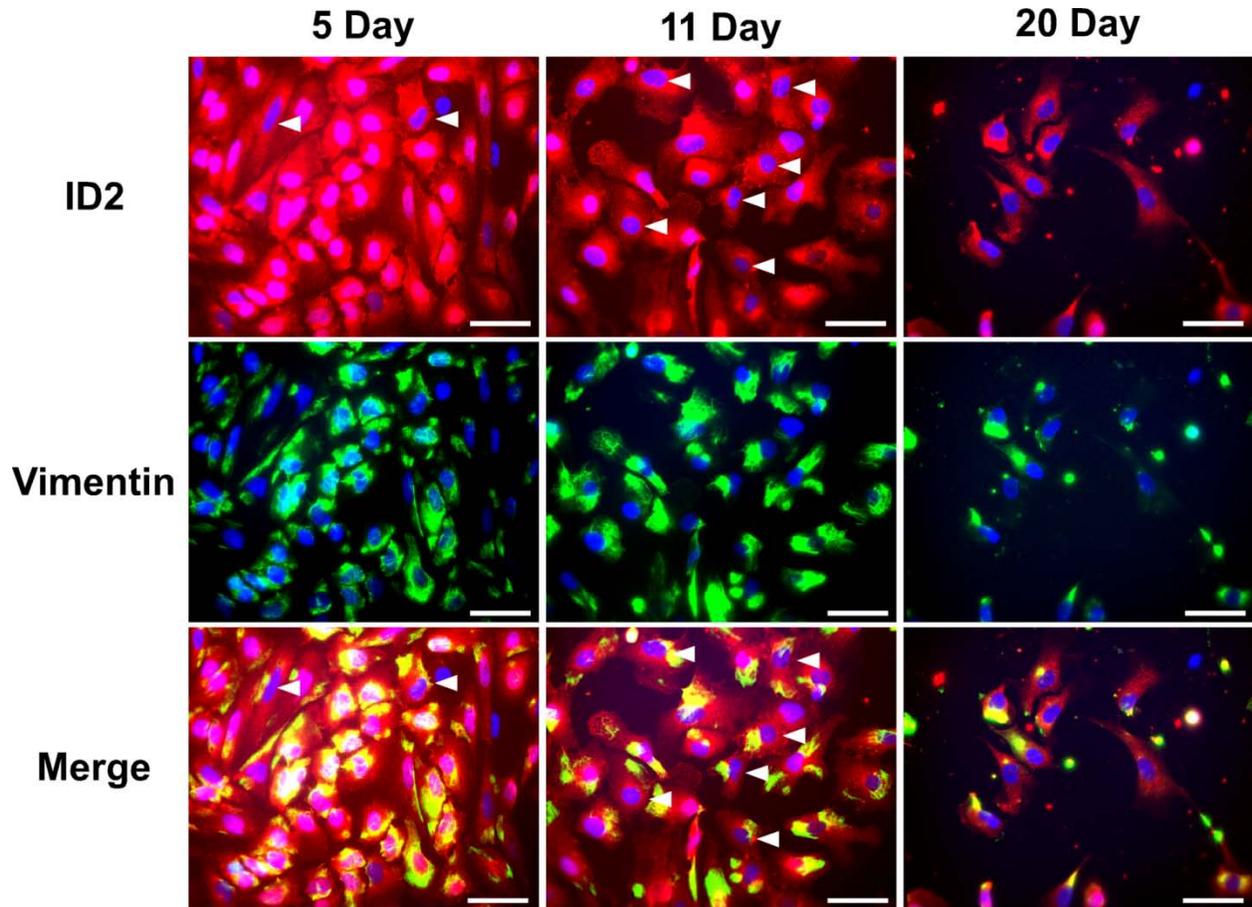


Figure 36. Immunostaining for ID2 Protein with Vimentin Co-localization in Cultured Sertoli Cells – Control for Figure 14.

Immunofluorescence of cultured 5, 11 and 20 day-old Sertoli cells was performed with antisera recognizing ID2 (top) as visualized with Alexa 488 fluorophore (red). Sertoli cells were identified by immunostaining with antisera against vimentin (middle) as visualized with the Cy5 fluorophore (green). Hoechst stains nuclei (blue). The bottom row of images merges the top two panels to show co-localization. Scale bars = 100 μ m.

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