Development of a self-proliferating Leydig cell line: A hyper-sensitive E-screening model

JAMES W. DuMOND Jr[1], KAMALESHWAR P. SINGH[1] and DEODUTTA ROY[2]

[1]Department of Biology, Texas Southern University, Houston, TX 77004; [2]Department of Environmental and Occupational Health Sciences, Florida International University, Miami, FL 33199, USA

Abstract. The mechanisms of estrogenic endocrine disruption on the male reproductive tract are poorly understood. In order to examine estrogenic properties of xenobiotic chemicals on male tissues, we have developed a mouse Leydig cell line (TM3-SF) that self-proliferates under serum-free conditions. This cell line was derived from ATCC's cell line, TM3. The development of TM3-SF was accomplished over a 4-month period by a progressive serum starvation of the original TM3 cells. The newly established cell line was maintained under serum-free conditions for 20 passages prior to testing. Sensitivity of the TM3-SF cells to estrogens was assayed by cell proliferation studies. A total of four compounds, diethylstilbestrol (DES), 17ß-estradiol, 17α-estradiol, and Bis-phenol A, were tested. Significant increases in cell proliferation occurred at various concentrations ranging from 1 pg/ml to 100 ng/ml for all four compounds. The order of potency observed was DES >Bis-phenol A > 17ß-estradiol and > 17α-estradiol. In addition, we investigated the mechanism for the self-proliferative properties of TM3-SF. The results of these trials indicate that either inhibin or activin is a primary growth factor for this cell line as a 50% inhibition of growth was noted when cell cultures were exposed to the anti-ßa subunit of inhibin/activin. Furthermore, the addition of the anti-ßa subunit of inhibin/activin blocked the DES-induced proliferation of TM3-SF. We conclude that the growth of TM3-SF cells is estrogen sensitive and that either inhibin or activin is involved in the self-regulation of growth.

Introduction

Interest in the role of environmental estrogens has increased during the last 15 years. This interest is the result of the identification of chemicals with estrogenic properties within our environment (1-6). Exposure to estrogenic chemicals has been implicated as a causal factor for decreases in sperm count, increases of hypospadias, and increased incidence of testicular cancer (7-16). In animal models, estrogen treatment can induce cancer in specific target tissue (17-19). Estrogen exposure in man results in testicular atrophy when administered at pharmacological doses (20-23). Furthermore, there are clinical cases of estrogen sensitive and/or estrogen secreting Leydig cell tumors (24-29).

The recent resurgence of interest in environmental estrogenic chemicals has resulted in the use of the E-screen assay that tests a chemical's ability to induce cell proliferation (30). MCF-7 cells, cloned from a human breast tumor, have been the primary choice for the E-screen assay. While the bulk of research on environmental estrogens has focused on the role they may play in breast cancer, interest has expanded to other target tissues such as testis, kidney, and liver in more recent years. This has resulted in the need and therefore the development of non-breast cell lines that are estrogen sensitive. We have previously shown that the normalized mouse Leydig cell line, TM3, is estrogen sensitive (31,32). However, our previous studies have failed to detect a significant increase in the proliferative response when TM3 cells are exposed to relatively weak estrogens (Du Mond and Roy, The Society of Toxicology International Conference, New Orleans, LA, 1998; Du Mond and Roy, The American Association for Cancer Research International Conference, Philadelphia, PA, 1998).

In our previous studies, we have revealed TM3's ability to proliferate for short periods of time after the growth medium was replaced with an unsupplemented serum-free medium. In addition, during routine cell culturing of TM3 cells, we observed their ability to withstand long periods of time without replacing the growth medium. Based on these observations, we postulated that TM3 cells would be a good candidate for development into a self-proliferating cell line using unsupplemented serum-free medium.

The research presented here focuses on the establishment of the new cell line (TM3-SF) and its ability to self-replicate. In addition, we have investigated TM3-SF's response to estrogens as measured by increased cell proliferation.

Materials and methods

Chemicals. Trypsin-EDTA (1X) and Ham's F12 and Dulbecco's modified Eagle's medium (DMEM) (1:1) mixture containing 1.2 g/l sodium bicarbonate, 15 mM HEPES, and 3.5 g/l glucose was purchased from Life Technologies, Grand Island, NY.

Correspondence to: Dr James W. DuMond Jr, Department of Biology, Texas Southern University, 210 Nabrit Science Building, 3100 Cleburne Avenue, Houston, TX 77004, USA
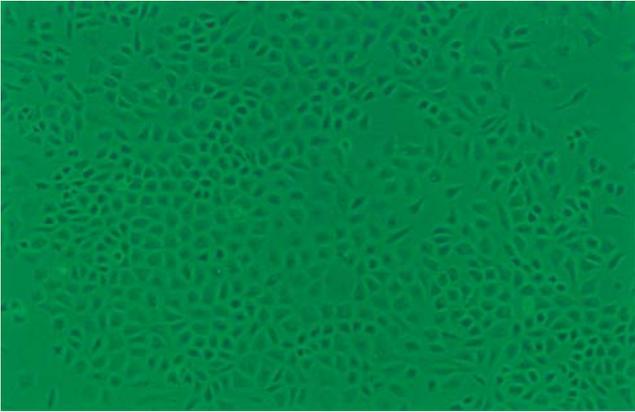E-mail: dumond_jw@tsu.edu

Figure 1. TM3-SF testicular Leydig cells.

Figure 4. TM3-SF cells 48 h after plating with 0.007% serum.
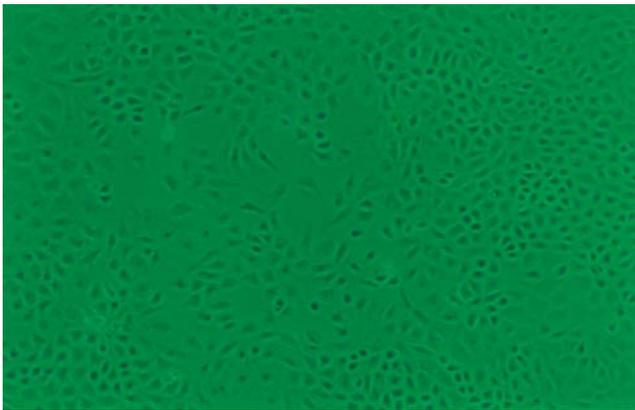

Figure 2. TM3 testicular Leydig cells.

Figure 5. TM3-SF cells 48 h after plating with 0.015% serum.

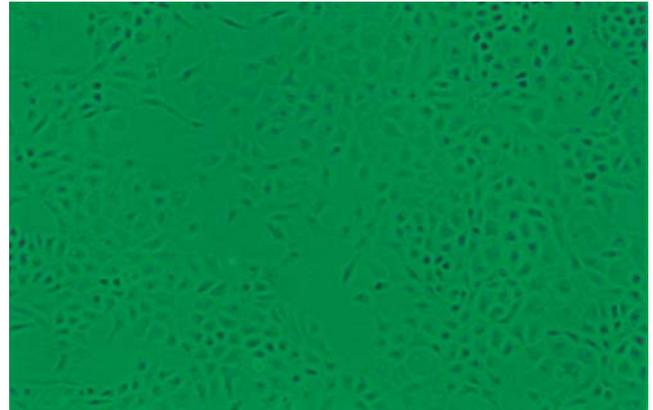Figure 3. TM3-SF cells 48 h after plating with 0.000% serum.

Figure 6. TM3-SF cells 48 h after plating with 0.100% serum.

Diethylstilbestrol (DES), tamoxifen, horse serum, fetal bovine serum, DMSO, and propidium iodide were purchased from Sigma Chemical Co., St. Louis, MO. TM3-Leydig cells # CRL-1714 were purchased from ATCC, Rockville, MD. A Corning® 75-cm² culture flask was purchased from Fisher Scientific, Pittsburgh, PA.

Cell culture preparation. Prior to treatment, TM3 Leydig cells were cultured using DMEM/F-12 medium supplemented with 5% horse serum and 2.5% fetal bovine serum. The growth medium was changed once every 2-3 days as needed. Cultures were sub-cultured (1-200) during late log phase growth.

cell line development. A progressive starvation of al TM3 cells was used to accomplish the development of the TM3-SF cell line. In phase 1, the cells were plated with the standard growth medium as defined above and grown to a hyperconfluent condition. The cells were then subcultured and the process was repeated. The cells were allowed to remain in the hyperconfluent state for gradually increasing time periods. Once the cells were able to maintain a ≥75% viability for a 2-week starvation period, they entered phase 2. In the second phase, the cells were plated with normal growth medium, which was replaced 24 h later with unsupplemented serum-free medium. Once the cells had reached their maximum growth potential, they were subcultured and grown for one cell cycle with normal growth medium. This cycle was repeated until the cells were able to withstand continuous growth in unsupplemented serum-free medium. Phase 3 was then initiated. In this phase, the amount of serum used in the plating medium was progressively decreased until the cells were weaned from requiring standard growth medium for plating. Upon completion of phase 3, the cells were self-proliferating and designated TM3-SF.

Cell proliferation assay. The cells (≅10,000) were placed into 48-well plates and allowed to attach for 24 h. The cells were then treated with DES, 17ß-estradiol, Bis-phenol A, or 17α-estradiol. The concentrations tested ranged from $100x10^{-7}$ to $1x10^{-12}$ g/ml in 10^{-1} intervals for a total of 6 concentrations. The treatment time for all assays was 5 days. Each trial was conducted in duplicate. After the growth period had expired, all cell cultures were harvested simultaneously and the yields were measured. This was accomplished by removing the medium and adding the cell lysing solution (1X trypsin-EDTA). The cells were then counted via the use of a hemato-cytometer.

Anti-inhibin/activin assay. The cells (≅10,000) were placed into 48-well plates, and allowed to attach for 24 h. Cells were then treated with one of 8 different concentrations of anti-ßa subunit of inhibin/activin, ranging from 2 to 40 µl/ml (10,000 units per µl). DES concentrations tested ranged from $100x10^{-7}$ to $1x10^{-12}$ g/ml in 10^{-1} intervals for a total of 6 con-centrations, and 6 µl of anti-ßa was used in the combination studies. The treatment time for all assays was 5 days. Each trial was conducted in duplicate. After the growth period had expired, all cell cultures were harvested simultaneously and the yields were measured. This was accomplished by removing the medium and adding the cell lysing solution (1X trypsin-EDTA). The cells were then counted via the use of a hemato-cytometer.

Statistical analysis. Cell proliferation data were analyzed using the Students' t-test along with an analysis of variation (ANOVA). The overall α was set at 0.05 for the Students' t-test, which was used in the analysis of variation.

Results

TM3-SF cell line development. The establishment of the TM3-SF cell line from the parent cell line, TM3, was accomplished over a 4-month period. Once TM3-SF cells were established,

they were allowed to grow for 20 passages before any analysis was completed. The TM3-SF cells remained morphologically similar to the parent cell line, TM3 (Figs. 1 and 2). These cells typically grow in a clonal monolayer, although over-lapping of the cells has been observed. The doubling time for TM3-SF cells is approximately 4-5 days, although this can vary with seeding density. This is in contrast to the 16-h doubling time of the TM3 parent cells. TM3-SF sensitivity to the effects of growth stimuli was assayed by exposing the cells to low doses of fetal bovine serum.

The TM3-SF cell line was responsive for increased proliferation, as an increase in proliferation was detected when the cells were exposed to low doses of serum, 0.007, 0.015 and 0.10% serum (Figs. 3-6). Short-term studies revealed that TM3-SF cells exposed to serum were able to return immediately to unsupplemented serum-free medium without any altered growth characteristics.

Influence of estrogen on growth of TM3-SF cells. The exposure to estrogenic chemicals resulted in a significant ($p < 0.05$) increase in proliferation when compared to the controls (Fig. 7). DES produced the greatest response as it resulted in a significant ($p < 0.05$) increase of cell proliferation with all concentrations <100 ng/ml. DES 100 ng/ml, resulted in a significant decrease in cell proliferation. Maximum pro-liferation was noted at 1 pg/ml DES (184.45%). A second peak in proliferation was noted at 1 ng/ml DES (166.57%). Bis-phenol A was the second most potent chemical as treatment resulted in peak proliferation at 1 pg/ml (145.26%) and 100 ng/ml (138.95%). Concentrations of 10 pg/ml to 1 ng/ml Bis-phenol A resulted in significant ($p < 0.05$) decreases in cell proliferation. TM3-SF cells were unresponsive to 17ß-estradiol at 1 pg to 10 ng/ml; however, 100 ng/ml resulted in a significant ($p < 0.05$) increase in cell proliferation. 17α-estradiol was the weakest of all compounds tested, although it did produce a significant ($p < 0.05$) increase in proliferation at 10 pg/ml.

Anti-inhibin/activin assay. The addition of activin/inhibin ßa subunit antibody to TM3-SF cells significantly reduced the ability of these cells to proliferate (Fig. 8). The addition of 2 µl/ml of the anti-ßa subunit antibody was sufficient to reduce growth by 35.9%. The peak reduction of cell growth was noted with the addition of 30 µl/ml of the anti-ßa subunit (58.95%). Co-administration of the anti-ßa subunit (6 µl/ml) and DES (1 pg to 100 ng/ml) resulted in a similar reduction in growth as seen with the addition of the anti-ßa subunit alone (Fig. 9).

Discussion

The development of the TM3-SF cell line is novel as currently there are no reports of any Leydig cell lines that can be maintained under unsupplemented serum-free conditions. A comparison of the cell growth of TM3-SF data presented here with precursor cells, TM3, showed that both cell lines differ in estrogen sensitivity. However, TM3-SF demonstrated a greater mitogenic activity with lower concentrations of DES and inhibition of proliferation at 100 ng/ml, whereas TM3 had its greatest response to DES at 100 ng/ml. The Bis-
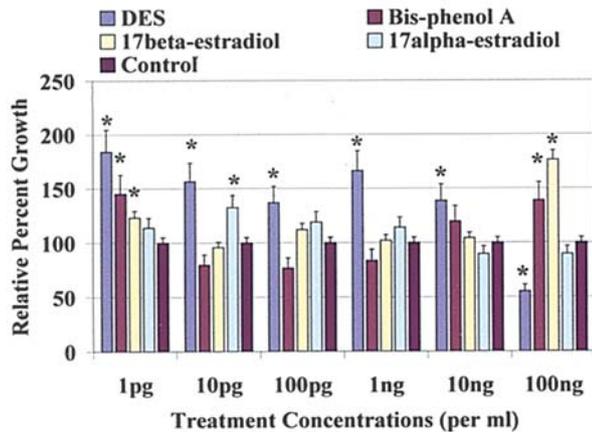
Figure 7. Estrogen-induced proliferation of TM3-SF cells. Data are presented as percent growth with controls set at 100%. Results are from 6 different treatment concentrations (1 pg/ml to 100 ng/ml). Exposure time was 5 days for all treatments. Asterisk indicates treatments significantly different from controls ($p<0.05$) using the Student's t-test. Error bars (T) represent standard deviations.
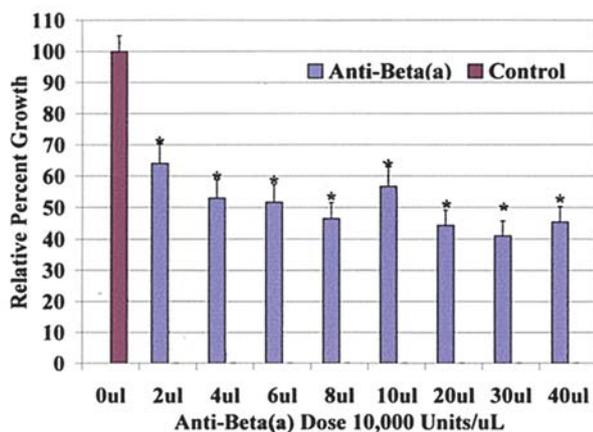

Figure 9. The effect of anti-ßa subunit of activin and inhibin on DES-induced proliferation of TM3-SF cells. Data are presented as percent growth with intra-trial controls set at 100%. Results are from a 5-day treatment period. Treatment concentrations ranged from 1 pg/ml to 100 ng/ml for DES, combination treatment consisted of the addition of 6 μl/ml (10,000 units/μl) of anti-ßa to the same DES treatments. Asterisk indicates treatments significantly different from controls ($p<0.05$) using the Student's t-test. Asterisks indicate a significant reduction of DES-induced proliferation of Leydig cells ($p<0.05$) using the Student's t-test. Error bars (T) represent standard deviations.

Figure 8. The inhibition of TM3-SF growth by the anti-ßa subunit of activin and inhibin. Data are presented as percent growth with intra-trial controls set at 100%. Results are from a 5-day treatment period. A total of 8 different concentrations, 2-40 μl/ml, (10,000 units/μl) of anti-ßa were administered. Asterisk indicates treatments significantly different from controls ($p<0.05$) using the Student's t-test. Error bars (T) represent standard deviations.

phenol A-mediated stimulation of cell growth of TM3-SF cells was novel, as Bis-phenol A had no effect on TM3 cells. We also observed a difference in 17ß-estradiol results, as the peak proliferation was noted at 100 ng/ml in TM3-SF cells. In TM3 cells, 17ß-estradiol had its greatest response at 10 ng/ml and was remarkably weaker in comparison. The response to 17α-estradiol also differs between the two cell lines, as 17α-estradiol is able to produce a significant mitogenic response in the TM3 cell line, whereas TM3-SF is non-responsive.

We find the anti-ßa subunit data both remarkable and intriguing, as it is suggestive of a possible mechanism for the self-proliferation of the TM3-SF cell line. It is known that Leydig cells are a primary target for both inhibin and activin. It is also believed that the primary source of activin is the seminiferous cells, whereas the Sertoli cells are the primary source for inhibin (33-35). However, the role of inhibin and
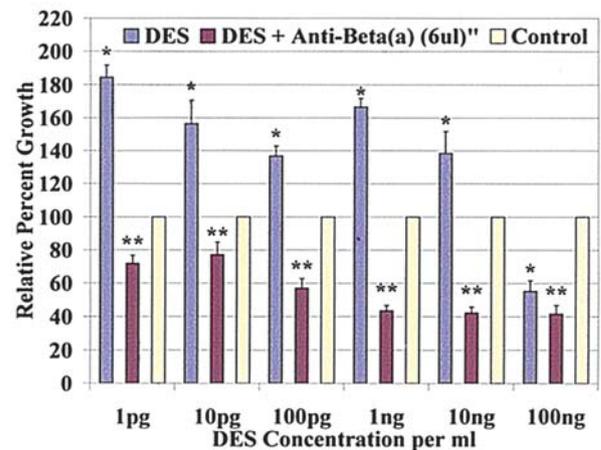
activin in regulation of cell growth is not clear. There are reports that suggest both Sertoli and Leydig cancer cells secrete increased levels of inhibin (36-39).

There are two possible explanations for the data we have presented. First, the gene for activin or inhibin may have been turned on and/or up-regulated in TM3-SF cells. Second, the original cell line, TM3, actually might be a co-culture of Leydig, Sertoli, and/or seminiferous cells. After reviewing the procedure used in the development of TM3, the latter possibility is not likely (40).

The ability for anti-inhibin/activin to block DES-induced proliferation is of interest, as it may indicate a possible mechanism for proliferation. DES exposure may up-regulate both the inhibin or activin gene, and one of the two or both might act as a second messenger for the observed mitogenic effects. This observation may also be explained by binding of DES by the anti-ßa subunit, thereby decreasing the bioavailability of DES. Currently, additional studies are being conducted using the newly established TM3-SF cell. These studies will investigate further the role of activin in the proliferation of these cells.

In summary, this report presents the novel mouse Leydig cell line, TM3-SF. TM3-SF cells are estrogen sensitive for a cell proliferation endpoint and the observed mitogenic response differs significantly from its precursor cells, TM3. Furthermore, the data presented here suggest that either inhibin or activin may act as a second messenger for DES-induced proliferation, although additional research is needed for the validation of this mechanism.

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