Transcriptional Regulation of FasL Expression and Participation of sTNF-α in Response to Sertoli Cell Injury*

Pei-Li Yao1, Yi-Chen Lin2, Pragati Sawhney3, and John H. Richburg1,§

From the 1Institute for Cellular and Molecular Biology and the 3Division of Pharmacology and Toxicology, College of Pharmacy, University of Texas, Austin, Texas 78712-1074

The Fas/FasL signaling pathway has previously been demonstrated to be critical for triggering germ cell apoptosis in response to mono-(2-ethylhexyl)phthalate (MEHP)-induced Sertoli cell injury. Although Sertoli cells ubiquitously express the FasL protein, MEHP-induced germ cell apoptosis appears to tightly correlate with increased levels of Sertoli cell FasL. Here we characterize the transcriptional regulation of the murine FasL gene in Sertoli cells after MEHP exposure. A serial deletion strategy for 1.5 kb of the 5′-upstream activating sequence of the Fasl promoter was used to determine transcriptional activity in response to MEHP. Luciferase activity of the FasL promoter in the rat Sertoli cell line ASC-17D revealed that two regions, −500 to −324 and −1250 to −1000, were necessary to drive the inducible transcription of FasL. Sequence analysis of these two regions revealed two cis-regulatory elements, NF-κB and Sp-1. By site-directed mutagenesis, electrophoretic mobility shift and chromatin immunoprecipitation assays, it was confirmed that MEHP-induced FasL expression is enhanced through the transcriptional regulation of both NF-κB and Sp-1. Experiments performed both in vitro and in vivo revealed that MEHP exposure results in an increased production of sTNF-α and that sTNF-α-mediated NF-κB activation causes robust increases in FasL levels in both the ASC-17D Sertoli cell line and in primary rat Sertoli cell/germ cell co-cultures. In the seminiferous epithelium, Sertoli cells express TNFR1, whereas germ cells produce TNF-α. Therefore, sTNF-α released by germ cells after MEHP-induced Sertoli cell injury acts upon Sertoli cell TNFR1 and activates NF-κB and Sp-1 that consequently causes a robust induction of FasL expression. These novel findings point to a potential “feed-forward” signaling mechanism by which germ cells prompt Sertoli cells to trigger their apoptotic elimination.

Germ cell apoptosis occurs spontaneously in the adult testis and likely serves as a physiological mechanism to control the number of germ cells in the testis and maintain functional spermatogenesis (1). Proper levels of germ cell apoptosis are particularly important during the first 2–3 weeks after birth in rodents (2, 3), a period representing “the first wave of spermatogenesis” when the initial spermatogonia divide to result in the production of the initial spermatocytes and spermatids. Although many studies in humans have associated alterations in the incidence of germ cell apoptosis with conditions of abnormal spermatogenesis and male infertility (4–6), the cellular processes that regulate germ cell apoptosis in the testis remain poorly characterized.

Fas ligand (FasL), a type II transmembrane protein belonging to tumor necrosis factor (TNF) superfamily, is a well-characterized apoptosis initiating protein (7, 8). An interaction between FasL and its cognate receptor Fas stimulates the formation of the death-inducing signaling complex that ultimately leads to the orderly death of the cell (9–11). Previous reports demonstrated that the Fas/FasL paracrine signaling mechanism between Sertoli cells and germ cells plays a key role in mediating germ cell apoptosis in the testis after Sertoli cell injury (12–14). In this model, Sertoli cell-expressed FasL acts on Fas-expressing germ cells to initiate their elimination by apoptosis. A number of studies indicate that Sertoli cells are the main cells expressing FasL in the seminiferous tubule (15–18) and that Fas is localized to specific germ cell subtypes (15). Two reports from the same laboratory indicate that some germ cell subtypes may also express FasL (19, 20).

Phthalates are a class of chemical compounds that are generally used as plasticizers to produce plastic wraps, toys, and bags, and have been shown to adversely affect spermatogenesis in juvenile male mammals and induce developmental variations and malformations in conception after maternal exposure (21). Consequently, the risk of phthalate exposure, especially to the developing mammal, is of concern. Immunohistochemical studies indicate that mice exposed to di-2-(ethylhexyl)phthalate reveal an increasing distribution of FasL in the Sertoli cell (22). Mono-2-(ethylhexyl)phthalate (MEHP), the active metabolite of di-2-(ethylhexyl)phthalate, is a well-known Sertoli cell toxicant (23–26). Exposure of rodents to MEHP causes an early induction in FasL mRNA expression (27, 28) and increase the FasL protein levels in the testis (26).

Several promoter studies indicated that transcriptional regulation of FasL is important to control the death signaling pathway in the testis, eyes, placenta, and brain (9, 29). Some transcription factors have been implicated in the regulation of FasL gene expression, including specificity protein-1 (Sp-1) (30),

*This work was supported by NIEHS National Institutes of Health Grant ES09145 (to J. H. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: 1 University Station, Austin, TX. 78712-1074. Tel.: 512-471-4736; Fax: 512-471-5002; E-mail: john_richburg@mail.utexas.edu.

2 The abbreviations used are: FasL, Fas ligand; TNF, tumor necrosis factor; MEHP, mono-(2-ethylhexyl)phthalate; Sp-1, specificity protein-1; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UAS, upstream activating sequence; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; MMA, mithramycin A.
interferon regulatory factor-1 (31), nuclear factor in activated T cells (32, 33), and nuclear factor-κB (NF-κB) (34). The constitutive basal regulation of FasL transcription in Sertoli cells has been reported to be controlled by Sp-1 (35). Although Sp-1 is sufficient to drive basal transcription of FasL in Sertoli cells, several studies also demonstrate that Sp-1 acts co-operatively with other transcription factors, such as activator protein-1, Ets-1, NF-κB, signal transducers and activators of transcription 1, and E2F (29, 36). However, the exact regulation of Sertoli cell FasL gene expression in response to MEHP, as well as the regulation of its inducible expression in the testis, is still unknown.

In this study, we investigate the transcriptional regulation of FasL in Sertoli cells in response to MEHP using in vitro and in vivo experimental approaches. Sequential deletion of the FasL promoter sequence was used to analyze differential transcriptional activity levels after MEHP treatment. We identify at least two transcription factors that participate in MEHP-induced FasL expression (NF-κB and Sp-1). Furthermore, we demonstrate that NF-κB controlled FasL expression in Sertoli cells is triggered by sTNF-α released from germ cells. These results reiterate the importance of paracrine interactions between Sertoli cells and germ cells after MEHP-induced Sertoli cell injury.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The adult rat Sertoli cell line, ASC-17D (a gift from Ann Clark, Serono Research Institute, Rockland, MA; originally created by the laboratory of Dr. Ken Roberts) (37), was cultivated at 33 °C in cell culture medium consisting of equal volumes of Dulbecco’s modified Eagle’s media and Ham’s F-12 supplemented with 4% fetal bovine serum and 1% antibiotic penicillin-streptomycin (all Invitrogen). ASC-17D cells were created with a temperature-sensitive mutant of the SV40 ori plasmid. Two transcription factors that participate in MEHP-induced FasL expression (4) were identified, and one of these factors is NF-κB. However, the exact regulation of Sertoli cell FasL in response to MEHP using in vivo and in vitro experimental approaches. Sequential deletion of the FasL promoter sequence was used to analyze differential transcriptional activity levels after MEHP treatment. We identify at least two transcription factors that participate in MEHP-induced FasL expression (NF-κB and Sp-1). Furthermore, we demonstrate that NF-κB controlled FasL expression in Sertoli cells is triggered by sTNF-α released from germ cells. These results reiterate the importance of paracrine interactions between Sertoli cells and germ cells after MEHP-induced Sertoli cell injury.

**Preparation of Primary Sertoli Cell/Germ Cell Co-cultures**—Primary co-cultures were prepared as previously described (27). Briefly, testes of 28-day-old Fisher rats were detunicated and subjected to a series of enzymatic digestions. Cells (2 × 10⁶) were seeded in a 10-cm culture dish containing Dulbecco’s modified Eagle’s media and Ham’s F-12 media supplemented with 1 ng/ml of epidermal growth factor (Sigma), 15 μg/ml of ITS plus premix (insulin, transferrin, selenious acid, bovine serum albumin, and linoleic acid; BD Biosciences), and 50 μg/ml of gentamicin (Invitrogen).

**Animals**—28-Day-old male wild-type C57BL/6j mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were acclimatized for 1 week before experiments. The climate of the animal room was kept at a constant temperature (22 ± 0.5 °C) at 35–70% humidity with a 12 light:12 dark photoperiod. Animals were given standard lab chow and water ad libitum. All procedures involving animals were performed in accordance with the guidelines of the University of Texas at Austin’s Institutional Animal Care and Use Committee in compliance with guidelines established by the National Institutes of Health.

**Transcriptional Regulation of FasL in Sertoli Cells**

**MEHP Treatment**—5 × 10⁵ ASC-17D cells and 2 × 10⁶ primary Sertoli cell/germ cell co-cultures were dosed with 200 μM MEHP (TCI America, Portland, OR) diluted in Me2SO for various time periods. The viability of MEHP-treated cells was evaluated to determine the appropriate dosage using the trypan blue dye exclusion method and AO/EB staining.

To evaluate MEHP-triggered gene expression in the testis in vivo, 28-day-old C57BL/6j mice were given a single dose of MEHP (1 g/kg) by oral gavage, a standard procedure for the investigation of MEHP-induced testicular toxicity (38). Animals were given MEHP in corn oil by oral gavage at a volume equal to 4 ml/kg. Control animals received a similar volume of vehicle (corn oil). Vehicle and MEHP-treated animals were killed by CO₂ inhalation. The testis was flash frozen in liquid N₂ and stored at −80 °C.

**Real-time Quantitative RT-PCR**—To measure the level of FasL mRNA expression in ASC-17D cells, real-time quantitative reverse transcription-PCR (RT-PCR) was performed. Total RNA was isolated using TRIzol reagent (Invitrogen Corp.). First strand cDNA was prepared using 2 μg of total RNA with SuperScript II reverse transcriptase and oligo(dT) primer (all Invitrogen Corp.). The detailed procedure of real-time quantitative RT-PCR described by Dr. Chen (39) was followed with minor modifications. Briefly, all reactions were carried in 50-μl volumes containing 25 μl of SYBR Green PCR master mixture (Applied Biosystems Inc., Foster City, CA). The forward and reverse primers used to detect the rat FasL expression levels were: F441, 5’-ACCAACACAGCCTTAGATCATCTAC’3’ and R493, 5’-TGTTAAGTGGCCACACTCCTT-3’. Glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) was quantified as an internal control using the forward and reverse primers: F811, 5’-AACCTGGGAATGTGACATC’3’ and R843, 5’-AACGGTGTTAGCAGCGG-3’. DNA amplification was carried out using an ABI 7700 Sequence Detection System (Applied Biosystems). Each assay including a standard curve and a non-template control was performed in triplicate. The relative expression level of FasL against that of GAPDH was defined as $\frac{\Delta \text{CT}}{\Delta \text{CT}_{\text{GAPDH}}} = \frac{\text{CT}_{\text{FasL}} - \text{CT}_{\text{GAPDH}}}{\Delta \text{CT}_{\Delta \text{CT}_{\text{GAPDH}}}}$.

**Preparation of Nuclear Extracts**—The procedure from Chen et al. (40) was used to isolate nuclear extracts, with slight modifications. Briefly, adherent ASC-17D cells were scraped into 1.5 ml of cold phosphate-buffered saline, pelleted by centrifugation, and resuspended in 400 μl of cold hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride). Cells were lysed on ice for 10 min and then centrifuged at 5,000 × g for 5 min at 4 °C. The nuclear pellet was resuspended in 80 μl of cold high salt buffer (20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride), incubated on ice for 20 min, and centrifuged at 17,800 × g for 5 min at 4 °C. The supernatant containing the nuclear extract was collected and flash frozen in liquid N₂ and stored at −80 °C.

**Total Protein Preparation and Western Blot Analysis**—A detailed description for total protein preparation from cultured cells and mice tissue, and Western blot analysis have been
Transcriptional Regulation of FasL in Sertoli Cells

Table 1: Nucleotide sequence of PCR primers used to generate sequential deletions in the upstream activating sequence of the murine Fas ligand gene

| Deletion clone  | Primer sequence*          |
|-----------------|---------------------------|
| FasL-Luc-P1500  | Forward: 5’-CAGCTAGGTCCTGTCCTTCTTTCGAG-3’ |
|                 | Reverse: 5’-CCAACTTCAGGCCCAGAAG-3’          |
| FasL-Luc-P1250  | Forward: 5’-CAGCTAGGTCCTGTCCTTCTTTCGAG-3’ |
|                 | Reverse: 5’-CCAACTTCAGGCCCAGAAG-3’          |
| FasL-Luc-P1000  | Forward: 5’-CAGCTAGGTCCTGTCCTTCTTTCGAG-3’ |
|                 | Reverse: 5’-CCAACTTCAGGCCCAGAAG-3’          |
| FasL-Luc-P500   | Forward: 5’-CAGCTAGGTCCTGTCCTTCTTTCGAG-3’ |
|                 | Reverse: 5’-CCAACTTCAGGCCCAGAAG-3’          |

* Restriction enzyme cutting sites located within PCR primers are underlined. NheI site, GCTAGC; HindIII site, AAGCTT.

described previously (41). Total cellular proteins and nuclear extracts were detected using primary antibodies against FasL (1:200, Abcam Inc., Cambridge, MA), TNF-α (1:500, Abcam Inc.), phospho-1xβα (1:500, Cell Signaling Technology, Inc., Beverly, MA), NF-κB (1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA), Sp-1 (1:500, Santa Cruz Biotechnology Inc.), and β-actin (1:500, Santa Cruz Biotechnology Inc.) coupled with horseradish peroxidase-conjugated secondary antibody (1:5000, Santa Cruz Biotechnology Inc.). The ECL chemiluminescent substrate (Amersham Biosciences) was used as the detection reagent and β-actin as the internal control for gel loading.

Construction of Luciferase Reporter Gene—Expression of the luciferase gene under the control of the FasL promoter and 5’-upstream activating sequence (5’-UAS) (GeneBankTM accession number AF045739) was used to evaluate the effects of MEHP exposure on Sertoli cell line ASC-17D. FasL-P1500, a 1500-bp region, upstream of the FasL promoter, was PCR amplified using a mouse BAC clone (RP24–253-H8, BACPAC Resources, The Children’s Hospital Oakland Research Institute, Oakland, CA). Varying lengths of the 5’ end of the FasL promoter, FasL-P500, FasL-P1000, and FasL-P1250, were generated by PCR using the FasL-P1500 fragment as the template. The common reverse primer and distinct forward primers used for each successive fragment are shown in Table 1. Primers were designed to contain overhanging sites for the restriction enzymes HindIII and NheI, respectively, which allowed for directional cloning into the multiple cloning site of the luciferase expressing vector plasmid pGL3-3-enhancer (Promega Corp.). FasL-P138, FasL-P253, FasL-P298, FasL-P309, FasL-P324, and FasL-P689 fragments cloned in pGL3 vector were kindly provided by Dr. Ratliff, University of Iowa (42), and were subcloned into pGL3-3-enhancer vector. The composition of all of the constructs was confirmed by restriction endonuclease digestion and DNA sequencing.

Transfection and Luciferase Activity Assay—All transfections were carried out in triplicate in six-well cell culture plates. 2 x 10^5 ASC-17D cells were transiently transfected using Lipofectamine™ Transfection Reagent (Invitrogen Corp.) according to the manufacturer’s instructions. 10 μg of the luciferase reporter constructs described above, along with the mock plasmid (promoter-less), were co-transfected with 1 μg of the β-galactosidase construct, pCMV-β (Promega Corp.). The cells were incubated in transfection mixture for 6 h and then harvested after 24 h in culture. Cell lysates were prepared in 200 μl of lysis buffer (Luciferase Assay Kit, Roche). Luminescence was estimated, in cell lysates after addition of luciferase substrates A and B, on a Victor2 1420 Multilabel Counter (Wallac, PerkingElmer Life Sciences). β-Galactosidase activity of the control vector pCMV-β was assayed on the same culture using the cell extract with β-galactosidase substrate reagents (β-Galactosidase Assay Kit, Roche). Luciferase activity was normalized by dividing the mean of relative luciferase units by the mean of relative β-galactosidase units. All experiments were performed in triplicate. Non-transfected and mock-transfected cells were used as negative controls.

DpnI-mediated Site-directed Mutagenesis—To confirm that the identified cis-regulatory element is indeed essential for MEHP-induced FasL up-regulation, we employed DpnI-mediated site-directed mutagenesis (43, 44) to alter its sequence. The DpnI endonuclease targeting sequence, 5’-GmATC-3’, is specific for methylated and hemimethylated DNA. The procedure outlined below was performed for disruption of NF-κB and Sp-1 binding sites. The designed sequence-specific primers overlapping with and flanking the NF-κB binding fragment at the 5’ end of the FasL-Luc-P1500 fragment (Fig. 1a) were 5’-actcctggctttattaac-3’ and 5’-tgggtcagggtcatgatttttaaaagaccaggattt-3’. For the Sp-1 binding site (Fig. 1a, binding site 2), the designed sequence-specific primers were 5’-agcctgttagctattttgggcccggggagggt-3’ and 5’-caccctccccggccccaaaatgatatcagcgtct-3’. The mutation sites are identified by underlines. pGL3-enhancer-FasL-P500 and pGL3-enhancer-FasL-P1250 plasmids were used as templates for the PCR: 12 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 1 min using the Electrophoretic Mobility Shift Assay kit (Invitrogen Corp.). The cells were incubated in transfection mixture for 6 h and then harvested after 24 h in culture. Cell lysates were prepared in 200 μl of lysis buffer (Luciferase Assay Kit, Roche). Luminescence was estimated, in cell lysates after addition of luciferase substrates A and B, on a Victor2 1420 Multilabel Counter (Wallac, PerkinElmer Life Sciences). β-Galactosidase activity of the control vector pCMV-β was assayed on the same culture using the cell extract with β-galactosidase substrate reagents (β-Galactosidase Assay Kit, Roche). Luciferase activity was normalized by dividing the mean of relative luciferase units by the mean of relative β-galactosidase units. All experiments were performed in triplicate. Non-transfected and mock-transfected cells were used as negative controls.

Electrophoretic Mobility Shift Assay (EMSA)—Double-stranded oligonucleotides containing Sp-1 and NF-κB binding sequences were designed for EMSA as follows: Sp-1, forward, 5’-CCTGGAATGATCAGGGTGGGCG-3’ and reverse, 5’-CGCCCCACCTGATATCCAG G-3’; NF-κB, forward, 5’-CTCTCTGTTGTTTTTCCCATGC-3’ and reverse, 5’-GCA-TGGGGAAAAGACCAAGAGG-3’. The forward and reverse oligonucleotides were annealed to each other to generate double-stranded binding fragments. Equal amounts of nuclear extract (5 μg) from ASC-17D cells, grown in the absence or presence of MEHP, were incubated with the double-stranded Sp-1 and NF-κB binding fragments at room temperature for 20 min using the Electrophoretic Mobility Shift Assay kit (Invitrogen Corp.), according to the manufacturer’s instructions, with
Transcriptional Regulation of FasL in Sertoli Cells

Antibody Neutralization by Anti-TNF-α Primary Antibody—Various concentrations of anti-TNF-α primary antibody (Santa Cruz Biotechnology, Inc.) were added to primary Sertoli cell/germ cell co-cultures for 12 h. Nuclear extracts and total protein were isolated as previously described. The NF-κB and FasL expression in the nucleus and total protein were detected, respectively, by Western blot analysis. Anti-rabbit IgG was used as a negative control.

Measurement of sTNF-α Level in Primary Co-cultures—To determine the production of sTNF-α from the testis, 2 × 10⁶ primary Sertoli cell/germ cells in co-culture were exposed to 200 μM MEHP. After 12 h incubation, the culture medium was collected, centrifuged to remove cellular debris, and the supernatant frozen at −80 °C until assayed for sTNF-α by enzyme-linked immunosorbent assay (R&D Systems, Inc., Minneapolis, MN). MEHP-exposed cells, remaining in 10-cm culture dishes, were rinsed twice with phosphate-buffered saline and counted. This cell number was used to normalize the sTNF-α protein level.
Transcriptional Regulation of FasL in Sertoli Cells

Expression assay by enzyme-linked immunosorbent assay. All experiments were performed in triplicate.

Statistical Analysis—All experiments were performed in triplicate and repeated at least three times. The results were presented as the individual mean ± S.E. All means were presented with standard deviations. The data were subjected to a parametric one-way analysis of variance. If the results of the analysis of variance were significant (p < 0.05), Fisher’s protected least significant difference test was applied to the data.

RESULTS

FasL Expression in ASC-17D Cells and C57BL/6J Mouse Testis Is Induced by MEHP in a Time-dependent Manner—Real-time quantitative RT-PCR was used to measure the influence of MEHP on FasL mRNA levels in the rat adult Sertoli cell line ASC-17D. Cells were exposed to 200 μM MEHP for the indicated time points (Fig. 1A). FasL mRNA expression level was significantly increased (~4-fold) after incubation for 8 h (Fig. 1A). Similar changes in protein levels (~3-fold at 8 h) were observed via Western blot analysis (Fig. 1B) using whole cell lysates from MEHP-treated ASC-17D cells. In the 28-day-old C57BL/6J mouse testis, Western blot analysis revealed a significant induction (~2-fold) of FasL expression as early as 3 h after exposure to 1 g/kg of MEHP (Fig. 1C).

Inducible Activity of FasL Promoter in ASC-17D Cells Is Mediated Through −500 to −324 and −1250 to −1000 Regions—To determine the regulatory region that controls MEHP-induced FasL gene expression in Sertoli cells, a series of fragments were generated in the 5′-UAS of the FasL promoter and ligated to the pGL3-enhancer vector plasmid. The nucleotide sequence for the murine FasL promoter used for these constructs is based on the sequence reported in the GenBank data base with accession number AF045739. Several constructs were generated based on the presence of putative transcription binding sites in the promoter region (Fig. 2). Each construct resulted in the deletion of specific regulatory elements in the 5′-UAS of the FasL promoter. Luciferase-expressing pGL3-enhancer plasmids were transiently co-transfected with pCMV-β into ASC-17D cells and assayed for luciferase activity after exposure to MEHP. Promoter activity of each construct was expressed as the ratio of luciferase activity units relative to the β-galactosidase activity. As shown in Fig. 3A, plasmids containing the 1250 bp upstream of the initiation codon (FasL-Luc-P1250) and the 500 bp upstream (FasL-Luc-P500) showed an approximately 40-fold increase in basal luciferase activity. Luciferase activity of the deletion constructs immediately downstream of FasL-Luc-P1250 and FasL-Luc-P500, and all other constructs was only sparingly different from the mock construct.

Upon exposure to MEHP, FasL-Luc-P1250 demonstrated a 1.4-fold increase in luciferase activity. On the other hand, luciferase activity of FasL-Luc-P500 was independent of MEHP stimulation. Thus, we hypothesized that the −1250 to −1000 and −500 to −324 regions of the FasL 5′-UAS may contain elements important for the inducible expression of FasL. Closer examination of the −1250 to −1000 and −500 to −324 regions revealed the presence of putative Sp-1, NF-κB, and NF-E2 transcription factor binding sequences, respectively (Fig. 2).

MEHP-induced FasL Expression Is Enhanced through Transcriptional Regulation of NF-κB and Sp-1—To determine whether DNA binding by multiple transcription factors is required for MEHP-induced expression of FasL, we generated two mutant constructs containing either the mutated Sp-1 or NF-κB binding sites to disrupt the direct binding of these transcription factors to the FasL 5′-UAS. Site-directed mutagenesis was performed by the DpnI-mediated PCR method to generate
Transcriptional Regulation of FasL in Sertoli Cells

FIGURE 3. Functional deletion mapping and activity of the 5′-UAS of FasL promoter. A, each construct (10 μg) was transiently co-transfected with 1 μg of pCMV-β into ASC-17D cells in the presence (solid bars) or absence (open bars) of MEHP and results were expressed as relative luciferase activity with arbitrary units. In the presence of MEHP, FasL-Luc-P1250 and FasL-Luc-P500 had a significant increase in luciferase activities, indicating that Sp-1 and NF-κB activity, both in the presence and absence of MEHP exposure, are essential for MEHP-mediated induction in ASC-17D cells. B, site-directed mutagenesis to mutated elements (Sp-1 and NF-κB) in FasL 5′-UAS for the reporter constructs were performed by the DpnI-mediated PCR method. As predicted, luciferase activity of P1250-ΔSp1 was decreased and similar results were also shown in P500-ΔNFκB in the presence of MEHP. Significant differences (p < 0.05) between groups are denoted by bars with different letters. Transfections were carried out in duplicate and the individual experiments were repeated three times.

FIGURE 4. NF-κB and Sp-1 binding activity to FasL promoter in response to MEHP. A, the influence of MEHP exposure on the transcriptional activity of FasL in ASC-17D cells was determined by EMSA. Nuclear extracts from untreated or MEHP-treated ASC-17D cells were reacted with double-stranded oligonucleotides containing Sp-1 or NF-κB binding sequences specific to FasL 5′-UAS (Sp-1 and NF-κB) and mutated oligonucleotides (ΔSp-1 and ΔNF-κB). The upper image of the EMSA gel shows staining with SYBR Green EMSA DNA stain. The three arrows indicate the three oligonucleotide-protein complexes that were increased after MEHP stimulation. The lower image of the same gel shows staining with SYPRO Ruby EMSA protein stain. B, detection of the presence of NF-κB and Sp-1 binding sites on FasL 5′-UAS in response to MEHP in ASC-17D cells by ChIP. PCR was performed on DNA extracted from lyses subjected to immunoprecipitation with anti-NF-κB or anti-Sp-1 antibody. Specific primers to FasL promoter amplified −483 to −329 and −1333 to −1163 regions containing NF-κB and Sp-1 binding sites, respectively. The input represents the amplification of the unprecipitated DNA. PCR amplification of irrelevant immunoprecipitation against anti-α-tubulin antibody and normal rabbit-IgG were used as negative controls.

the mutant plasmids, P1250-ΔSp1 and P500-ΔNFκB. P1250-ΔSp1 and P500-ΔNFκB constructs were co-transfected with pCMV-β into ASC-17D cells as previously described, and assayed for relative luciferase activity. As shown in Fig. 3B, both mutant constructs demonstrated substantially reduced luciferase activity, both in the presence and absence of MEHP exposure, indicating that Sp-1 and NF-κB were both essential for MEHP-mediated induction of FasL expression in ASC-17D cells.

An additional transcriptional factor binding site for NFE2 was identified immediately downstream of the NF-κB binding site (Fig. 2). DpnI-mediated site-directed mutagenesis of this putative binding site (P500-ΔNFE2) was used to evaluate NFE2-dependent FasL expression and it was demonstrated that NFE2 is not important for regulating either the basal or MEHP-inducible expression of ASC-17D cells (data not shown).

In addition, EMSA were performed to determine whether Sp-1 and NF-κB could bind to these putative binding sites and if their transcriptional activity was altered after exposure to MEHP. Double-stranded DNA fragments encompassing the putative Sp-1 (−1234 to −1222) and NF-κB binding sites (−374 to −362) in the FasL 5′-UAS were incubated with nuclear extracts from ASC-17D cells grown with or without MEHP, and resolved on a nondenaturing polyacrylamide gel as described. Fig. 4A reveals the specific binding of both Sp-1 and NF-κB proteins to the putative binding site sequences. This was
Transcriptional Regulation of FasL in Sertoli Cells

FIGURE 5. MEHP-induced FasL expression in ASC-17D cells is partially mediated by NF-κB regulation. A, nuclear extract (30 μg) from ASC-17D cells treated with 200 μM MEHP was subjected to 10% SDS-PAGE and analyzed by Western blot analysis using antibodies against NF-κB. After incubation for 12 h, NF-κB expression was increased to 1.66-fold (compared with 0 h control). B, suppression of FasL induction in ASC-17D cells by NF-κB activation inhibitor (SN50) was assessed by Western blot analysis. The indicated final concentration of SN50 was added to 5 × 10⁵ ASC-17D cells and incubated for 12 h. FasL protein expression was inhibited by SN50 in a dose-dependent manner (down to 0.21-fold, compared with control). C, similar analysis was performed to measure the dose-dependent inhibition of the FasL level in ASC-17D cells by the Sp-1 inhibitor (MMA) (down to 0.05-fold, compared with control). β-Actin served as the loading control.

Further confirmed by the observation that neither protein was able to bind DNA fragments containing mutated binding sites.

In addition, in response to MEHP treatment, increased DNA-binding activity of all three Sp-1 and NF-κB isoforms was observed. These observations indicate that functional Sp-1 and NF-κB were present in the nuclear extracts of ASC-17D cells and that their DNA-binding activity is inducible by MEHP exposure. It is thus conceivable that these putative binding sites are indeed active in testicular Sertoli cells and that specific exposure. It is thus conceivable that these putative binding sites and that their DNA-binding activity is inducible by MEHP (Fig. 4B).

FasL Expression in ASC-17D Cells Is Controlled by Sp-1 and NF-κB Pathways in the Presence of MEHP—NF-κB and Sp-1 protein levels in the nucleus were measured in ASC-17D cells after MEHP treatment. Fig. 5A shows that NF-κB and Sp-1 expression in ASC-17D cells were elevated in a time-dependent manner after treatment with MEHP. After incubation for 12 h, NF-κB expression in the nucleus was increased by 1.66-fold, whereas Sp-1 expression was elevated 2.08-fold after incubation for 6 h. Addition of specific inhibitors of Sp-1 (MMA) and NF-κB (SN50) resulted in a dose-dependent reduction in the expression of FasL in ASC-17D Sertoli cell extracts (Fig. 5, B and C), indicating that both proteins influence expression of FasL in vitro.

MEHP Exposure Leads to Up-regulation of TNF-α Expression in Vivo—To determine the effect of MEHP on TNF-α expression in vivo, TNF-α production was measured in whole testis homogenates by Western blot analysis. Within 1.5 h of exposure, TNF-α expression was increased by 2.67-fold (Fig. 6A) as compared with vehicle-treated controls. Additionally, increased secretion of sTNF-α was observed from MEHP-exposed Sertoli cell/germ cell co-cultures in a time-dependent manner (Fig. 6B) with an almost 4-fold increase observed at 24 h post-exposure.

Induction of FasL Expression in the Testis Is Mediated by TNF-α through NF-κB Regulation—To evaluate the influence of TNF-α on FasL expression levels in the testis, NF-κB and FasL protein expression were measured by Western blot analysis. ASC-17D cells stimulated with various concentrations of mouse TNF-α recombinant protein demonstrated an ~12-fold increase in nuclear NF-κB (Fig. 7A). The addition of anti-TNF-α antibody resulted in a dose-dependent reduction in TNF-α-induced nuclear localization of NF-κB in primary Sertoli cell/germ cell co-cultures (Fig. 7C). Furthermore, downstream effects of TNF-α-dependent NF-κB activation were demonstrated by assessing FasL protein expression. Addition of specific inhibitors of Sp-1 (MMA) and NF-κB (SN50) resulted in a dose-dependent reduction in the expression of FasL in ASC-17D Sertoli cell extracts (Fig. 5, B and C), indicating that both proteins influence expression of FasL in vitro.
could be inhibited by the addition of anti-TNF-α antibody after incubation for 12 h (reduced to 57.8, 20.3, 19.6, and 15.1%, respectively). Anti-rabbit IgG was used as a negative control.

Influence of sTNF-α on NF-κB and FasL expression. 5 × 10⁵ ASC-17D cells were incubated with the indicated final concentration of mouse sTNF-α recombinant protein for 12 h. NF-κB and FasL protein levels were measured in both nuclear extracts and in total protein (each 30 μg) by Western blot analysis. NF-κB levels in ASC-17D cells were significantly induced after sTNF-α treatment in a dose-dependent manner (A). Similar results occur with the levels of FasL and phospho-κBα (B). The indicated final concentration of anti-TNF-α antibody was added to primary Sertoli cell/germ cell co-cultures for 12 h to neutralize the effect of sTNF-α. A dose-dependent reduction in NF-κB and FasL expression levels were detected in both nuclear extract (C) and total protein (D) preparations by Western blot analysis.

of various concentrations of recombinant mouse TNF-α protein resulted in increased expression of FasL (Fig. 7B), which could be inhibited by the addition of anti-TNF-α antibody in a dose-dependent manner (Fig. 7D). These data indicate that the increase in FasL protein expression in the testis is regulated by TNF-α through the NF-κB pathway.

To test the relative contribution of TNF-α and MEHP on induction of FasL expression, ASC-17D cells were exposed to MEHP in the presence or absence of recombinant mouse TNF-α protein (50 ng/ml) and FasL protein expression was measured by Western blot analysis. Fig. 8A illustrates that both TNF-α and MEHP alone triggered an almost 5-fold increase in FasL protein expression with respect to the vehicle control. In combination, TNF-α and MEHP resulted in a slight hyper-stimulation of FasL expression (~5.6-fold) with respect to the vehicle control.

In primary Sertoli cell/germ cell co-cultures, the level of FasL protein expression was significantly suppressed by inhibitors of Sp-1 (MMA, ~58%) or NF-κB (SN50 or pyrrolidine dithiocarbamate, 80%) or the anti-TNF-α antibody (15%) (Fig. 8B). Taken together, these data indicate that Sertoli cell FasL expression in response to MEHP is controlled by more than one signaling pathway, and that TNF-α plays a key role in driving the MEHP-inducible expression of FasL in Sertoli cells.

DISCUSSION

Our previous studies have indicated that MEHP-induced Sertoli cell injury leads to an increase in the incidence of testicular germ cell apoptosis (12, 14, 26, 49, 50). The functional participation of Fas/FasL signaling in directly triggering germ cell apoptosis after MEHP-induced Sertoli cell injury has been described (12, 14, 27, 28, 51). Although Sertoli cells express a basal level of FasL protein, a robust increase in FasL levels appears to be a prerequisite for MEHP-induced increases in germ cell apoptosis. The goal of this study was to determine the mechanisms responsible for initiating the increased transcription of FasL in Sertoli cells after MEHP-induced injury. Here, we describe a direct effect of MEHP on Sertoli cells that enhances the transcriptional regulation of FasL through the activation of transcription factors Sp-1 and NF-κB and an indirect effect triggered by the increased production and release of sTNF-α from germ cells resulting in the further activation of NF-κB in Sertoli cells through the activation of TNFRI signaling pathways. These observations provide further insights into the mechanisms by which germ cell numbers are actively reduced in the testis to compensate for the compromised supportive capacity of the MEHP-injured Sertoli cells to achieve populations of germ cells that can be supported by the Sertoli cell.

To understand the molecular mechanisms controlling the expression of FasL in Sertoli cells, we identified the cis-regulatory elements that are important for the transcriptional regulation of the MEHP-inducible expression of FasL. Deletional
analysis of the 5′-UAS of Fasl indicated that two specific regions −1250 to −1000 bp and −500 to −324 bp are important for MEHP-induced Fasl expression (Fig. 3A). Alteration of putative binding sites for Sp-1 and NF-κB, identified in these regions, by site-directed mutagenesis, abrogated FasL protein expression altogether (Fig. 3B), indicating that the identified Sp-1 and NF-κB binding sites were necessary for both the basal and MEHP-inducible Fasl expression. Although the luciferase activity of FasL-Luc-P500 was only increased by 1.5-fold in response to MEHP treatment (Fig. 3A), loss of this activation upon mutation of the NF-κB binding site established that NF-κB indeed recognizes and binds to the specific motif identified on the 5′-UAS of Fasl promoter and that NF-κB activation could result in the activation of the expression of Fasl (Fig. 3B).

Our previous studies have indicated that the induction of FasL protein expression above the ubiquitous basal expression is critical for initiating large increases in germ cell apoptosis after Sertoli cell injury (14, 27, 28). This is a concept that has been uniquely revealed with the MEHP-induced Sertoli cell injury model. However, understanding the mechanisms that specifically control the induced expression of Fasl over the basal levels may be especially important for understanding the regulation of germ cell apoptosis that occurs in the testis during critical periods of testicular development, such as during the first wave of spermatogenesis (2). Although others have reported that the basal expression of Fasl in Sertoli cells is regulated by Sp-1 (35), our observations indicate that additional transcription factors participate in regulating the MEHP-inducible expression of Fasl in Sertoli cells. Interestingly, a recent report recognized that the transcription factors, Sp-1 and NF-κB, are co-activated and co-bind to proximal and distal regulatory regions of the monocye chemoattractant protein-1 promoter and activate its expression (52). It was also shown that the binding of Sp-1 is a prerequisite for NF-κB binding to the distal activating region. This results in facilitating the transcription of NF-κB-dependent downstream genes. We have shown, using EMSA and ChIP assay that activated Sp-1 and NF-κB are present in the nuclei of ASC-17D cells challenged by MEHP, and that both transcription factors bind their specific cis-regulatory elements in the 5′-UAS of the Fasl promoter (Fig. 4). Basal transcription of Fasl was influenced by the activation of both Sp-1 and NF-κB (Figs. 3 and 4). Therefore, these observations indicate the novel insight that Sp-1 and NF-κB are believed to be responsible for both basal and stress-inducible expression of the Fasl gene in Sertoli cells. It is also possible that the two Sp-1 binding sequences in the −1250 to −1000 bp region aid in enhancing the response to MEHP. Precedent for this idea comes from two recent reports that have indicated a TFIID-enhanced, Sp-1-required, transcriptional regulation of IGF-binding protein 3 and cyclin D1 in granulosa and kidney cells (53, 54). Hence, it is possible that TFIID with its putative transcription binding site found in the Fasl −1250 to −1000-bp promoter region, could cooperate with Sp-1 in regulating the expression of Fasl in Sertoli cells.

NF-κB has been shown to be a common mediator of FasL induction in response to various stress stimuli in T cells (34, 55) and Kupffer cells (56). The expression and nuclear translocation of NF-κB subunits, p65 and p50, in rat Sertoli cells has been previously described (57). A recent study demonstrated that MEHP induces the activation and nuclear translocation of NF-κB in rat Sertoli cells (58). We have further demonstrated a significant increase in the activation and nuclear localization of NF-κB in ASC-17D Sertoli cells in response to MEHP treatment (Fig. 5A). Dose-dependent inhibition in Fasl expression (−80%) after the addition of the NF-κB inhibitory peptide, SN50, established the involvement of the NF-κB signaling pathway in the regulation of Fasl expression (Fig. 5B). However, the involvement of other transcription factors cannot be excluded as the inhibition due to SN50 was incomplete in each independent experiment. Similarly, addition of MMA caused a reduction in Fasl expression confirming that the regulation by Sp-1 was important for inducible Fasl expression (Fig. 5C). Taken together, these data indicate that MEHP-inducible expression of Fasl in Sertoli cells is regulated by transcription factors NF-κB as well as Sp-1.

It is well known that the NF-κB signaling pathway is activated by TNF-α in many types of cells (59–61), including primary rat Sertoli cells and the MSC-1 Sertoli cell line (57, 62). In whole testis homogenates, MEHP stimulation caused a robust early increase in TNF-α expression (Fig. 6A). In the testis, TNF-α is released from specific germ cell subtypes (spermatocytes or round spermatids (63)), or from activated macrophages in the interstitial space (64). However, testes of C57BL/6 mice did not show any evidence of macrophage infiltration into the seminiferous epithelium after MEHP exposure was observed (Fig. 6B). This further supports the idea that germ cells are the source for the secretion of sTNF-α.

TNF-α has been reported to regulate mammalian spermatogenesis (63), modulate Leydig cell steroidogenesis (65–67), and influence the expression of cell-cell adhesion molecules (68, 69). To determine the functional role of TNF-α in Sertoli cells exposed to MEHP, the influence of sTNF-α on NF-κB activation and Fasl production were examined. The present study demonstrated that recombinant sTNF-α induces a dose-dependent increase in both NF-κB translocation and expression of Fasl protein in ASC-17D cells (Fig. 7 A and C). A dose-dependent suppression of NF-κB translocation and Fasl expression was observed in the presence of an anti-TNF-α antibody in primary Sertoli germ cell cultures (Fig. 7 B and D). In the seminiferous epithelium, Sertoli cells are known to express TNFR1 but not TNFR2 (70). Interestingly, a preliminary analysis of the testis of TNF p55/p75 double knock-out mice (71) reveal that they have an increase in testis weight reflecting an expansion of the germ cell population (data not shown) further suggesting that the TNFR-mediated pathway plays a role in the regulation of cell apoptosis. Thus, we hypothesize that TNF-α is secreted as a paracrine factor from germ cells in response to MEHP-induced Sertoli cell injury. In effect, this allows for the regulation of MEHP-induced germ cell apoptosis by increasing Fasl expression in Sertoli cells, through the TNFR1−NF-κB-dependent pathway. The role of sTNF-α in Fasl expression and apoptotic regulation is still considered
controversial. In primary human keratinocytes treated with TNF-α, interleukin-1β, or interferon-γ a significant increase in cellular and membrane FasL expression was observed in time- and dose-dependent manners (72). Injection of TNF-α in mice caused a rapid induction of FasL mRNA in intestinal epithelial cells via a NF-κB regulatory motif in FasL promoter (73). On the other hand, some reports show that TNF-α is responsible for the inhibition of testicular germ cell death in the human seminiferous epithelium (74), and implicate a role for TNF-α in the down-regulation of FasL expression (75). Therefore, a detailed investigation of the TNF p55/p75 double knock-out mice in future experiments will allow us to further distinguish the biological role of the TNFR/TNF-α signaling pathway in the testis in response to Sertoli cell injury.

Metalloproteinases are essential for the processing of the TNF-α precursor (76–79) and a number of metalloproteinases in the testis have been described (80–83). Interestingly, the TNF-α-induced disruption of Sertoli cell tight junctions has been associated with the production of extracellular matrix proteins, including MMP-9, collagen, and TIMP-1 (83, 84). Therefore, TNF-α may be involved in the physical and metabolic cooperation between Sertoli cells and germ cells.

NF-κB activation-mediated apoptosis under a variety of stimulators or stress in a number of cell types has been previously established (85, 86). Nuclear localization and NF-κB DNA-binding activity in Sertoli cells are increased during testicular stress-induced germ cell apoptosis (87). Our results suggest that Sertoli cell NF-κB is a key regulator in triggering the pro-apoptotic process in germ cells in response to either TNF-α or MEHP-induced signaling. Similarly, in the testis, NF-κB has recently been reported to serve as a pro-apoptotic factor to modulate germ cells in response to radiation stress (88). Whereas most agents that cause apoptosis also activate NF-κB, recent studies have pointed out the anti-apoptotic effect of NF-κB is mediated by the regulation of expression of genes favoring cell survival (89–91). Thus, the balance between apoptosis and survival pathways in the testis appears to involve a highly complex inter-connected network.

During our experiments, the expression of FasL was up-regulated by 6 h after MEHP exposure of cultured Sertoli cells in vitro (Fig. 1B). In vivo results, however, show that MEHP caused an increase in FasL levels at an earlier (1.5 h) time point (Fig. 1C), suggesting that cellular responses to MEHP exposure are more responsive in the intact testis where both Sertoli cells and germ cells are present. Furthermore, sTNF-α production was stimulated as early as 1.5 h in vivo (Fig. 6A), indicating that germ cells are very responsive to MEHP-induced Sertoli cell injury. These observations illustrate the temporal differences between MEHP-induced changes in FasL levels instigated by direct effects within Sertoli themselves as well as the participation and influence of germ cell factors on modulating Sertoli cell FasL levels.

Our results show that both MEHP and TNF-α induce a significant increase in FasL expression. In combination, TNF-α and MEHP result in only a slight additive effect on FasL expression. Using primary Sertoli cell/germ cell co-cultures, we established that inhibition of the TNF-α-triggered NF-κB activation pathway significantly inhibits FasL expression in the presence of MEHP (Fig. 8B). Blocking the Sp-1 pathway, however, does not completely suppress FasL expression. These observations indicate that TNF-α secreted from germ cells may play a key role in MEHP-induced FasL expression in Sertoli cells. However, the differential participation of the direct effect of MEHP on Sertoli cells versus the indirect effect of germ cell TNF-α on Sertoli cells and consequent germ cell apoptosis remains to be investigated.

Fig. 9 illustrates diagrammatically the two identified mechanisms for increasing Sertoli cell FasL transcription and ultimately leading to increases in germ cell apoptosis in response to Sertoli cell injury. Sertoli cell injury results in reduced support for developing germ cells and leads, through an as yet described mechanism, to the likely activation of matrix metalloproteinases and increased production and release of sTNF-α. MEHP can directly influence the activation and nuclear translocation of NF-κB and Sp-1 within Sertoli cells, leading to the up-regulation of FasL expression. However, the differential contribution between the direct effects of MEHP on Sertoli cells and the indirect effect of germ cell factors such as TNF-α on FasL expression increases cannot be concluded at this time. The two predominant questions arising from our previous and the current investigations are (a) what is the primary cellular target for MEHP in Sertoli cells and how does this lead to the nuclear translocation of NF-κB and Sp-1 and (b) what are the mechanisms that explain the increased production of sTNF-α from germ cells? Does this response from germ cells serve as an
amplification loop for sensitizing germ cells that lack Sertoli cell support to FasL-mediated apoptosis?

In summary, we report that the transcriptional regulation by both Sp-1 and NF-κB is necessary for the MEHP-induced expression of FasL in Sertoli cells. We also report the novel finding that the production of sTNF-α from germ cells can act to up-regulate NF-κB-dependent FasL expression resulting in robust increases in Sertoli cell FasL levels. To the best of our knowledge, this is the first description of a potential "feed-forward" signaling mechanism by which germ cells prompt Sertoli cells to trigger their apoptotic elimination. By rigorously defining the critical cellular determinants expressed by Sertoli cells and germ cells that are required for triggering germ cell apoptosis we expect to appreciably expand our knowledge of testis biology and gain decisive insights into mechanisms by which environmental toxicants can lead to testicular disease.

REFERENCES

1. Print, C. G., and Loveland, K. L. (2000) Bioessays 22, 423–430
2. Wang, R. A., Nakane, P. K., and Koji, T. (1998) Biol. Reprod. 58, 1250–1256
3. Russell, L. D., Ettlin, R. A., Sinha Hikim, A. P., and Erkkila, K. (1997) Toxicol. Appl. Pharmacol. 144, 142–148
4. Dunkel, L., Hirvonen, V., and Erkkila, K. (1997) Cell Death Differ. 4, 171–179
5. Hadziselimovic, F., Geneto, R., and Emmons, L. R. (1998) Toxicol. Lett. 119, 1–10
6. Lin, W. W., Lamb, D. J., Wheeler, T. M., Lipshultz, L. I., and Kim, E. D. (1997) Fertil. Steril. 68, 1056–1069
7. Nagata, S. (1997) Cell 88, 355–365
8. Ashkenazi, A., and Dixit, V. M. (1998) Science 281, 1310–1308
9. Pinkoski, M. J., and Green, D. R. (1999) Cell Death Differ. 6, 1174–1181
10. Nagata, S., and Golstein, P. (1995) Science 267, 1449–1456
11. Okada, H., and Mak, T. W. (2004) EMBO J. 23, 953–964
12. Chen, J. J., Liu, Y., Hsu, S. C., Wu, C. C., Hwang, Y. C., Lai, M. Z., and Yang, P. C. (2001) J. Biol. Chem. 276, 16631–16636
13. Davis, L. A., and Shattil, S. J. (1991) Blood 78, 1515–1527
14. Chinnici, G., Mancini, M. V., Balestri, M. G., Cucchi, E., and Di Renzo, G. C. (1993) Endocrinology 133, 389–396
15. Xiong, Y., and Hales, D. B. (1993) Endocrinology 133, 2568–2573
16. Mauduit, C., Gasnier, F., Rey, C., Chauvin, M. A., Stocco, D. M., Louisot,
P., and Benahmed, M. (1998) *Endocrinology* **139**, 2863–2868
66. Xiong, Y., and Hales, D. B. (1993) *Endocrinology* **132**, 2438–2444
67. Morales, V., Santana, P., Diaz, R., Tabraue, C., Gallardo, G., Lopez Blanco, F., Hernandez, I., Fanjul, L. F., and Ruiz de Galarreta, C. M. (2003) *Endocrinology* **144**, 4763–4772
68. Ziparo, E., Riccioli, A., Filippini, A., De Cesaris, P., and Barbacci, E. (1995) *Ital. J. Anat. Embryol.* **100**, Suppl. 1, 553–562
69. Riccioli, A., Filippini, A., De Cesaris, P., Barbacci, E., Stefanini, M., Starace, G., and Ziparo, E. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5808–5812
70. Lysiak, J. J. (2004) *Reprod. Biol. Endocrinol.* **2**, 9
71. Ruby, J., Bluethmann, H., and Peschon, J. J. (1997) *J. Exp. Med.* **186**, 1591–1596
72. Arnold, R., Seifert, M., Asadullah, K., and Volk, H. D. (1999) *J. Immunol.* **162**, 7140–7147
73. Pinkoski, M. J., Droin, N. M., and Green, D. R. (2002) *J. Biol. Chem.* **277**, 42380–42385
74. Suominen, J. S., Wang, Y., Kaipia, A., and Toppari, J. (2004) *Eur. J. Endocrinol.* **151**, 629–640
75. Pentikainen, V., Erkkila, K., Suomalainen, L., Otala, M., Pentikainen, M. O., Parvinen, M., and Dunkel, L. (2001) *J. Clin. Endocrinol. Metab.* **86**, 4480–4488
76. Gearing, A. J., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., and et al. (1994) *Nature* **370**, 555–557
77. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Cerretti, D. P. (1997) *Nature* **385**, 729–733
78. Ohta, S., Harigai, M., Tanaka, M., Kawaguchi, Y., Sugiuura, T., Takagi, K., Fukasawa, C., Hara, M., and Kamatani, N. (2001) *J. Rheumatol.* **28**, 1756–1763
79. Gearing, A. J., Beckett, P., Christodoulou, M., Churchill, M., Clements, J. M., Crimmin, M., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., and et al. (1995) *J. Leukocyte Biol.* **57**, 774–777
80. Kim, T., Oh, J., Woo, J. M., Choi, E., Im, S. H., Yoo, Y. J., Kim do, H., Nishimura, H., and Cho, C. (2006) *Biol. Reprod.* **74**, 744–750
81. Slongo, M. L., Zampieri, M., and Onisto, M. (2002) *Biol. Chem.* **383**, 235–239
82. Siu, M. K., and Cheng, C. Y. (2004) *Biol. Reprod.* **70**, 945–964
83. Siu, M. K., and Cheng, C. Y. (2004) *Bioessays* **26**, 978–992
84. Siu, M. K., Lee, W. M., and Cheng, C. Y. (2003) *Endocrinology* **144**, 371–387
85. Hu, X., Tang, M., Fisher, A. B., Olashaw, N., and Zuckerman, K. S. (1999) *J. Immunol.* **163**, 3106–3115
86. Ivanov, V. N., and Ronai, Z. (2000) *Oncogene* **19**, 3003–3012
87. Pentikainen, V., Suomalainen, L., Erkkila, K., Martelini, E., Parvinen, M., Pentikainen, M. O., and Dunkel, L. (2002) *Am. J. Pathol.* **160**, 205–218
88. Rasoulpour, R. J., and Boekelheide, K. (2006) *Biol. Reprod.* , in press
89. Mattson, M. P., and Meffert, M. K. (2006) *Cell Death Differ.* **13**, 852–860
90. Shishodia, S., and Aggarwal, B. B. (2002) *J. Biochem. Mol. Biol.* **35**, 28–40
91. Oya, M., Ohsubo, M., Takayanagi, A., Tachibana, M., Shimizu, N., and Murai, M. (2001) *Oncogene* **20**, 3888–3896

**Transcriptional Regulation of FasL in Sertoli Cells**

FEBRUARY 23, 2007 • VOLUME 282 • NUMBER 8 • JOURNAL OF BIOLOGICAL CHEMISTRY 5431