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## Estrogen Receptor Signaling is an Unstable Feature of the Gonadotropic L $\beta$ T2 Cell Line

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## Abstract

The murine, gonadotrophic L $\beta$ T2 cell line was assessed as a potential *in vitro* model to analyze estrogen receptor (ER)-mediated regulation of luteinizing hormone (LH) synthesis and secretion. In agreement with limited literature data, repeated exposure to (sub) physiological concentrations of gonadotropin-releasing hormone enhanced LH $\beta$ -subunit gene expression, being the rate-limiting step of LH synthesis, and the corresponding LH secretory response. However, in the same subclone of the L $\beta$ T2 cell line, we observed that LH production was not affected following exposure to E<sub>2</sub>, which is in contrast to previously reported weak or modest effects. One explanation may be the absence of measurable ER $\alpha$  protein expression on the one hand and impaired ER signal transduction on the other. Furthermore, an alternative ER $\alpha$  mRNA splicing variant was detected in the L $\beta$ T2 cell line, which (theoretically) encodes for a protein that may alter ER $\alpha$  transcriptional activity, depending on the cellular context.

The studied L $\beta$ T2 subclone did not show a generalized impairment of nuclear receptor function, as we observed androgen- and glucocorticoid-induced gene transcription, together with enhanced LH secretory response following dexamethasone treatment. Since its development, the gonadotrophic L $\beta$ T2 cell line served as a reference model to study gonadotroph-specific effects because of its mature properties. Nevertheless, this cell line does not seem to be a suitable *in vitro* model for the study of estrogenic regulatory effects at the level of the pituitary gonadotrophs in view of the unstable nature of ER signaling in L $\beta$ T2 cells.

## Introduction

Reproductive competence depends on the interplay between the different compartments

of the hypothalamic-pituitary-gonadal axis. The episodic release of hypothalamic gonadotropin-releasing hormone (GnRH) results in a pulsatile pattern of luteinizing hormone (LH) secretion by the pituitary gonadotrophs (Wildt et al., 1981). Luteinizing hormone is responsible for the synthesis and the release of gonadal steroids, which in turn alter gonadotropin secretion through feedback interactions at the level of the hypothalamus and the pituitary (Belchetz et al., 1978; Haisenleder et al., 1994; Bousfield, 2004). In males, both testosterone (T) and its aromatization product 17- $\beta$ -estradiol (E<sub>2</sub>) contribute to the regulation of LH secretion (Hayes et al., 2000; Tilbrook and Clarke, 2001). In females, E<sub>2</sub> exerts a negative feedback on LH release for the greater part of the ovarian cycle, at least in part through direct actions at the pituitary level. This inhibitory action is transiently reversed into marked facilitation of pituitary LH secretion during the mid-cycle LH surge (Knobil, 1988; Herbison, 1998; Kerdelhué et al., 2002; Moenter et al., 2003)

The role of E<sub>2</sub> in the GnRH-mediated regulation of LH release by pituitary gonadotrophs has been extensively investigated using primary pituitary cell cultures and isolated perfused pituitaries (Emons et al., 1989; Ortmann et al., 1992a; Ortmann et al., 1992b). However, gonadotrophs represent only 10-15% of the anterior pituitary cell population (Wang, 1988). It has been established that paracrine interactions between gonadotrophs and other pituitary cell types, including lactotrophs and somatotrophs, may influence basal or GnRH-induced LH secretion (Cheung, 1983; Deneff and Andries, 1983; Andries et al., 1995; Gregory et al., 2004), hereby complicating data interpretation. The need for *in vitro* models to study estrogenic effects at the pituitary level is further emphasized by the increasing interest for the therapeutic use of phytoestrogens and the development of selective estrogen receptor modulators on the one hand and growing concern for potentially disrupting effects of chemical pollutants with estrogenic actions on reproductive function on the other hand (Eertmans et al., 2003)

The murine, gonadotropic L $\beta$ T2 cell line shows important similarities with mature gonadotrophs *in vivo*, including the expression of the GnRH receptor (GnRH-R) and the production of both gonadotropins LH and follicle stimulating hormone (FSH) (Mellon et al., 1991; Alarid et al., 1996; Thomas et al., 1996; Graham et al., 1999) Therefore, this cell line is a useful *in vitro* model to unravel cellular mechanisms involved in LH synthesis and secretion and thus potentially also for the study of estrogen-specific regulation at the level of the pituitary gonadotrophs.

In the present study, LH secretion and the corresponding LH $\beta$ -subunit (LH $\beta$ -su) gene expression were assessed in the L $\beta$ T2 cells following exposure to GnRH alone or in combination with E<sub>2</sub>.

## Materials and Methods

### Plasmids, chemicals and materials

The reporter plasmid rLH $\beta$ -Luc (Rosenberg and Mellon, 2002) was kindly provided by Dr. D. Coss (University of San Diego, La Jolla, CA, USA). The ERE-tk-Luc vector (Harnish et al., 2000) was a kind gift of Dr. W. Vanden Berghe (Ghent University, Ghent, Belgium). The pSG-hER $\alpha$ 66 (HEGO) vector (Green et al., 1994), encoding for the human estrogen receptor  $\alpha$  (hER $\alpha$ ) was a kind gift of Dr. J. A. Gustafsson (Karolinska Institute, Stockholm, Sweden). The MMTV-Luc reporter plasmid (De Vos et al., 1993) was kindly provided by Dr. F. Claessens (Faculty of Medicine, University of Leuven, Leuven, Belgium). The pSV- $\beta$ -Gal vector, encoding for the enzyme  $\beta$ -galactosidase, was purchased from Promega (Leiden, The Netherlands). Gonadotropin-releasing hormone (GnRH), 17- $\beta$ -estradiol (E<sub>2</sub>), dexamethasone (Dex) and primary antibodies against  $\alpha$ -tubulin were obtained from Sigma (Bornem, Belgium). All cell culture products and Lipofectamine Plus were purchased from Invitrogen (Merelbeke, Belgium). The rat LH

RIA, the anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase and ECL Western Blotting Detection Reagents were from Amersham Biosciences (Roosendaal, The Netherlands). RNAlater solution and the RNeasy mini-kit were purchased from Westburg (Leusden, The Netherlands). The Taqman PCR core reagent kit was obtained from Applied Biosystems (Lennik, Belgium). The RevertAid First Strand cDNA Synthesis Kit was from Fermentas (Sint Leon-Rot, Germany). PCR primers were purchased from Operon (Leiden, The Netherlands). Primary antibody against ER $\alpha$  (NCL-L-ER-6F11) was from Novocastra (Newcastle, United Kingdom).

### **Cell culture**

The murine, gonadotropic  $\alpha$ T3-1 and L $\beta$ T2 cell lines were kindly provided by Dr. P. Mellon (University of San Diego, La Jolla, CA, USA). The human cervical Hela cell line and the human breast cancer cell line MCF-7 (subclone AZ) were a gift from Dr. P. Briand (Jack Bell Research Center, British Columbia, Canada). Lung fibroblasts and pituitaries, isolated from C57BL/6 mice, were provided by Dr. K. Vermaelen (Ghent University Hospital, Ghent, Belgium). All cell lines were grown as a monolayer in a 5% CO<sub>2</sub>, humidified atmosphere (37° C) and weekly passaged in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS), 100 units/ ml penicillin G and 100  $\mu$ g/ ml streptomycin, except for the murine Sertoli TM4 cell line, which was grown in DMEM: Nutrient Mix F12 supplemented with 5% horse serum, 2.5% FCS, 100 units/ ml penicillin G and 100  $\mu$ g/ ml streptomycin.

### **LH secretion studies**

L $\beta$ T2 cells ( $5 \times 10^5$  cells/ well) were seeded in 24 well plates and incubated for 48 hours, after which culture medium was replaced by phenol red-free DMEM: Nutrient Mix F12 supplemented with 5% dextran-coated charcoal (DCC) treated FCS and antibiotics, containing GnRH (0.1-1000 nM), alone or in combination with E<sub>2</sub> (0.2 nM) and Dex (20



nM), either alone or combined. Two stimulation setups were used whereby in a first approach, a 15 min exposure was followed by a 75 min collection period in GnRH-free vehicle. In the second protocol, the cells were incubated continuously for several hours (1h-6h). "Conditioned medium" was collected at the end of each incubation and stored at -80°C until analysis. Cells were washed and incubated overnight in fresh steroid-free medium or medium containing one or both steroid hormones. Both stimulation protocols were repeated for the next two days. At day 5 (third day of stimulation), following medium collection, cells were counted and then stored at -80°C in a mixture of 50 µL of phosphate-buffered salines (PBS) and 250 µL of RNAlater solution until total RNA extraction. For all experiments, experimental medium containing 0.1% ethanol, served as vehicle control, indicated by C in the figures. LH was measured using a commercial RIA (Amersham Biosciences, Roosendaal, The Netherlands) according to the manufacturer's protocol.

### **RNA isolation, real time PCR analysis and reverse transcription PCR**

Total RNA was extracted from LβT2 and TM4 cells, and from lung fibroblasts and total pituitaries, freshly isolated from C57BL/6 mice using the RNeasy mini-kit, treated with DNase and frozen at -20°C until analysis.

Quantitative expression of LHβ-subunit (LHβ-su) messenger RNA (mRNA) with mouse β-actin as the internal standard was performed using the Taqman® real-time analysis method on a Perkin-Elmer ABI Prism 7700 sequence detection system (Applied Biosystems, Lennik, Belgium). A LHβ-su-specific 120 bp fragment was amplified using forward primer (fp) 5'-CATCACCTTCACCACCAGCAT-3' and reverse primer (rp) 5'-GAGGCGAAGCGCAGCTC-3', in combination with the TaqMan® probe 5'-FAM-CCTCCCGTGCCTCAGCCAGTGT-TAMRA-3'. A mouse β-actin specific 138 bp fragment was amplified using fp, 5'-AGAGGGAAATCGTGCGTGAC-3' and rp, 5'-

CAATAGTGATGACCTGGCCGT-3', in combination with Taqman® probe 5'-FAM-CACTGCCGCATC CTCTTCCTCCC-TAMRA-3'. All primer/ probe sets were developed using the Primer Express software (Applied Biosystems, Lennik, Belgium), based on genbank entries **NM\_012858** (LH $\beta$ -su) and **NM\_007393** ( $\beta$ -actin). PCR conditions were as follows: a reverse transcription step at 48°C for 30 min and an initial denaturation step at 91°C for 10 minutes, followed by 40 cycles consisting of a denaturation step at 91°C for 1 min, a 1 min annealing step at 65°C and a 2 min extension step at 72°C. An elongation step of 10 min at 72°C finalized the amplification. LH $\beta$ -su mRNA levels were quantified against a standard curve, prepared by a serial dilution (1/10) of 50 ng total RNA extracted from L $\beta$ T2 cells;  $\beta$ -actin mRNA was quantified as a reference gene against a separate standard curve. Threshold cycle ( $C_T$ ) values for the LH $\beta$ -su and  $\beta$ -actin were obtained for each sample, the corresponding dilutions were determined from both standard curves and the dilution ratio for LH $\beta$ -su and  $\beta$ -actin was calculated. Values for treated cells are expressed relative to the control (untreated cells), set to 100%.

For qualitative reverse transcription PCR analysis of the androgen receptor (AR), the glucocorticoid receptor (GR) and the ER $\alpha$  pituitary-specific variant truncated estrogen receptor product 1 (TERP-1), total RNA was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit, according to the manufacturer's instructions. The following PCR primers were used (fp= forward primer, rp=reverse primer): a 203 bp-specific amplicon for AR fp, 5'-CTCTTCTTCCTGGCATACTCTCTT-3', AR rp 5'-CTGGTGGAGTTGTGAACAGAGTAC-3', a 461 bp-specific amplicon for the GR fp, 5'-GCCTGGTGTGCTCCGATGAA-3', GR rp, 5'-CACTGCGGCAATCACTTGGC-3', a 370 bp-specific amplicon for TERP-1, TERP-1 fp, 5'-CCATTTCTTGAGCTTGTTGAACAG-3', TERP-1 rp, 5'-GTGTCTGTGATCTTGCCAGGAC-3' (adapted from Schreihofner *et al.*, 2000) and a 238 bp-specific amplicon for  $\beta$ -actin fp, 5'-CTGGCACCCACCTTCTA-3',  $\beta$ -

actin rp, 5'-GGGCACAGTGTGGGTGAC-3'. Primers were based on genbank entries NM\_013476, NM\_008173, **NM\_007956** and **NM\_007393** for AR, GR, ER $\alpha$  respectively  $\beta$ -actin. Two microlitres of cDNA were amplified as follows: an initial denaturation step at 92°C for 10 min was followed by 40 cycles consisting of a denaturation step at 92°C for 30 sec, a 30 sec annealing step (58°) and a 45 sec extension step at 72°C. An elongation step of 10 min at 72°C finalized the amplification. PCR products were analyzed using a 2% agarose gel electrophoresis, followed by ethidium bromide staining.

Expression of full length ER $\alpha$  and possible ER $\alpha$  mRNA splicing variants was analyzed using two primer sets. The first primer set was taken from Swope et al. (2002) and consists of a common FP and two different RPs, which are able to distinguish between full length ER $\alpha$  (516 bp) and a variant message (480 bp). Following RT-PCR analysis and agarose gel electrophoresis, densitometric analysis was performed using Digimizer image analysis software (Medcalc Software, Mariakerke, Belgium) to quantify full length ER $\alpha$  and the variant messages. The ratio of full length ER $\alpha$  to the variant message was calculated for each tested cell line and corrected for  $\beta$ -actin, which was amplified in parallel and served as a control for PCR efficiency on the one hand and as loading control on the other. The murine, gonadotropic  $\alpha$ T3-1 cell line and the murine TM4 Sertoli cell line served as a positive control, while mouse lung fibroblasts served as an alleged negative control.

The second primer set (fp, 5'-GTCTGGTCCTGCGAAGGCTGC-3' and rp 5'-TGACGTAGCCAGCAACATGTCAAAG-3'), adapted from the one described by Pasqualini and colleagues (1999) in the rat, yields an amplicon of 702 bp for full length ER $\alpha$ , while alternative splicing messages correspond to an amplicon of 585 bp (exon 4 deletion), 366 bp (exon 5 deletion) and 249 bp (exon 4 and 5 deletion), respectively. PCR conditions were similar to the conditions described above, differing only in

annealing temperature of the reverse primer (55°C instead of 45°C).

### **Western Blot**

Lysates were prepared of MCF-7 cells (untransfected) and of LβT2 and Hela cells, prior to and following transfection with a human estrogen receptor α (hERα) expression vector (50 ng). Cells were washed twice with PBS and lysed in Laemmli buffer (0.25 M Tris, 1.92 M glycine and 1 % sodium dodecyl sulphate in aqueous solution). Lysates were centrifuged at 14000 rpm for 30 min at 4 °C, and protein concentration was determined by Lowry assay. All samples were diluted with sample buffer (62 mM Tris–HCl, pH 6.8, 10% glycerol, 5.0% β-mercaptoethanol, 2.0% SDS, 0.0012% bromophenol blue) and heated at 94 °C for 5 min. A total of 25 µg of protein was loaded for each sample. Electrophoresis was carried out on a 10% separating gel at 100 V for 1 h in a Mini-Protean II electrophoresis system (BioRad). After electrophoresis, proteins were transferred from polyacrylamide gels onto nitrocellulose membranes by electroblotting (0.8 mA / cm<sup>2</sup> constant current, 60 min, room temperature). Following transfer, membranes were blocked with 5.0% dehydrated nonfat milk in TBS-T (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 0.10% Tween-20) for 60 min at room temperature. Membranes were then washed three times in fresh TBS-T followed by a 1 h incubation with primary antibody. Following washing (3x TBS-T, 5 min), membranes were incubated for 1 h in secondary antibody. Immunodetection was performed with an enhanced chemiluminescence (ECL) system according to manufacturer's specifications. Chemiluminescence was captured on photographic film. Each Western blot was exposed for 15 s, 30 s and 60 s to guarantee linearity of the film.

### **Transient transfection studies**

Cells were grown in 24-well plates at a density of  $1.25 \times 10^5$  cells per well (LβT2 cell line) respectively  $3 \times 10^4$  cells per well (Hela and TM4 cell lines) in identical conditions as for

the secretion experiments. Following overnight incubation, cells were transfected with rLH $\beta$ -Luc (250 ng) or MMTV-Luc (50 ng) using Lipofectamine Plus according to the instructions of the manufacturer. The ERE-TK-Luc (250 ng) reporter plasmid was transfected in L $\beta$ T2 cells, alone or in combination with the HEO expression vector, encoding for the hER $\alpha$  (50 ng). In each experiment, a reporter plasmid, encoding for the enzyme  $\beta$ -galactosidase ( $\beta$ -gal; 20ng) was co-transfected in order to control for transfection efficiency. Twenty-four hours later, cells were treated for 16h with test compounds. After cell lysis, luciferase and  $\beta$ -gal activities were determined using a Packard Lumicount Microplate luminometer (Packard Instrument Company Inc., Meriden, CT, USA). Luciferase values were normalized for  $\beta$ -gal values and the obtained results for treated cells were expressed relative to the control (untreated cells), set to 100%.

### **Statistical analysis**

Values in the figures are presented as the average  $\pm$  SD. Curve analysis (four parameter logistic regression) was performed using Sigmaplot 8.0 (SPSS Inc., Chicago, USA). Statistical analysis was carried out by oneway ANOVA using the SPSS 11 statistical package (SPSS Inc., Chicago, USA). Significant differences were determined by the Dunnet's post hoc test; statistical significance was inferred at  $P < 0.05$ .

## **Results**

### **LH secretion and LH $\beta$ -subunit (LH $\beta$ -su) gene expression following GnRH challenge**

A short pulse setup was applied to investigate the LH secretory response of L $\beta$ T2 cells following repeated GnRH treatment. L $\beta$ T2 cells were exposed to 15 min GnRH, followed by a 75 min collection of GnRH-free medium. On the first treatment day, LH secretion, as measured by RIA, was not induced by raising concentrations of GnRH (0.1-1000 nM).

On treatment day 2, LH release was significantly induced, reaching a plateau at 10 nM of GnRH. At day 3, maximal induction of secretion was shown at 10 nM of GnRH followed by a declining LH response at higher GnRH concentrations, the secretion at 1  $\mu$ M being no longer different from the blank. When investigating the effect of varying the duration of GnRH exposure from 1h up to 6h, stimulation for 4h was found to result in the largest difference between stimulated and unstimulated cells. Despite the fact that LH medium concentrations were approximately 1.55 fold higher for cells treated with GnRH for 4h in comparison to L $\beta$ T2 cells exposed for 15 min, no differences were observed in the LH release profile between both stimulation setups (Fig. 1A and 1B). In a next series of experiments, the short pulse setup was used to investigate the effects of E<sub>2</sub> (0.2 nM) and Dex (20 nM) on LH secretion as assessed on the third day of repeated exposure to GnRH. 17- $\beta$ -estradiol alone did not alter cell number or the LH secretory response. In contrast, a significant ( $P < 0.001$ ) decrease of 20% in cell number was observed in Dex-exposed L $\beta$ T2 cells, with a significant ( $P = 0.002$ ) increase in LH release, corrected for cell number, when compared to cells exposed to GnRH (10 nM) alone. We observed a comparable 19% reduction in cell number (fig. 2) and a borderline significant ( $P = 0.06$ ) induction of LH secretion, corrected for cell number, in L $\beta$ T2 cells, treated with both E<sub>2</sub> and Dex, in comparison to cells treated with GnRH (10 nM) alone. Results are shown in figure 3.

LH $\beta$ -subunit (LH $\beta$ -su) messenger RNA (mRNA) expression, as determined using real-time PCR, was significantly ( $P < 0.05$ ) induced by GnRH in a dose-dependent manner; at 1  $\mu$ M of GnRH, mRNA levels were approximately 3 fold higher compared to the control. In the presence of E<sub>2</sub> and Dex, LH $\beta$ -su mRNA levels remained unchanged in comparison to cells treated with GnRH alone (Fig. 4). The expression of mouse  $\beta$ -actin mRNA, which served as an internal standard, was not affected by any treatment (data

not shown).

Finally, LH $\beta$ -su promoter activity was studied following treatment with GnRH using the reporter plasmid rLH $\beta$ -Luc. Luciferase activity was dose-dependently induced by GnRH, while co-incubation with E<sub>2</sub> and Dex did not affect promoter functioning (Fig. 5).

### **Gonadotropin subunits and hormone receptor expression**

Expression of the mRNAs encoding for the GnRH-receptor and the gonadotropin subunits was demonstrated in our subclone of the L $\beta$ T2 cell line and in C57BL/6 mouse pituitaries, serving as a positive control (data not shown).

Expression of full length ER $\alpha$  and possible ER $\alpha$  mRNA splicing variants was analyzed in L $\beta$ T2 cells using different primer sets. The first primer set distinguishes between full length ER $\alpha$  (516 bp) and a variant message (482 bp), which results from inappropriate splicing at the 3'-end of exon 4. The levels of full length ER $\alpha$  were 1.6-fold higher in comparison to the variant message. In contrast, the ratio of full length ER $\alpha$  to the variant message was significantly ( $P < 0.05$ ) higher (3.7- and 3.5-fold, respectively) in the murine, gonadotropic,  $\alpha$ T3-1 cell line and in the murine, TM4 Sertoli cell lines, both serving as a positive control. No bands could be detected in the mouse, lung fibroblast. An example of a representative gel electrophoresis is illustrated in figure 6A.

In a second series of experiments, a primer set was used which is able to detect both full length ER $\alpha$  mRNA and splicing variants, which result from deletion of exon 4, exon 5 or the combination of both exons. Following RT-PCR analysis, only full length ER $\alpha$  was amplified, while no splicing variants could be detected in the L $\beta$ T2 cell line. Observations were identical in the  $\alpha$ T3-1 and TM4 cell lines, showing higher expression of full length ER $\alpha$  mRNA when compared to the L $\beta$ T2 cells (Fig. 6B). Furthermore, we were unable to demonstrate expression of the pituitary-specific truncated estrogen receptor product-1 (TERP-1) in the L $\beta$ T2 cell line, while positive results were obtained using C57BL/6 mouse pituitaries (data not shown).

Following Western analysis, neither ER $\alpha$  protein (Fig. 6) nor the (theoretically) expected protein of 42.3 kDa could be detected. Similar negative results were obtained for the demonstration of full length ER $\alpha$  (66 kDa) in L $\beta$ T2 cells which were grown in DMEM containing varying concentrations (1-10 %) of either non-treated or DCC-treated FCS, respectively (data not shown).

Transfection with an expression vector, encoding for the human ER $\alpha$  (HEO), strongly induced receptor expression. Lysates from human MCF-7 breast cancer cells and from human cervical Hela cells, transfected with the HEO expression vector, served as a positive control (Fig. 7). In contrast to the low ER $\alpha$  mRNA abundance, the glucocorticoid receptor (GR) and the androgen receptor (AR) were strongly expressed in the L $\beta$ T2 cell line. For all amplicons of interest, all primer sets yielded negative results when applied to mouse fibroblast mRNA which served as an alleged negative control. Mouse  $\beta$ -actin expression was used to control for PCR efficiency (data not shown).

### **Assessment of hormone receptor-mediated gene expression**

No response could be observed in L $\beta$ T2 cells, transiently transfected with an ER-driven reporter construct (ERE-TK-Luc), following exposure to E<sub>2</sub> (16h; 10 pM – 1  $\mu$ M). In addition, L $\beta$ T2 cells did not respond to E<sub>2</sub> following co-transfection with an expression vector encoding for the human ER $\alpha$ . As a positive control, the human Hela cell line was transfected in parallel. E<sub>2</sub> was able to stimulate luciferase activity in a dose-dependent manner, with an EC<sub>50</sub> of 1.68 pM. At the highest concentration of E<sub>2</sub> (10 nM) tested, ER-mediated luciferase induction was 22.55-fold in comparison to vehicle control. Results are presented in figure 8.

AR- and GR-mediated gene transcription was investigated in L $\beta$ T2 cells using a MMTV-Luc reporter construct. At a concentration of 1  $\mu$ M, MMTV promoter activity was significantly ( $P < 0.05$ ) stimulated by dihydrotestosterone (DHT) and Dex (1.83 fold and



2.5 fold, respectively, in comparison to vehicle control) in the L $\beta$ T2 cell line. As a positive control, the mouse Sertoli TM4 cell line was transfected in parallel. Luciferase activity showed a significant ( $P<0.05$ ) 2.21 fold and 4.56 fold increase when compared to vehicle control following treatment with DHT and Dex respectively. Results are shown in fig. 9.

## Discussion

Whereas the results of the present study confirm that the murine, gonadotropic L $\beta$ T2 cell line displays characteristics of mature gonadotrophs *in vivo* with gonadotropin-releasing hormone (GnRH) inducible luteinizing hormone (LH) secretion, in our hands, extensive experiments failed to demonstrate regulatory effects of 17- $\beta$ -estradiol ( $E_2$ ).

Using a short pulse setup, daily repeated exposure to (sub) physiological concentrations of GnRH enhanced LH release by L $\beta$ T2 cells, except on the first treatment day, showing lack of secretory response following GnRH challenge. These findings suggest that the previously well documented self-priming effect of GnRH (Aiyer et al., 1974; Waring and Turgeon, 1980) was preserved in the L $\beta$ T2 cell line. Turgeon et al. (1996) and Nicol et al., (2002) also reported increased responsiveness of L $\beta$ T2 cells, although there was already a significant response on day 1. In our experiment, increasing the exposure time to 4h, although resulting in higher basal medium LH concentrations, still failed to show a significant response to GnRH challenge on day 1. In addition, the LH secretion pattern in response to GnRH, seen on the following treatment days was identical for the short and longer stimulation setups.

On the third day of exposure, we observed a maximal effect at 10 nM of GnRH, while higher concentrations resulted in a decreased secretory response, which is in agreement with the findings of Nicol (Nicol et al., 2002). Currently, the mechanism underlying the latter phenomenon remains unknown, but post-transcriptional processes or vesicle

storage of LH may be impaired in the L $\beta$ T2 cell line at these supraphysiological GnRH concentrations. Indeed, in our experiments, a diminished response to higher GnRH concentrations was not seen for LH $\beta$ -subunit (LH $\beta$ -su) promoter activity and the corresponding messenger RNA (mRNA) expression, which were both dose-dependently stimulated by GnRH, hereby reaching a maximal effect at 1 $\mu$ M.

On the third day of repeated exposure to 10 nM of GnRH, we did not observe any changes in cell number and the LH secretory response of L $\beta$ T2 cells in the presence of E<sub>2</sub>. In contrast, dexamethasone (Dex) treatment decreased cell number while stimulating the LH secretory response. Results for the combination of both steroids were similar as for Dex alone. Turgeon and colleagues reported that a significant effect of E<sub>2</sub> was seen only on the fourth day of repeated exposure. Furthermore, in their experiments, the GnRH-induced secretory response to Dex was approximately 2-fold higher in comparison to the E<sub>2</sub>-treated group and was comparable to the effects they observed in E<sub>2</sub>- and Dex-treated (E<sub>2</sub>/Dex) cells (Turgeon et al., 1996). In contrast, at day 3 of repeated exposure, Nicol and colleagues reported that LH secretion was significantly stimulated by all steroid treatments, with a stronger induction in the Dex-group in comparison to E<sub>2</sub>-treated cells (Nicol et al., 2002). Similar to the observations of Turgeon (1996) the combination of both steroids had no additional effects above the Dex-mediated response.

In our hands, the combination of E<sub>2</sub> and Dex did not influence GnRH-induced LH $\beta$ -su gene expression, being the rate-limiting step of LH synthesis (Evans, 1999; Yamada et al., 2004). Our data are in agreement with the findings of Turgeon (1996), but are at variance with the observations of Nicol (2002), showing inconsistent effects on LH $\beta$ -su mRNA expression. The lack of estrogenic response in our L $\beta$ T2 cell line may plausibly be attributed to the rather weak abundance of the mRNA encoding for the ER $\alpha$  and the absence of demonstrable receptor protein. Similar findings were reported by the group of

Niswender (2003), who transfected ER $\alpha$  in the L $\beta$ T2 cell line because of the absence of the receptor (Colorado AES Projects COL00220; ref. 32)

Our data are conflicting with the observations of another group, reporting strong ER $\alpha$  mRNA and protein expression in the L $\beta$ T2 cells (Schreihofner et al., 2000). In our hands, different culture conditions did not affect ER $\alpha$  mRNA and protein expression.

Furthermore, we demonstrated that our L $\beta$ T2 cell line preserved the main properties of mature gonadotrophs in vivo, including mRNA expression of the GnRH receptor (GnRH-R) and the gonadotropin subunits, which is in agreement with other reports (Turgeon et al., 1996; Lawson et al., 2001). L $\beta$ T2 cells also showed a strong expression of the mRNAs encoding for the androgen receptor (AR) and the glucocorticoid receptor (GR). Dedifferentiation in function of time and passage number may be a possible explanation for the loss of ER expression in our subclone. This phenomenon was reported by Kim and colleagues (Kim et al., 2000) in immortalized hepatocytes, which were generated using simian virus 40 (SV40) T-antigen oncogenesis. The authors demonstrated that dedifferentiation was caused by chromosomal damages, induced by raising T-antigen levels following continuous passages. As SV40 T-antigen oncogenesis was used to develop the L $\beta$ T2 cell line (Alarid et al., 1996), we cannot exclude that similar effects occurred in these cells, resulting in the loss of cell characteristics, including ER $\alpha$  expression. Another explanation could have been overexpression of the truncated estrogen receptor product 1 (TERP-1) in our cell line. This pituitary-specific ER-isoform is able to modulate E<sub>2</sub>-mediated gene transcription, depending on the ER over TERP-1 concentration ratio (Resnick et al., 2000). However, TERP-1 expression appeared to be also absent in our L $\beta$ T2 cell line. Furthermore, another group reported that TERP-1 expression in the L $\beta$ T2 cells was considerably lower when compared to ER $\alpha$  and also varied between experiments (Schreihofner et al., 2000).

Although we were not able to demonstrate TERP-1 expression in the L $\beta$  T2 cell line, we did show the expression of an alternative ER $\alpha$  mRNA, which results from inappropriate splicing at the 3'-end of exon 4, whereby an intronic sequence is incorporated at this 3'-end and downstream exons are deleted. Theoretically, this mRNA variant encodes for a protein of 42.4 kDa, which lacks most of the ER $\alpha$  ligand binding domain (LBD) (Swope et al., 2002). In the mouse, the exact function of this protein remains currently unknown but a similar variant has already been described in humans, where it was able to enhance or inhibit full length ER $\alpha$  transcriptional activity, depending on the cellular context (Bollig et al., 2000). Although we were not able to demonstrate the expression of this truncated protein in the L $\beta$  T2 cell line with the applied primary antibody, one can postulate that the observed lack of estrogenic effects in the L $\beta$  T2 cell line might be related to the low ratio of full length ER $\alpha$  to the variant message. These findings are in contrast to our observations in the gonadotrophic  $\alpha$  T3-1 and TM4 Sertoli cell lines, which are both estrogen-responsive. In the latter cell lines, substantially higher levels (3.7- and 3.5-fold) of full length ER $\alpha$  in comparison to the variant message were measured. In the rat, Pasqualini and colleagues (2001) have described ER $\alpha$  splicing variants showing deletions of exon 3 and/ or exon 4, which can alter ER transcriptional activity

either positively or negatively. Furthermore, these variant mRNAs were expressed in a stage- and region-specific manner (Pasqualini et al., 1999) in the presence of full length ER $\alpha$  mRNA. We have analyzed the expression of corresponding ER $\alpha$  splicing variants in the L $\beta$  T2 cell line. One important difference is the fact that the rat ER $\alpha$  gene contains only 8 exons, which is in contrast to the mouse gene, which consists of 9 exons. In other words, the murine ER $\alpha$  gene exons 4 and 5 correspond to rat exons 3 and 4. We have performed RT-PCR analysis using a primer set which was derived from the one described by Pasqualini and colleagues (1999). However, no ER $\alpha$  splicing variants were detected in the L $\beta$  T2 cell line nor in the other two tested murine cell lines ( $\alpha$  T3-1 and TM4 cell line)

In order to by-pass the apparent absence of ER $\alpha$  protein, L $\beta$  T2 cells were transiently transfected with an expression vector encoding for the human ER $\alpha$  in combination with an ER-driven reporter construct (ERE-tk-Luc). Despite strong expression of ER $\alpha$  protein, as demonstrated using Western blot analysis, E<sub>2</sub> treatment still failed to stimulate estrogen response element (ERE)-mediated luciferase expression. In contrast, Dex and dihydrotestosterone (DHT) were able to stimulate the mouse mammary tumor virus (MMTV) promoter, which is under transcriptional control of the GR and the AR.

These observations, together with the demonstrated mRNA expression of both receptors, suggest a specific problem of ER signaling in the L $\beta$  T2 cell line, rather than a generalized impairment of nuclear receptor functioning.

It should be noticed that we transfected human ER $\alpha$  into a murine cell line, and thus that a species-specific incompatibility might underlie our negative transfection results in the L $\beta$  T2 cell line. However, similar experiments were performed in the mouse TM4 Sertoli cell line, whereby transfection with a HEO vector and ERE-LUC resulted in a significant increase in estrogenic response above cells, transfected with ERE-Luc alone (data not shown).

Recently, Turgeon and Waring (2006) demonstrated that progesterone receptor (PR) A and B expression in L $\beta$ T2 cells was not affected by E<sub>2</sub>, which was in contrast to their observations in rat and mouse pituitary cultures, showing increased expression of both PR isoforms following E<sub>2</sub>-exposure. Another group reported a decrease in GnRH-induced aromatase promoter activity in L $\beta$ T2 cells treated with E<sub>2</sub> (Galmiche et al., 2006), however at a 50.000-fold higher concentration (10  $\mu$ M) in comparison to our and other experiments (Turgeon et al., 1996; Nicol et al., 2002).

The present results indicate the importance of careful analysis of data, obtained in highly specific cell lines. In the case of the L $\beta$ T2 cell line, the previously reported estrogenic effects were rather modest or weak (Turgeon et al., 1996; Nicol et al., 2002) Moreover, variable outcome may occasionally be observed even for cell lines yielding much more robust estrogenic responses than those previously described for L $\beta$ T2 cells, such as in

the human MCF-7 breast cancer cell line, which has been widely used in validated *in vitro* assays to assess estrogenic effects (Rasmussen and Nielsen, 2002). One can postulate that ER-mediated signaling is an unstable feature of the L $\beta$ T2 cell line, which is vulnerable to dedifferentiation

In conclusion, in our hands, extensive investigations failed to demonstrate any regulatory effect of E<sub>2</sub> on the GnRH-induced LH secretory response and the corresponding LH $\beta$ -su gene expression in the L $\beta$ T2 cell line. Underlying causes appear to be situated at different levels involved in ER signal transduction, including rather weak ER $\alpha$  mRNA expression, the absence of a functional ER $\alpha$  protein and lack of estrogenic response following co-transfection with an ER $\alpha$  expression vector. Furthermore, the relative strong expression of an ER $\alpha$  mRNA splicing variant in comparison to full length ER $\alpha$  mRNA suggests a possible role for this variant in abnormal ER signaling in the L $\beta$ T2 cell line. In view of the inconsistencies between laboratories or subclones in the expression of ER-mediated signaling, the L $\beta$ T2 cell line does not seem to be a suitable *in vitro* model for the study of estrogen regulation of gonadotropin synthesis and secretion. It seems relevant to point out this limitation in view of the unique position of the L $\beta$ T2 cell line as an immortalized cell line reflecting essential properties of mature gonadotrophs and a well established and useful model for *in vitro* studies of gonadotropin regulation.

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Figure 1A

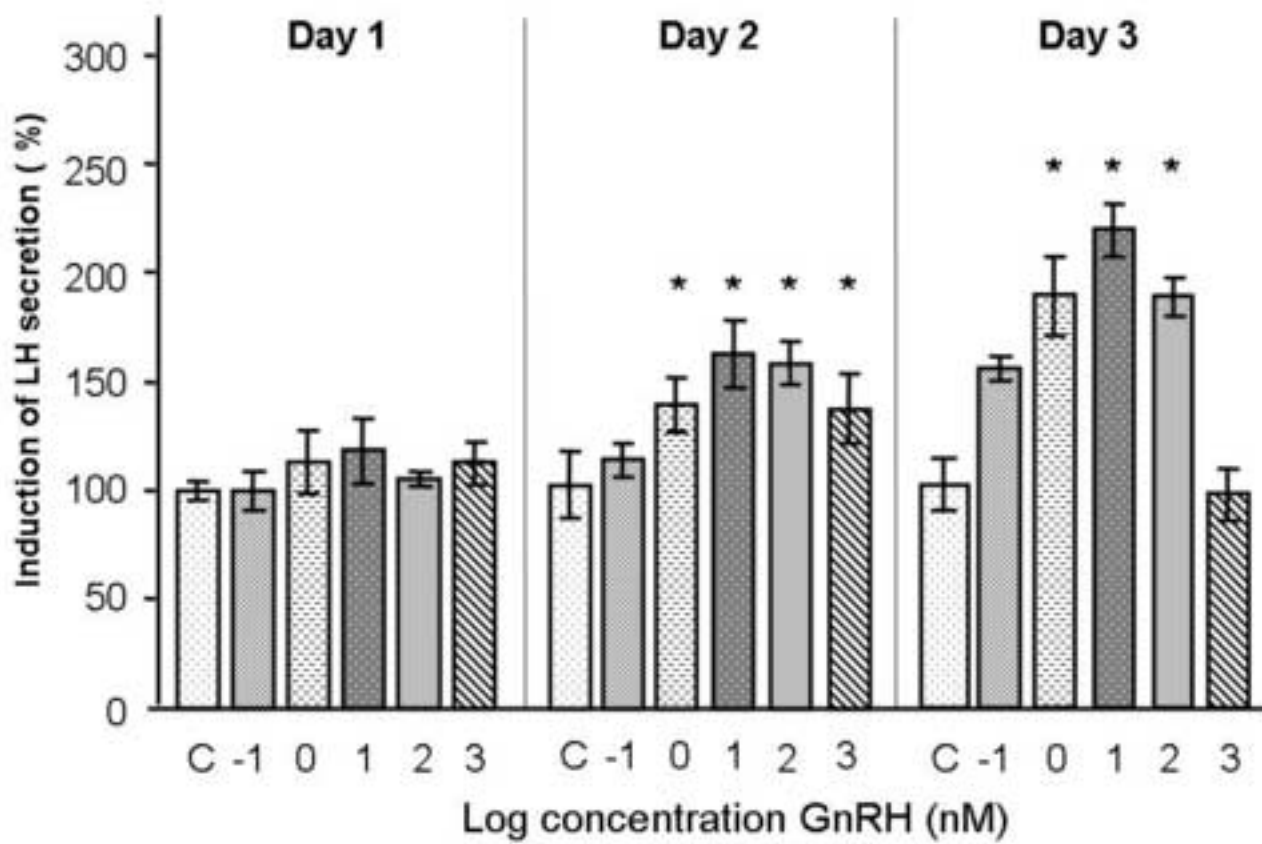


Figure 1B

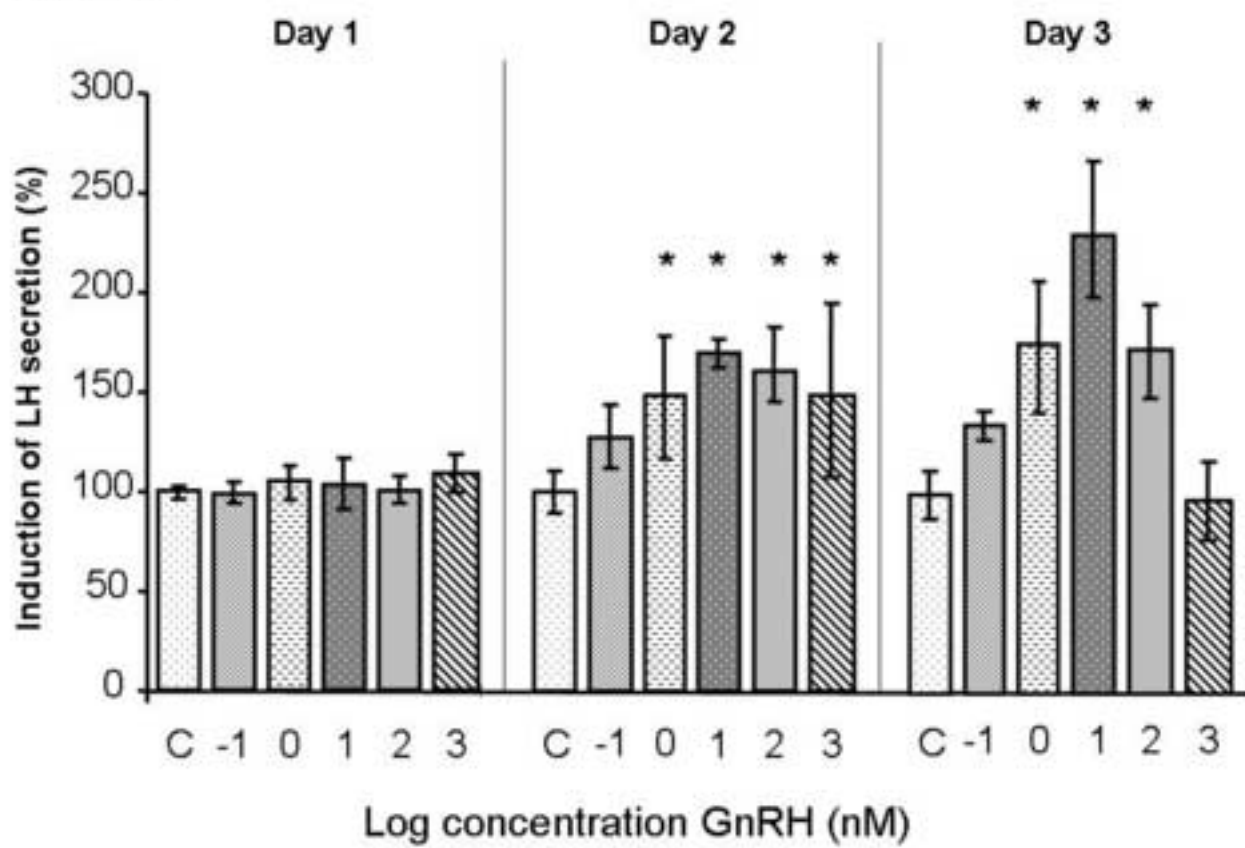
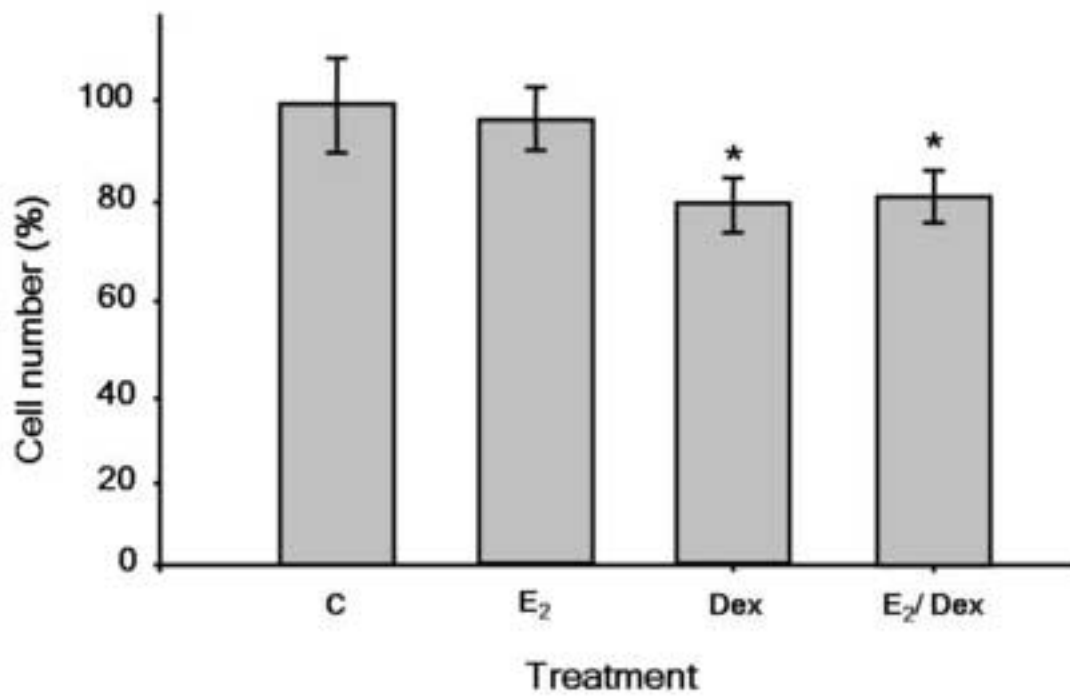




Figure 2



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Figure 3

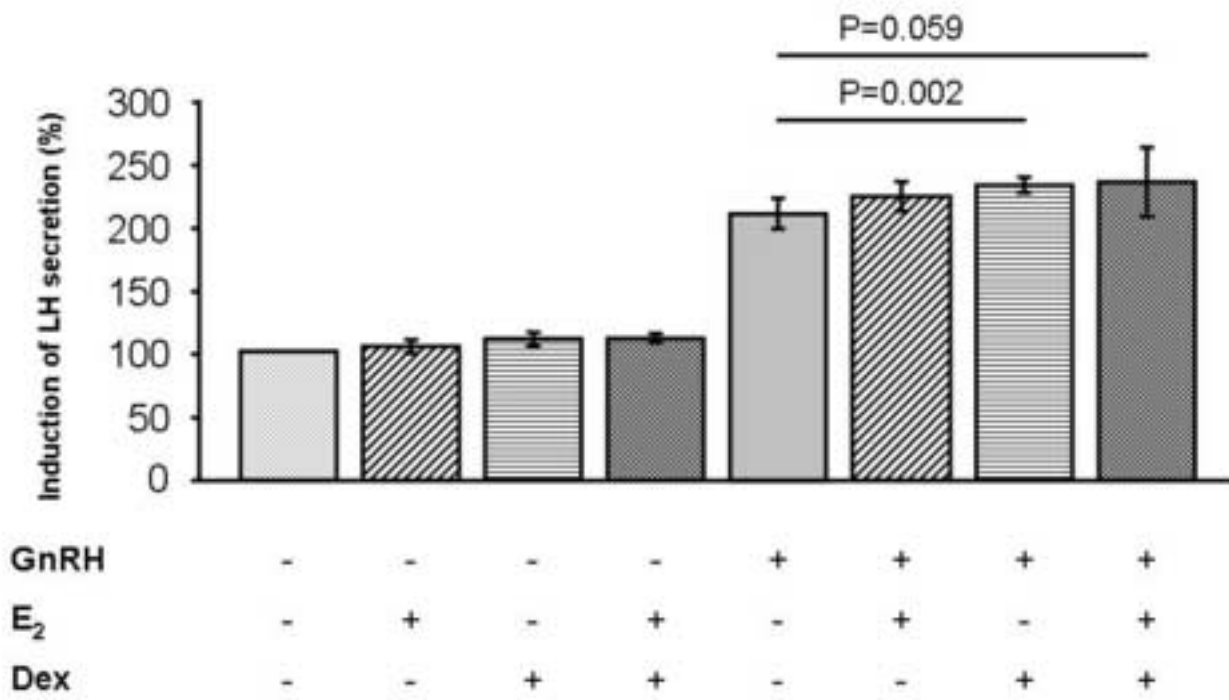


Figure 4

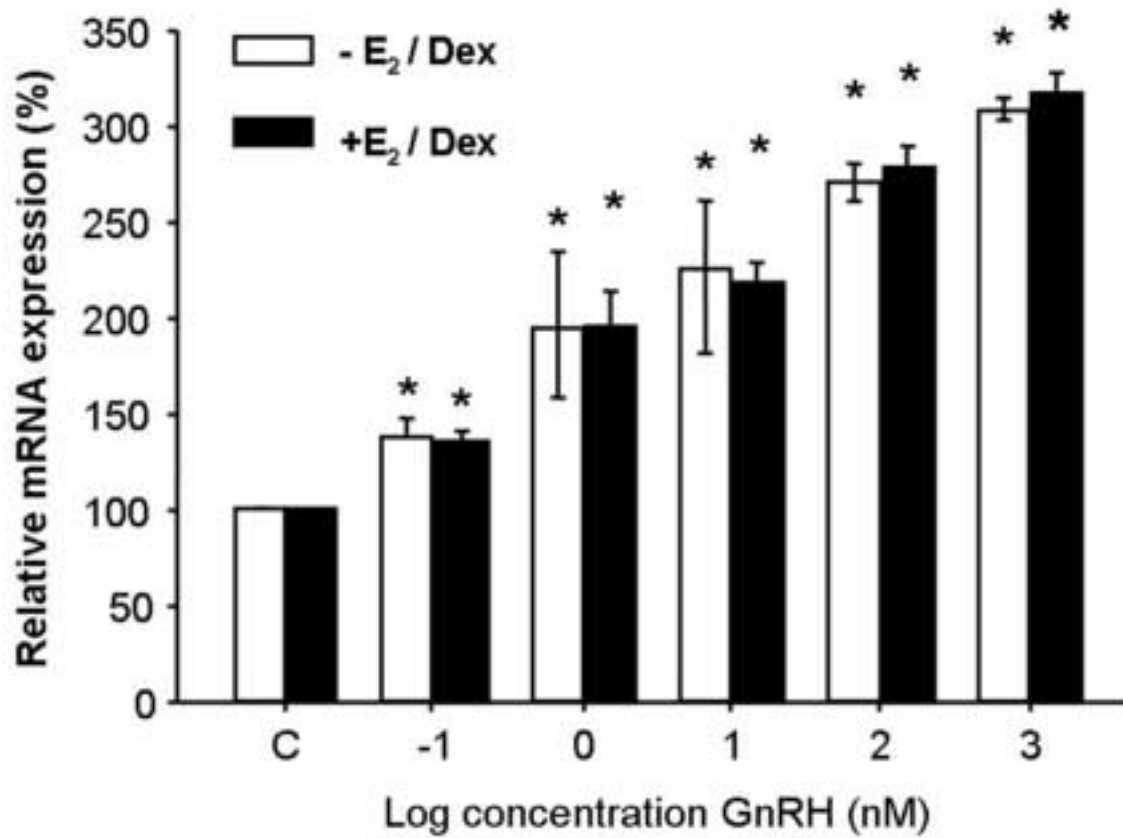
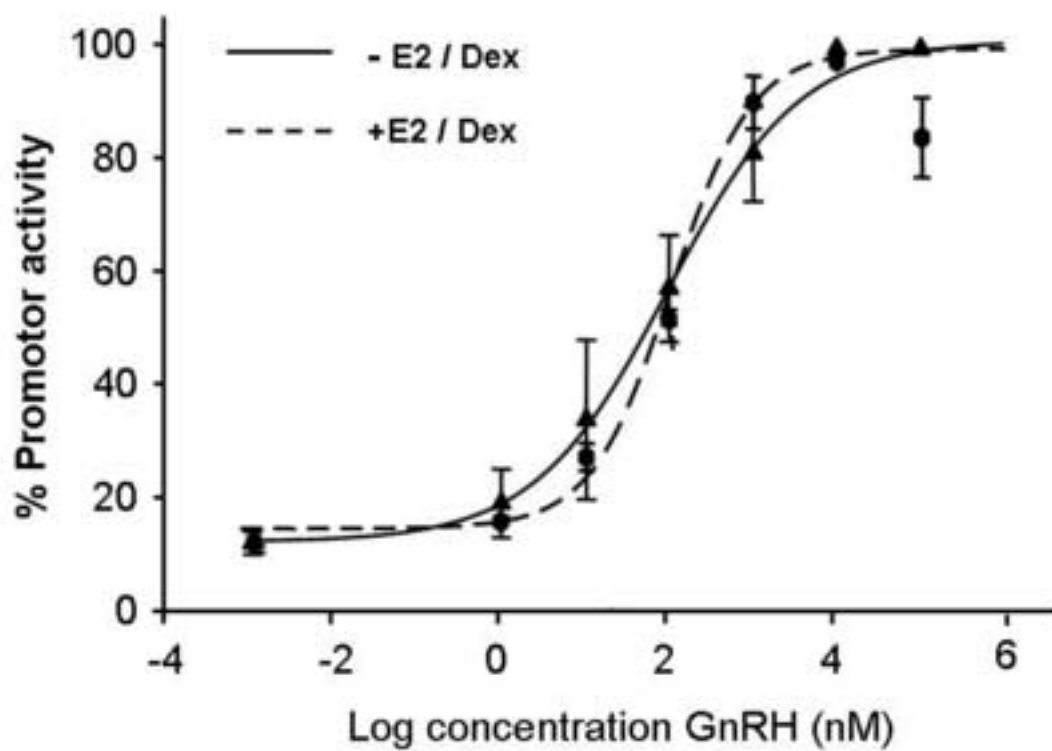


Figure 5

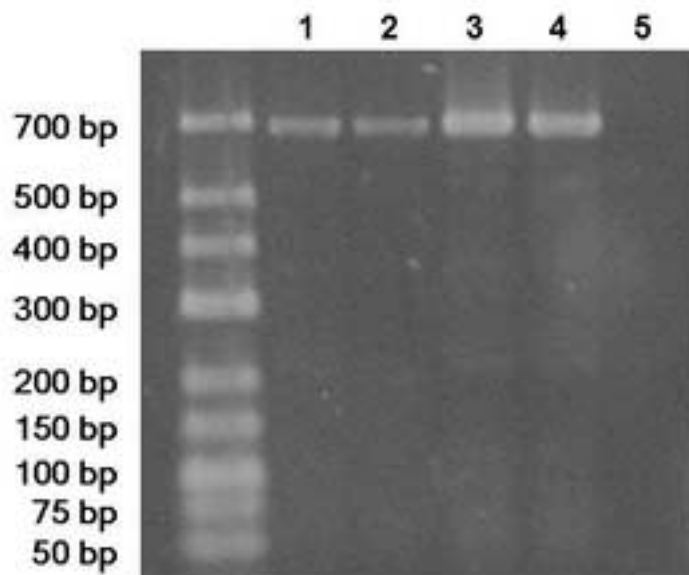


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Figure 6A



Figure 6B



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Figure 7

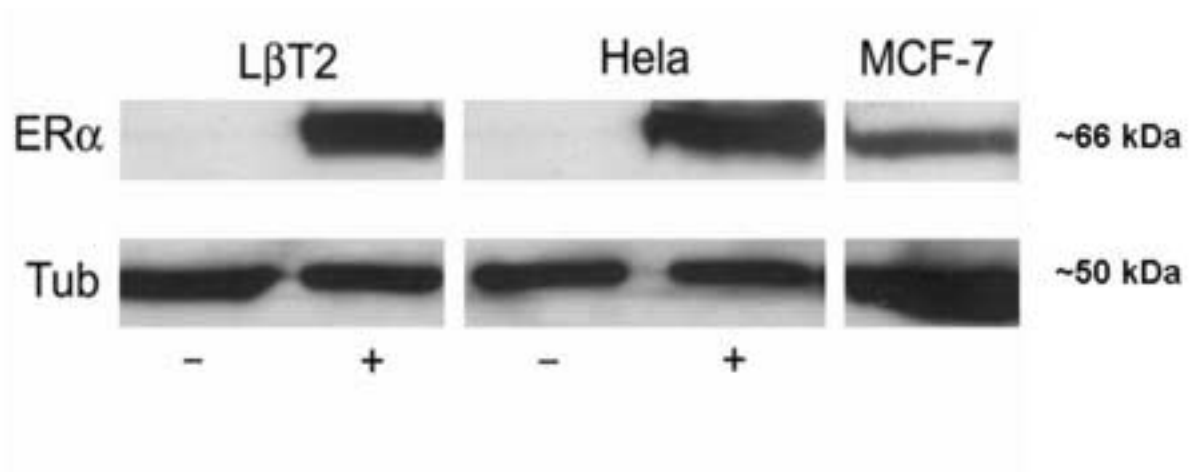


Figure 8

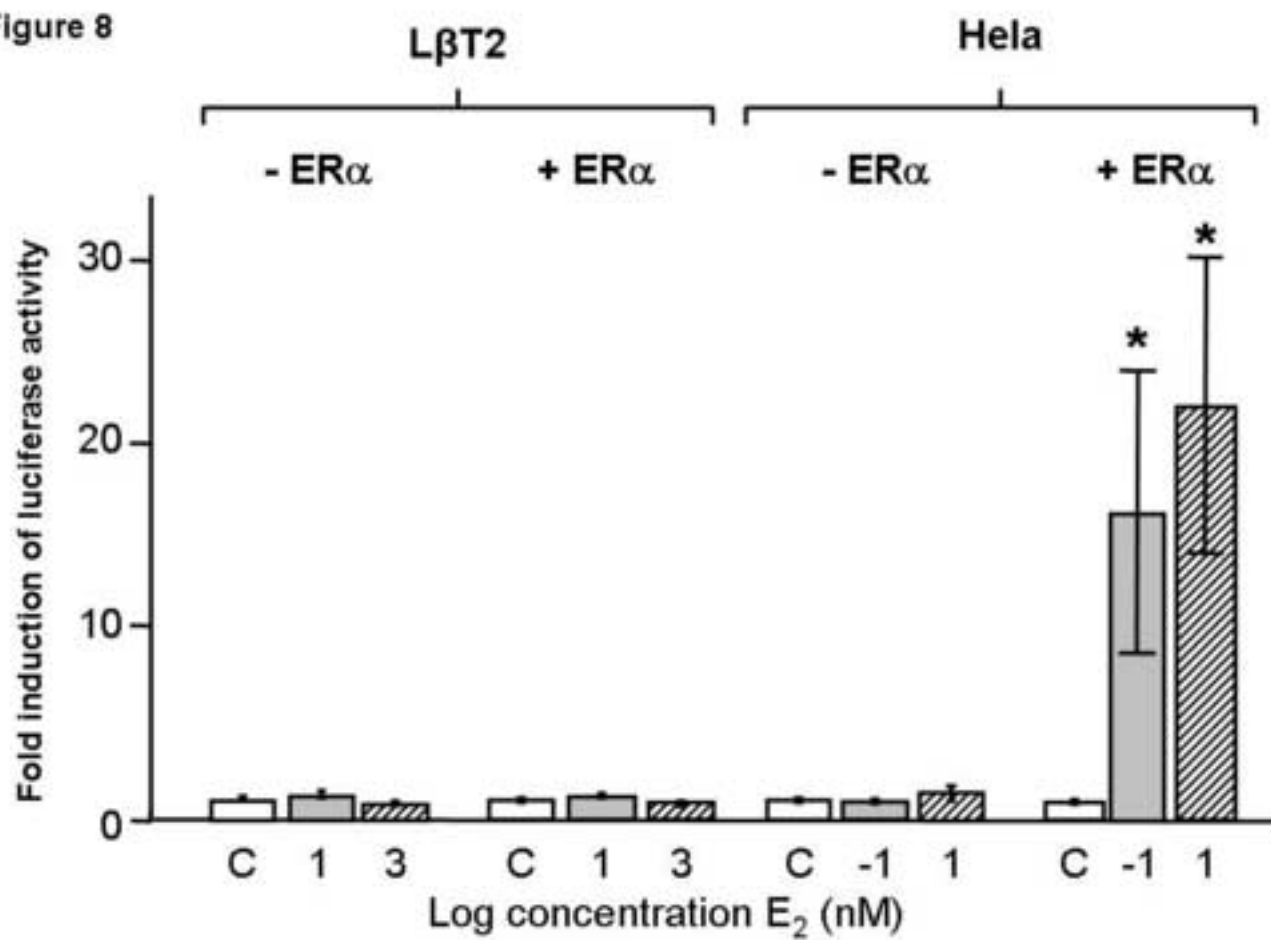
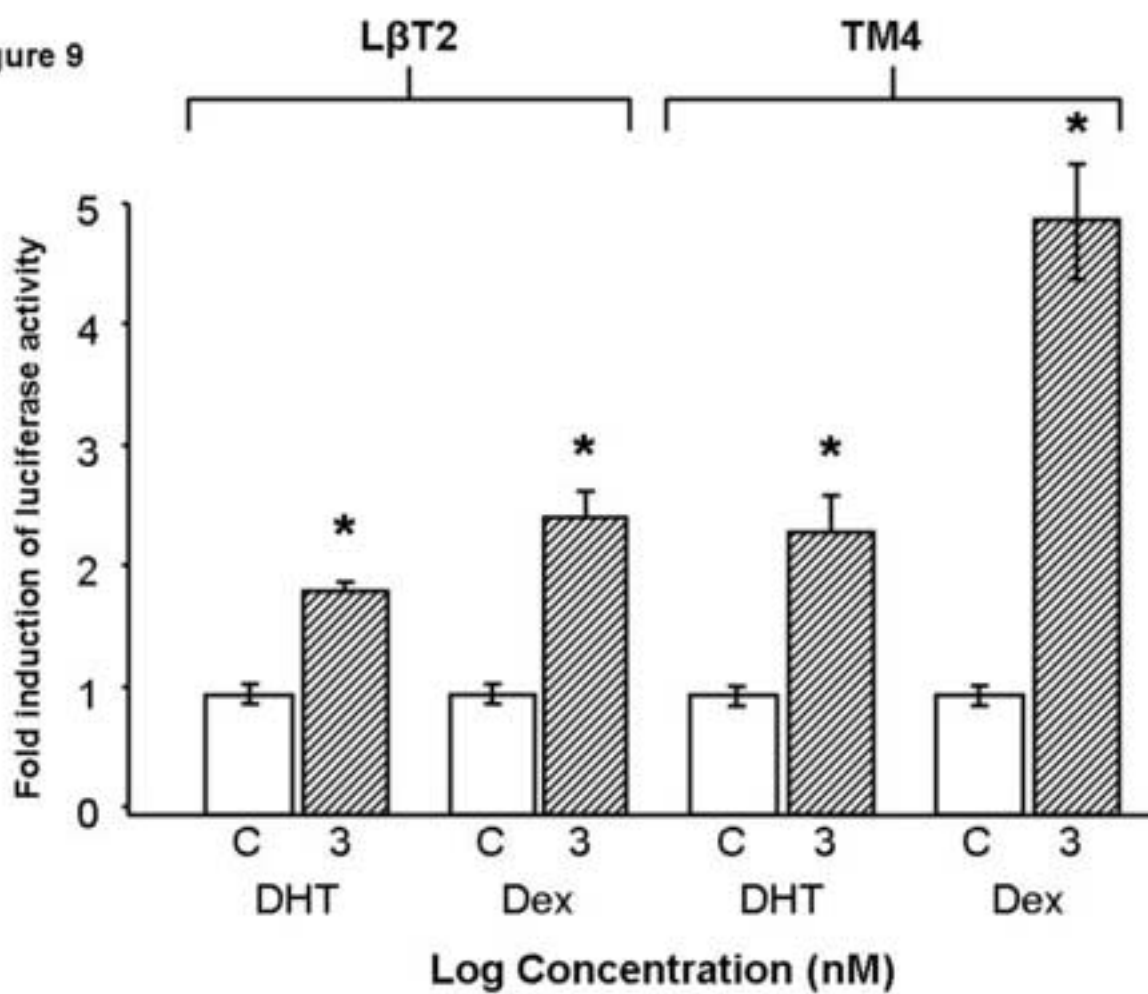


Figure 9





## Legends

### Figure 1:

#### **Gonadotropin-releasing hormone (GnRH)-induced luteinizing hormone (LH)-secretion in the L $\beta$ T2 cell line.**

L $\beta$ T2 cells were exposed daily to GnRH (0.1–1000 nM) during three consecutive days using a short pulse setup (Fig. 1A; 15 min GnRH - 75 min collection of GnRH-free medium) or a 4h exposure setup (Fig. 1B). Values for treated cells were corrected for cell number and are expressed relative to vehicle control (experimental medium containing 0.1% ethanol), set at 100%. Results are presented as the mean  $\pm$  SD from three separate experiments. \* =  $P < 0.05$  versus vehicle control (C).

### Figure 2:

#### **Effect of E<sub>2</sub> (0.2 nM) and/ or Dex (20 nM) treatment on cell proliferation in the L $\beta$ T2 cell line.**

At the end of the LH secretion experiments (third day of repeated exposure to GnRH), cell number was determined using the trypan blue exclusion method. Results were expressed relative to vehicle control (C), set at 100%. Results are presented as the mean  $\pm$  SD from three separate experiments. \* =  $P < 0.05$  versus vehicle control (C).

### Figure 3:

#### **Effects of different steroid treatments on LH secretion following repeated exposure to 10 nM of GnRH.**

L $\beta$ T2 cells were exposed daily to 10 nM of GnRH during three consecutive days using a short pulse setup (15 min GnRH – 75 min collection of GnRH-free medium) in the absence or presence of 0.2 nM E<sub>2</sub> and 20 nM Dex, alone or combined. At treatment day

3, LH medium concentrations were determined and corrected for cell number. Shown are also controls (in absence of GnRH). Values are expressed relative to vehicle control (experimental medium containing 0.1% ethanol), set at 100%. Results are presented as the mean  $\pm$  SD from three separate experiments. \* =  $P < 0.05$  versus vehicle control.

Figure 4:

**Dose-dependent stimulation of rat LH $\beta$ -subunit (LH $\beta$ -su) mRNA expression by GnRH in the L $\beta$ T2 cells.**

L $\beta$ T2 cells were exposed daily to GnRH (0.1–1000 nM), alone or in the presence of E<sub>2</sub> (0.2 nM) and Dex (20 nM) during three consecutive days. At the final day, L $\beta$ T2 cells were harvested and total RNA was extracted for real-time PCR analysis. Values for treated cells are expressed relative to their respective control (experimental medium containing 0.1% ethanol and experimental medium supplemented with E<sub>2</sub> (0.2 nM) and Dex (20 nM) respectively), set at 100%. Results are presented as the mean  $\pm$  SD from three separate experiments. \* =  $P < 0.05$  versus respective control (C); GnRH-induced LH $\beta$ -su mRNA expression was not significantly altered following treatment E<sub>2</sub>/Dex-treatment.

Figure 5:

**Modulation of rLH $\beta$ -su promoter activity by GnRH.**

L $\beta$ T2 cells were transiently transfected with the rLH $\beta$ -Luc construct (250 ng), which consists of an 1800 bp fragment of the 5' flanking region of the rLH $\beta$ -su gene, coupled to a gene encoding for the luciferase enzyme. L $\beta$ T2 cells were exposed to GnRH (0.1–10000 nM), alone (full line) or in combination (dashed line) with E<sub>2</sub> (0.2 nM) and Dex (20 nM) for 16h. Results are presented as the mean  $\pm$  SD from three separate transfections.

Figure 6:

**Reverse-transcription PCR analysis of ER $\alpha$  expression in the L $\beta$ T2 cell line.**

Fig. 6A: The expression of ER $\alpha$  and an alternative splice variant was investigated using reverse transcriptase polymerase chain technique (RT-PCR). Full length ER $\alpha$  and the alternative message correspond to an amplicon of 516 bp and 480 bp, respectively. The estrogen-responsive TM4 Sertoli cell line and the gonadotropic  $\alpha$ T3-1 cell line served as a positive control, while lung fibroblasts were used as an alleged negative control.

Fig. 6B: RT-PCR was applied to analyze the expression of alternative ER $\alpha$  mRNA splicing variants, which contains an exon 3 and/ or exon 4 deletion. Full length ER $\alpha$  mRNA corresponds to a 702 bp amplicon. Sample 1 and 2 represent two different RNA extracts of the L $\beta$ T2 cell line. Sample 3 and 4 correspond to the  $\alpha$ T3-1 and TM4 cell lines, respectively. Murine, lung fibroblast RNA (lane 5) served as an alleged negative control.

Figure 7:

**Western Blot analysis of ER $\alpha$  expression in the L $\beta$ T2 and the Hela cell line.**

Lysates were prepared from L $\beta$ T2 and Hela cells, without (-) and with (+) prior transfection with a human ER $\alpha$  expression vector, and analyzed for the presence of ER $\alpha$  protein (66 kDa fragment). The human MCF-7 cell line served as a positive control. Mouse tubulin (Tub.; 50 kDa fragment) was used as loading control.

Figure 8:

**Assessment of estrogen receptor (ER)-mediated gene transcription in the L $\beta$ T2 and the Hela cell line.**

L $\beta$ T2 and Hela cells were transiently transfected with the ERE-tk-Luc construct alone, or in combination with a human ER $\alpha$  expression vector. The ERE-tk-Luc reporter plasmid consists of two copies of the *Xenopus laevis* vitellogenin A2 ERE (58-GGTCACAGTGACC-38), linked to the thymidine kinase (tk) promoter and coupled to a

gene encoding for the luciferase enzyme. Cells were exposed to E<sub>2</sub> (10 nM and 1000 nM for LβT2 cells; 0.1 and 10 nM for Hela cells) for 16h. Results are presented as the mean ± SD from three separate transfections. \* =  $P < 0.05$  versus vehicle control (C).

Figure 9:

**Differential response of the MMTV-promoter in the LβT2 and TM4 cell lines.**

Both cell lines were transiently transfected with the AR- and GR-driven MMTV-Luc reporter plasmid and treated with Dex (1 μM) or dihydrotestosterone (DHT; 1 μM) for 16h. Results are presented as the mean ± SD from three separate transfections. \* =  $P < 0.05$  versus vehicle control(C).

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