Identification of estradiol/ER α -regulated genes in the mouse pituitary

Hyun Joon Kim^{1,2,*}, Mary C Gieske^{1,3,*}, Kourtney L Trudgen^{1,*}, Susan Hudgins-Spivey¹, Beob Gyun Kim⁴, Andree Krust^{5,6}, Pierre Chambon^{5,6}, Jae-Wook Jeong⁷, Eric Blalock⁸ and CheMyong Ko^{1,3}

¹Division of Reproductive Sciences, Department of Clinical Sciences, University of Kentucky, Lexington, Kentucky 40536, USA

²Department of Anatomy and Neurobiology, Institute of Health Sciences, Medical Research Center for Neural Dysfunction, School of Medicine, Gyeongsang National University, Jinju, Korea

³Department of Biology, University of Kentucky, Lexington, Kentucky 40536, USA

⁴Department of Animal Science and Environment, Konkuk University, Seoul 143-701, Korea

⁵Institut de Genetique et de Biologie Moleculaire et Cellulaire (CNRS, INSERM, ULP, College de France), 67404 Illkirch Cedex, Strasbourg, France

⁶Institut Clinique de la Souris, BP10142, 67404 Illkirch Cedex, Strasbourg, France

⁷Department of Obstetrics, Gynecology and Reproductive Biology, Michigan State University College of Human Medicine, Grand Rapids, Michigan 49503, USA

⁸Department of Molecular and Biomedical Pharmacology, University of Kentucky, Lexington, Kentucky 40536, USA

(Correspondence should be addressed to C Ko who is now at Division of Clinical and Reproductive Sciences, College of Health Sciences, University of Kentucky, Lexington, Kentucky 40536, USA; Email: cko2@uky.edu)

*(H J Kim, M C Gieske, and K L Trudgen contributed equally to this work)

Abstract

Estrogen acts to prime the pituitary prior to the GnRH-induced LH surge by undiscovered mechanisms. This study aimed to identify the key components that mediate estrogen action in priming the pituitary. RNA extracted from the pituitaries of metestrous (low estrogen) and proestrus (high estrogen) stage mice, as well as from ovariectomized wild-type and estrogen receptor α (ER α) knockout mice treated with 17 β -estradiol (E₂) or vehicle, was used for gene expression microarray. Microarray data were then aggregated, built into a functional electronic database, and used for further characterization of E₂/ER α -regulated genes. These data were used to compile a list of genes representing diverse biological pathways that are regulated by E₂ via an ER α -mediated

pathway in the pituitary. This approach substantiates ER α regulation of membrane potential regulators and intracellular vesicle transporters, among others, but not the basic components of secretory machinery. Subsequent characterization of six selected genes (*Cacna1a*, *Cacna1g*, *Cited1*, *Abepl1*, *Opn3*, and *Kcne2*) confirmed not only ER α dependency for their pituitary expression but also the significance of their expression in regulating GnRH-induced LH secretion. In conclusion, findings from this study suggest that estrogen primes the pituitary via ER α by equipping pituitary cells with critical cellular components that potentiate LH release on subsequent GnRH stimulations.

Journal of Endocrinology (2011) **210**, 309–321

Introduction

The ovarian steroid estradiol (E₂) plays a critical physiological role in inducing the LH surge by acting on both the hypothalamus and the pituitary (Clarke 2002, Christian *et al.* 2005). While much focus has been placed on the role of estrogen in the hypothalamic GnRH surge, less work has been done concerning the actions of estrogen on the pituitary. Estrogen has been shown to be involved in priming or sensitizing the pituitary gonadotroph to GnRH stimulus (Clarke & Cummins 1984, Clarke 1995a) by increasing expression of GnRH receptor (GnRH-R) in gonadotrophs (Liu & Yen 1983, Leung & Peng 1996, Strauss & Barbieri 2009), mobilizing secretory granules to the periphery of the cell (Thomas & Clarke 1997, Thomas *et al.* 1998), and recruiting the number of gonadotrophs to the pool of those that are capable of responding to GnRH stimulation

(Smith *et al.* 1984). It has also been shown that estrogen downstream pathways include cytoskeleton rearrangement (Powers 1986, Sapino *et al.* 1986, DePasquale 1999), regulation of ion channels (Clarke 2002), and energy metabolism (Simpson *et al.* 2005, Jones *et al.* 2006). The molecular mechanisms by which pituitary priming occurs remain largely unknown, but these functions may play a part. Both estrogen receptor (ER) subtypes ER α and ER β are expressed throughout the pituitary (Mitchner *et al.* 1998). However, diverse lines of evidence indicate that ER α is the predominant mediator of estrogen action in the pituitary. Agonists for ER α , but not ER β , were capable of inducing increased LH secretion in estrogen-primed GnRH-stimulated rat pituitaries *in vitro* (Sanchez-Criado *et al.* 2004, 2005). ER α activation was shown to be primarily responsible for the reorganization of the disrupted organelle morphology seen in the gonadotroph after ovariectomy

(Sanchez-Criado *et al.* 2006). In addition to ER α knockout (ER α KO) female mice exhibiting complete infertility and lack of ovulation (Dupont *et al.* 2000, Hewitt & Korach 2003), we recently reported that targeted deletion of ER α in the gonadotroph caused infertility in female mice (Gieske *et al.* 2008).

In the gonadotroph, binding of GnRH to GnRH-R, a G-protein-coupled receptor, activates intracellular signaling pathways causing membrane depolarization and a rapid change in intracellular Ca²⁺ concentration, which subsequently elicits multiple intracellular events that facilitate LH secretion (Ghosh *et al.* 1996, Shacham *et al.* 2001). The well-timed and rapid nature of LH secretion on GnRH stimulation at the time of surge suggests the need for synchronization of the cellular secretory machinery. Considering the evidence that estrogen priming of the pituitary is required for the induction of the LH surge (Liu & Yen 1983, Strauss & Barbieri 2009), we hypothesize that estrogen via ER α primes the pituitary by equipping gonadotrophs and other pituitary cells with key regulators of the LH secretion machinery.

This study aimed to identify components that play critical roles in estrogen priming of the pituitary. For this purpose, genes that are differentially regulated in the pituitary under various estrogen and ER α backgrounds were identified, and the expression patterns and functional roles of six selected genes were characterized. Future study on the functional roles of the identified genes will begin to reveal the molecular mechanism of pituitary estrogen priming for the GnRH-induced LH surge.

Materials and Methods

Animals and treatments

Animal handling procedures were carried out in accordance with the University of Kentucky Animal Care and Use Committee. Mice were maintained with food and water made available *ad libitum* in a 14 h light:10 h darkness cycle at 24 °C. All mice used in this study were of C57BL/6 genetic background. ER α KO mice were produced as described previously (Gieske *et al.* 2008). For microarray analysis, wild-type (WT) mice were divided into two subgroups at the age of 7 weeks after birth. The first group of WT mice was ovariectomized (OVX), kept for 3 weeks, and injected (s.c.) with 10 µg E₂ per mouse or 100 µl sesame oil (vehicle (veh)) at 0900 h for two consecutive days. On the second day, the mice were killed at 1500 h by carbon dioxide inhalation, and the pituitaries were harvested and frozen on dry ice. The second group of WT mice was kept for a week, and their estrous cycling patterns were determined by daily vaginal smear for next 2 weeks using a standard procedure (Becker *et al.* 2005). During the second week of vaginal smear, mice were killed on proestrus or metestrus at 1500 h and pituitaries were collected. The pituitaries of ER α KO mice were collected at 1500 h at the age of 10 weeks. ER α KO mice did not cycle but displayed a consistent pattern of diestrus. For the histological analyses,

cardiac perfusion was performed on 10-week-old mice using 4% neutralized buffered paraformaldehyde. After postfixation with the same fixative, tissues were stored in 20% sucrose and then frozen in OCT compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA). For primary pituitary culture, 10-week-old cycling WT female mice were used.

Reagents

Antibodies raised in rabbit for adipocyte enhancer binding protein 1 (AEBP1; ARP31592_P050, AVIVA Systems Biology, San Diego, CA, USA), Ca_v2.1 (encoded by *Cacna1a*; ACC-001, Alomone Labs, Jerusalem, Israel), Ca_v3.1 (encoded by *Cacna1g*; ACC-021, Alomone Labs), potassium voltage-gated channel, Isk-related subfamily, gene 2 (KCNE2; APC-054, Alomone Labs), Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1 (Cited1; XAV-8490, ProSci, Inc., CA, USA), and encephalopsin, opsin 3 (Opn3; NLS2134, Novus Biologicals, Inc., CO, USA) were purchased from indicated company. Polyclonal antiserum for mouse pituitary LH was purchased from the National Hormone and Pituitary Program (Harbor-UCLA Medical Center, Torrance, CA, USA). GnRH and E₂ were purchased from Sigma. Molecular reagents were purchased from Invitrogen. Cell culture reagents including DMEM, gentamicin, BSA, HEPES, trypsin, trypsin inhibitor, and DNase I were purchased from Sigma. Other reagents including ITS, fungizone, and fetal bovine serum were purchased from Gibco-BRL. ω-Agatoxin TK (selective blocker of Ca_v2.1 channel), r-Kurtosin (selective blocker of Ca_v3.1), and E-4031 (selective blocker of HERG K⁺ channel) were purchased from Alomone Labs.

Gene expression microarray

Gene expression microarray was performed with total RNA (5 µg/group) at the University of Kentucky DNA Microarray Core Facility using the Affymetrix Mouse 430 2.0 oligonucleotide array set (Affymetrix, Santa Clara, CA, USA). Briefly, the total RNA was extracted from the pituitaries of mice in six groups: naturally cycling WT mice in either metestrus (group 1) or proestrus (group 2), OVX WT mice treated with vehicle (group 3) or E₂ (group 4), and OVX ER α KO mice treated with vehicle (group 5) or E₂ (group 6). Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Inc.) and purified using an RNeasy Kit (Qiagen, Inc.). The integrity of RNA was checked by visualizing 28S and 18S rRNA bands on a 1.5% agarose gel. For each group, total RNA extracted from at least three different mice were pooled together for microarray. Completely different sets of mice were used for generating triplicate samples. Subsequently, microarray was performed for *n* = 2 samples. RNA was labeled and hybridized according to the standard Affymetrix procedures. Data were prestatistically filtered as reported previously (Kadish *et al.* 2009). Briefly, the MAS5 algorithm was used to generate quality

control metrics, produce presence/absence calls, and calculate signal intensity values. Results were transferred to Excel spreadsheets, filtered to remove genes rated absent (>2 presence calls across the study), and statistically analyzed (see section below) using the Multi-Experiment Viewer (Saeed *et al.* 2003).

RT-PCR

The gene expression patterns of selected genes were confirmed by real-time RT-PCR analysis using the total RNA (1 μ g/group) used for microarray. The primers used were as follows: AEBP1, forward (5'-AGA CAC ACC CTT CCC AAA TG-3') and reverse (5'-GTG GGC ATC TCA GTC TCC TC-3'); Ca_v2.1, forward (5'-AGG CAC CCT TTT GAT GGA G-3') and reverse (5'-GCG GAT GTA GAA ACG CAT TC-3'; Xu *et al.* 2007); Ca_v3.1, forward (5'-TGC TGT GGA AAT GGT GGT GA-3') and reverse (5'-AGC ATC CCA GCA ATG ACG AT-3'; Nordskog *et al.* 2006); KCNE2, forward (5'-GCA TGT TCT CGT TCA TCG TG-3') and reverse (5'-CCT TGG AGT CTT CCA GAT GC-3'); Cited1, forward (5'-CAT CCT TCA ACC TGC ATC CT-3') and reverse (5'-ACC AGC AGG AGG AGA GAC AG-3'; Howlin *et al.* 2006); Opn3, forward (5'-TCT TCA TGA ACA GAA AGT TTC G-3') and reverse (5'-CCT GTC CCC ATC TTT CTG TGA C-3'; Henkel *et al.* 2006); and L7 ribosomal protein, forward (5'-TCA ATG GAG TAA GCC CAA AG-3') and reverse (5'-CAA GAG ACC GAG CAA TCA AG-3'; Jeong *et al.* 2005). The L7 ribosomal protein gene was used as internal control. Real-time RT-PCR was performed using SyberGreen Master Mix (Ambion, Austin, TX, USA), and all of the triplicate samples that were originally prepared were used for each gene. C_t values used were each automatically generated by PCR machine software (Bio-Rad iQ5, version 2.0). The relative mRNA amount (RMA) was calculated by the following equation: $RMA = 1000 \times 2^{(-\Delta\Delta C_t)}$, $\Delta\Delta C_t = \Delta C_t$ (target gene) $-\Delta C_t$ (internal L7 control; Livak & Schmittgen 2001).

Immunohistological analysis

For all immunohistological analyses, tissues were fixed and processed as described previously (Kim *et al.* 2005). Metestrus WT, proestrus WT, and diestrus ER α KO pituitary sections were mounted on the same slide for procedural control purposes. Tissue sections were incubated with 5% normal serum for 30 min at room temperature followed by incubation with specific antibody overnight at 4 °C. Immunopositive signals were then visualized by ABC method (Vector, Burlingame, CA, USA) and hematoxylin counterstaining was used on all slides except that for Cited1 (as this is a nuclear protein). Densitometric analysis was performed using the analyzeSIS TS, OLYMPUS Soft Imaging Solutions Software (Münster, Germany). Relative signal intensities were calculated and proestrus and ER α KO sections were standardized to metestrus sections, which were given a relative intensity

of 1 ($n=4$ for each gene). For double-immunofluorescent detection, sections were blocked by 10% normal serum (5% normal goat + 5% normal horse serum) and then anti-LH β (1:500) and specific antibody against each of the selected gene products (1:50 for Ca_v2.1, Ca_v3.1, Kcne2, and AEBP1; 1:100 for Opn3; and 1:300 for Cited1) were co-treated overnight at 4 °C. The Alexa Fluor 488-conjugated anti-rabbit IgG and the Alexa Fluor 594-conjugated anti-guinea pig IgG (both at 1:500) were incubated to detect each selected gene and LH signal respectively. After washing with distilled water, sections were mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes, Eugene, Oregon, USA). Photographs were taken using a fluorescent microscope (Olympus) and a digital camera (DP70, Olympus). The proportions of double-positive signals were calculated as described previously (Kim *et al.* 2007).

Cell cultures and treatment for LH assay

Anterior pituitary lobes were dissected from whole pituitaries of 10-week-old female C57BL/6 mice after carbon dioxide inhalation. Pituitaries were pooled, cells were isolated, and then maintained as described previously (Kim *et al.* 2007). For assessment of the effect of specific channel blockers on LH secretion, primary pituitary cells were counted and plated (1×10^5 cells/well) in 96-well plates coated with poly-L-lysine. After 2 days of culture, incubation media were exchanged for medium supplemented with 10% charcoal-treated fetal bovine serum and cultured for an additional 2 days. The cells were then treated with either 0.00001% ethanol or 1 nM E₂ in 0.00001% ethanol for 48 h. Cells were then treated with ω -Agatoxin TK (50 and 200 nM), r-Kurtotoxin (50 and 200 nM), and E-4031 (100 and 1000 nM) for 30 min followed by GnRH (10 nM) challenge in the presence of the blockers for an additional 2 h. Media were snap-frozen and stored at -80 °C until assay. RIA of LH concentration was performed using a mouse LH sandwich assay by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (NICHD (SCCPRR) Grant U54-HD28934, University of Virginia, VA, USA). Channel blocker concentrations were chosen based on the Alomone company assay for E-4031, and cited literature for ω -Agatoxin (Barral *et al.* 2001) and r-Kurtotoxin (Chuang *et al.* 1998), and slight variations on these concentrations.

Statistical analysis

For densitometry and RIA data analyses, data were analyzed using one-way ANOVA and the Student–Newman–Keuls method or *t*-test. Microarray data were analyzed using one-way ANOVA to test each comparison group individually (metestrus versus proestrus, WT OVX veh versus E₂, and ER α KO OVX veh versus E₂). The probe sets were then standardized to maintain variability shifted about zero. Those probe sets that were found significant with ANOVA ($P<0.05$) were compared in a *post hoc* all-pairwise strategy

Table 1 Estradiol (E₂)/estrogen receptor α -regulated genes (upregulation)

Gene	Gene title	Pro/Met	WT E ₂ /veh	KO E ₂ /veh
<i>Abcb6</i>	ATP-binding cassette, sub-family B (MDR/TAP), member 6	1·61	2·30	0·92
<i>Accn1</i>	Amiloride-sensitive cation channel 1, neuronal (degenerin)	2·14	5·73	0·85
<i>Adam12</i>	A disintegrin and metallopeptidase domain 12 (meltrin alpha)	3·62	13·81	1·56
<i>Aebp1*</i>	AE binding protein 1	3·60	9·41	1·21
<i>Ammecr1</i>	Alport syndrome, mental retardation, midface hypoplasia and elliptocytosis chromosomal region gene 1 homolog (human)	1·83	2·89	1·48
<i>Amn</i>	Amnionless	30·38	25·42	0·60
<i>Ank1</i>	Ankyrin 1, erythroid	1·64	7·14	0·84
<i>Arhgap24</i>	Rho GTPase activating protein 24	1·66	2·56	0·96
<i>Asah1</i>	N-acylsphingosine amidohydrolase (acid ceramidase)-like	1·47	1·61	0·96
<i>Asns</i>	Asparagine synthetase	1·19	1·50	1·00
<i>Bcan</i>	Brevican	1·46	1·72	1·03
<i>Cacna1a**</i>	Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	2·01	3·60	0·95
<i>Cacna1g*</i>	Calcium channel, voltage-dependent, T type, alpha 1G subunit	1·89	7·74	0·66
<i>Ccdc123</i>	Coiled-coil domain containing 123	1·70	2·52	1·26
<i>Cckar</i>	Cholecystokinin A receptor	5·82	126·33	0·75
<i>Cgref1</i>	Cell growth regulator with EF hand domain 1	2·09	1·49	0·85
<i>Cited1**</i>	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	3·13	15·94	1·40
<i>Dbh</i>	Dopamine beta hydroxylase	1·62	3·03	1·22
<i>Fbxo31</i>	F-box protein 31	1·57	2·14	0·95
<i>Fbxw7</i>	F-box and WD-40 domain protein 7, archipelago homolog (<i>Drosophila</i>)	1·50	1·66	0·78
<i>G6pd2</i>	Glucose-6-phosphate dehydrogenase 2	1·55	3·87	1·19
<i>G6pdx</i>	Glucose-6-phosphate dehydrogenase X-linked	1·55	2·93	0·95
<i>Gadd45g</i>	Growth arrest and DNA-damage-inducible 45 gamma	2·56	4·52	0·95
<i>Gcs1</i>	Glucosidase 1	1·41	2·27	1·10
<i>Ggt1a1</i>	Gamma-glutamyltransferase-like activity 1	3·81	3·42	1·13
<i>Hif1a</i>	Hypoxia inducible factor 1, alpha subunit	1·23	1·74	1·02
<i>Hnt</i>	Neurotrimin	1·34	2·40	0·98
<i>Hspb8</i>	Heat-shock protein 8	2·14	2·05	0·96
<i>Immt</i>	Inner membrane protein, mitochondrial	1·20	1·18	1·09
<i>Impdh1</i>	Inosine 5'-phosphate dehydrogenase 1	1·65	1·93	0·83
<i>Impg1</i>	Interphotoreceptor matrix proteoglycan 1	2·13	25·50	1·71
<i>Isg20l1</i>	Interferon stimulated exonuclease gene 20-like 1	1·26	1·28	0·99
<i>Jtv1</i>	JTV1 gene	1·35	1·56	0·94
<i>Kcne2**</i>	Potassium voltage-gated channel, Isk-related subfamily, gene 2	3·97	44·10	0·56
<i>Lamb3</i>	Laminin, beta 3	2·34	5·35	1·15
<i>Lancl3</i>	LanC lantibiotic synthetase component C-like 3 (bacterial)	1·80	2·08	0·80
<i>Mars</i>	Methionine-tRNA synthetase	1·37	1·67	1·07
<i>Mcm2</i>	Minichromosome maintenance deficient 2 mitotin (<i>Saccharomyces cerevisiae</i>)	1·26	2·15	0·96
<i>Mesdc2</i>	Mesoderm development candidate 2	1·28	1·46	1·01
<i>Mife8</i>	Milk fat globule-EGF factor 8 protein	1·36	1·81	1·04
<i>Myc</i>	Myelocytomatosis oncogene	2·60	6·75	1·11
<i>Nhlh2</i>	Nescient helix loop helix 2	3·10	3·92	1·06
<i>Nol5a</i>	Nucleolar protein 5A	1·87	2·60	1·12
<i>Nomo1</i>	Nodal modulator 1	1·31	1·41	1·03
<i>Nudt19</i>	Nudix (nucleoside diphosphate linked moiety X)-type motif 19	1·81	2·84	0·88
<i>Opn3*</i>	Opsin (encephalopsin)	2·01	29·49	0·89
<i>Oxtr</i>	Oxytocin receptor	3·03	7·17	1·13
<i>Pcsk6</i>	Proprotein convertase subtilisin/kexin type 6	1·81	3·49	0·99
<i>Peo1</i>	Progressive external ophthalmoplegia 1 (human)	1·52	1·57	1·04
<i>Perp</i>	PERP, TP53 apoptosis effector	1·96	2·88	0·91
<i>Pgm2</i>	Phosphoglucomutase 2	1·38	1·56	1·05
<i>Phyhl1</i>	Phytanoyl-CoA hydroxylase interacting protein-like	1·24	1·82	1·18
<i>Plod1</i>	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1	1·54	2·33	1·10
<i>Prmt3</i>	Protein arginine N-methyltransferase 3	1·41	1·43	0·98
<i>Ptpn5</i>	Protein tyrosine phosphatase, non-receptor type 5	4·04	25·08	0·30
<i>Rnd2</i>	Rho family GTPase 2	1·45	2·19	0·94
<i>Rybp</i>	RING1 and YY1 binding protein	1·30	1·90	1·10
<i>Scnn1g</i>	Sodium channel, nonvoltage-gated 1 gamma	2·75	3·16	0·55
<i>Slc10a3</i>	Solute carrier family 10 (sodium/bile acid cotransporter family), member 3	1·26	1·55	1·18
<i>Srebf1</i>	Sterol regulatory element binding factor 1	1·32	1·54	0·98
<i>Stat5a</i>	Signal transducer and activator of transcription 5A	2·47	5·19	1·10
<i>Tbl3</i>	Transducin (beta)-like 3	1·50	1·62	1·24
<i>Tcam1</i>	Testicular cell adhesion molecule 1	2·72	4·09	0·17
<i>Tmem86a</i>	Transmembrane protein 86A	1·81	3·01	0·88
<i>Zfp804a</i>	Zinc finger protein 804A	3·07	6·92	1·20

Asterisks (*,**) indicate genes chosen for further study (see text).

with Fisher's protected least significant difference (pLSD). Stringency in the pLSD analysis was tuned until 90% of genes found significant by ANOVA yielded at least one significant contrast between treatments. Real-time PCR data were compared using *t*-test. In consideration of small sample size ($n=2$) for each microarray group, the presented S.E.M. should be given less weight than others of samples sizes larger than $n=3$.

Results

Identification of estrogen/ER α -regulated genes in the pituitary

To identify the genes that are under the regulation of estrogen via ER α , microarray analyses were performed using pituitaries collected from six different experimental groups. Pituitaries were collected from naturally cycling WT mice on the afternoons (1500 h) of metestrus (low circulating estrogen levels; group 1) and proestrus (high circulating estrogen levels; group 2). Pituitaries were also collected from OVX WT mice following treatment with vehicle (group 3) or E₂ (group 4), as well as OVX ER α KO mice following treatment with vehicle (group 5) or E₂ (group 6). A gene was determined to be E₂/ER α regulated if its pattern of expression met all of the following criteria: 1) significantly higher or lower expression on proestrus compared with metestrus, 2) significantly higher or lower expression in E₂-treated group compared with veh-treated group in the OVX WT mice, and 3) no significant difference in expression between E₂-treated and veh-treated OVX ER α KO mice. Statistical analysis of the raw microarray data, as described above, revealed 64 E₂-inducible/ER α -dependent genes (Table 1) and 17 E₂-repressible/ER α -dependent genes (Table 2).

Further characterization of microarray-identified ER α -inducible genes

The validity of the microarray and statistical analyses was evaluated using six genes. From the final list, we chose three genes (Table 1): *Cacna1g* (encodes protein Ca_v3.1; voltage-dependent calcium channel, T type, alpha 1G subunit), *Aebp1*, and *Opn3*. These three genes (marked with asterisk in Table 1) were selected on the basis of increased gene expression with high-estrogen environments found significant by the previously stated statistical analyses. In addition, selection of these genes was supported by the known function of their encoded proteins in other tissues. *Cacna1g* was chosen on the basis of its well-established regulation of ion transport, which has been thought to be an important regulatory mechanism in release of hormones and neurotransmitters (Douglas & Rubin 1961, Stojilkovic *et al.* 2005, Qiu *et al.* 2006, Stojilkovic 2006), and has previously been reported to be under estrogenic regulation in the pituitary (Bosch *et al.* 2009). *Aebp1* has been shown to be estrogen/ER α -regulated in other tissues (Zhang *et al.* 2005). The *Aebp1*-encoded protein is also an augments of MAPK function, which is significant considering that MAPK family downstream regulators are known to be activated by GnRH (Kim *et al.* 2001, Navratil *et al.* 2010). *Opn3* was chosen because of its known role in exocytosis in other cell types (Henkel *et al.* 2006).

Interestingly, we found a large number of genes that, while significantly different within treatment groups by ANOVA (metestrus versus proestrus, veh versus E₂), were not found to be significant by our *post hoc* analysis among the groups. We reasoned that the basal level of estrogen in metestrus in the cycling mice would be enough to induce or maintain the expression of some estrogen-inducible genes at relatively higher levels, which might reduce the gene expression differential between metestrus and proestrus. Therefore, the fold changes shown in the naturally cycling pituitaries, while significant

Table 2 Estradiol (E₂)/estrogen receptor α -regulated genes (downregulation)

Gene	Gene title	Pro/Met	WT E ₂ /veh	KO E ₂ /veh
<i>Caps2</i>	Calcyphosphine 2	0.54	0.31	0.99
<i>Chrna6</i>	Cholinergic receptor, nicotinic, alpha polypeptide 6	0.36	0.18	1.00
<i>Ctsf</i>	Cathepsin F	0.78	0.80	1.10
<i>Cyp39a1</i>	Cytochrome P450, family 39, subfamily a, polypeptide 1	0.67	0.74	0.91
<i>Fbp2</i>	Fructose biphosphatase 2	0.50	0.46	0.97
<i>Gla2</i>	Glycine receptor, alpha 2 subunit	0.63	0.27	0.92
<i>Gpm6a</i>	Glycoprotein m6a	0.71	0.58	0.90
<i>Hmgcll1</i>	3-Hydroxymethyl-3-methylglutaryl-coenzyme A lyase-like 1	0.77	0.72	0.97
<i>Oasl2</i>	2',5'-Oligoadenylate synthetase-like 2	0.65	0.62	1.17
<i>Ociad2</i>	OCIA domain containing 2	0.76	0.66	0.92
<i>Rod1</i>	ROD1 regulator of differentiation 1 (<i>Schizosaccharomyces pombe</i>)	0.82	0.72	1.03
<i>St8sia2</i>	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2	0.60	0.56	1.04
<i>Tacstd1</i>	Tumor-associated calcium signal transducer 1	0.82	0.74	1.00
<i>Tmem51</i>	Transmembrane protein 51	0.63	0.43	0.88
<i>Tmhs</i>	Tetraspan transmembrane protein, hair cell stereocilia	0.75	0.60	0.97
<i>Trdmt1</i>	tRNA aspartic acid methyltransferase 1	0.85	0.84	1.01
<i>Vangl1</i>	Vang-like 1 (van gogh, <i>Drosophila</i>)	0.70	0.66	0.95

themselves by ANOVA between metestrus and proestrus, were relatively low compared with the higher fold changes seen in the OVX group, thus eliminating them from the list of genes that were found significant with *post hoc* analysis. Among the list of the genes in this category, we selected three (marked with

double asterisk in Table 1) for further characterization: *Cited1*, *Cacna1a* (encodes protein Ca_v2.1; voltage-dependent calcium channel, P/Q type, α 1A subunit), and *Kcne2*. *Cited1* was chosen because of its well-established role as a mediator of ER action (Yahata *et al.* 2001). *Cacna1a* and *Kcne2*, as with *Cacna1g*, were chosen because of their known roles as ion transporters.

The expression levels of the six genes from the microarray analysis are shown in Fig. 1a. Real-time RT-PCR assay using RNA extracted from metestrus, proestrus, OVX veh, and OVX E₂ pituitaries (Fig. 1b) shows gene expression patterns closely resembling the microarray data for all six genes. For the scope of this study, only E₂/ER α -inducible genes were chosen for further evaluation. Additional characterization of the significant E₂/ER α -repressible genes (Table 2) will be intriguing topics for future investigation.

Localization of ER α -regulated genes in the gonadotroph

In order to examine whether protein expression of these genes correlated with their mRNA expression pattern, immunohistochemistry was performed on pituitary sections from metestrus and proestrus females, and ER α KO females constitutively displaying diestrus vaginal cytology (Fig. 2). All six proteins showed a significant increase in positive staining in proestrus sections compared with their respective metestrus and ER α KO sections, as determined by densitometric analysis (Fig. 2, right panels). All proteins are located to cytoplasmic compartments, with the exception of *Cited1*, which is mostly nuclear. To investigate whether these proteins are localized in gonadotrophs, further analysis was performed using double immunofluorescence on proestrus pituitaries with LH β antibody and antibody against each specific protein (Fig. 3). Each of the six proteins was found to be localized in the LH β containing cells. Interestingly, the proteins were not homogeneously expressed in all gonadotrophs, but rather in a subset of LH β -positive cells. Likewise, protein expression of the six genes was detected in cell types other than gonadotroph. Quantification of the percent co-localization indicated that 85% of LH β -positive gonadotrophs also show *Cited1* expression, approximately half stain positive for Ca_v2.1, *Kcne2*, AEBP1, and *Opn3*, and about 34% show positive signals for Ca_v3.1 (Fig. 3).

Functional validation of ER α -regulated genes in the primary pituitary cell culture

Validity of LH secretion being influenced by the expression of these ER α -regulated genes in pituitary cells was further tested using the three channel proteins (Ca_v2.1, Ca_v3.1, and *Kcne2*) as subjects of functional confirmation. Cells dissected from anterior pituitary were pre-treated with E₂ for 48 h, and the efficacy of GnRH-induced LH release was measured in the presence or absence of specific blockers of the chosen channel proteins. ω -Agatoxin TK was used to selectively block Ca_v2.1 (P/Q type) channels (Teramoto *et al.* 1993), r-Kurtoxin to

Figure 1 mRNA expression profiles of estrogen/ER α -regulated genes. Six of the E₂-upregulated/ER α -dependent genes were chosen for further confirmation and study. Gene expression profiles of the microarray data are shown for each gene (a). Gene expression profiles were compared by ANOVA within treatment groups for WT metestrus (met) and proestrus (pro) mice, as well as OVX WT and OVX ER α KO, each treated with vehicle or E₂. Data presented as mean $\pm$ S.E.M. ($n=2$; * $P\leq 0.05$). RNA expression was confirmed by real-time RT-PCR analysis for six selected genes (b). The y-axis represents the range of signal intensity detected by DNA microarray. Total RNA were extracted from WT Pro and Met, WT OVX with E₂- or veh-treated pituitaries. Data presented were mean $\pm$ S.E.M. ($n=3$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$).

Figure 2 Immunohistochemical analysis of six selected genes using pituitaries from wild-type metestrus, proestrus, and ER α KO mice. Tissue sections from WT metestrus, WT proestrus, and ER α KO (diestrus) pituitaries were sectioned and incubated with antibodies against each specified protein and immunopositive signals were detected by ABC method. Scale bar is 25 μ m. The right panel shows relative density of each protein on metestrus (Met), proestrus (Pro), and ER α KO pituitaries, which was calculated by dividing mean density of proestrus and ER α KO by mean value of metestrus. Data presented were mean $\pm$ S.E.M. ($n=4$). All the relative density values from proestrus of each protein are statistically significant compared with those from metestrus and ER α KO ($*P < 0.05$).

block Ca $_v$ 3.1 (T-type) channels (Chuang *et al.* 1998), and E-4031 to selectively block HERG K $^+$ channels, with which KCNE2 is associated (Spector *et al.* 1996). Treatment with E $_2$ alone or with the channel blockers, without GnRH challenge, did not yield a change in basal secretion of LH (non-GnRH-induced secretion; Fig. 4). On GnRH stimulation, E $_2$ -treated cells produced significantly larger amount of LH (18%) compared with the control group (Fig. 5). Channel blockers at concentrations of 200 nM ω -Agatoxin, 50 nM r-Kurtoxin, and 1000 nM E-4031 showed complete negation of the priming effect of E $_2$ on GnRH-stimulated LH secretion, whereas other concentrations of the blockers showed no significant difference from E $_2$ + GnRH-treated cells.

Discussion

The aim of this study was to examine genome-wide pituitary gene expression profiles in order to decipher the molecular networks involved in the process of estrogen priming in the

pituitary prior to the LH surge. Preceding the surge period, it is well known that the gonadotrophs exhibit increased sensitivity to GnRH and maintain capacity to release a comparable amount of LH on each GnRH stimulus for an extended period (Gallo 1981, van Dielen & de Koning 1995, Hoeger *et al.* 1999). There is evidence that estrogen plays a role in the increased gonadotroph responsiveness to GnRH (Tilbrook *et al.* 1995, Clarke 2002). In regard to the mechanism, E $_2$ -induced increase in GnRH-R expression has been postulated as a key event (Liu & Yen 1983, Leung & Peng 1996, Strauss & Barbieri 2009). Estrogen facilitates redistribution of secretory granules, positioning them to be readily secreted on GnRH stimulation (Thomas & Clarke 1997, Thomas *et al.* 1998). ER α has been implicated as the major mediator of this E $_2$ action in the pituitary. The ER α agonist PPT elicits increased LH secretion from rat pituitaries in response to consecutive GnRH challenges *in vitro*, comparable to that induced by E $_2$ (Sanchez-Criado *et al.* 2004). Others and ourselves recently generated mouse models that lack functional ER α in the gonadotrophs (ER $\alpha^{\text{lox/lox}}$ α GSU $^{\text{Cre}}$ mouse; Gieske *et al.* 2008, Singh *et al.* 2009).

Figure 3 Localization of estrogen/ER α -regulated genes in the gonadotroph. Detection of the six selected genes in gonadotrophs in proestrous pituitary was achieved using immunofluorescence with antibodies against each gene and LH β . Green color represents each selected gene as detected by each specific antibody and red color represents LH β immunopositive signals. Yellow color, further indicated by white arrows, represents double-positive signaling. Numbers in parentheses indicate the proportion of gonadotrophs that stain double positive for the indicated gene (mean % $\pm$ S.E.M., $n=4$). Scale bar = 200 μ m.

Based on the infertility and irregular estrous cycles observed in these mice, it was hypothesized that gonadotroph ER α is necessary for the positive feedback action of estrogen. Interestingly, the absence of gonadotroph ER α did not affect

the mRNA expression of LH β , α GSU, or FSH β (Gieske *et al.* 2008). Therefore, estrogen/ER α may rather regulate other cellular processes enhancing the responsiveness of proestrous gonadotrophs to GnRH, presumably by regulating a cohort of ER α downstream genes (Hoeger *et al.* 1999, Turgeon & Waring 2001).

In this study, we identified estrogen-responsive/ER α -dependent genes in the pituitary (Tables 1 and 2) and suggest that those genes may play a role in the sensitization of the pituitary to GnRH stimuli during the surge period. The localization of these proteins in the proestrous gonadotrophs (Fig. 3), in combination with their increased expression at proestrus compared with metestrus (Fig. 2), implicates putative functional roles of those proteins in gonadotroph function and LH secretion. However, our data also show expression of the selected genes in pituitary cell types other than gonadotroph, suggesting that E₂/ER α regulation of these genes in other cell types may play indirect roles on gonadotroph function and LH secretion, or that their expression may influence other pituitary functions. The anterior pituitary is a complex organ with a great degree of heterogeneity in the physiology of the cell types that reside there. The intricacy of paracrine interactions between these cell types is only beginning to be teased apart. A growing volume of evidence indicates that pituitary cells form extensive networks for long-distance communication and coordination (Fauquier *et al.* 2002, Bonnefont *et al.* 2005). Estrogen facilitates this organization and interaction between cells prior to and during the LH surge, even after GnRH levels have fallen (Lyles *et al.* 2010). An apparent connectivity exists between gonadotrophs and lactotroph cells, exhibited by the presence of adherent and gap junctions (Horvath *et al.* 1977, Morand *et al.* 1996). Prolactin (PRL), the hormone product of lactotroph cells, acts on gonadotrophs cells to modulate the secretion of LH (Cheung 1983, Hodson *et al.* 2010a,b). Lactotrophs are also estrogen sensitive, and gonadotrophs express PRL receptors (Henderson *et al.* 2008). This evidence further signifies that, in addition to their function in the gonadotroph cells, the genes presented in this study may further modify the LH surge through their actions in lactotroph physiology.

While diverse molecular events would be necessary to increase the sensitivity to GnRH and the secretion of LH from gonadotrophs, the regulation of membrane potential via ion conductivity has been expected to be a key regulatory mechanism (Stojilkovic *et al.* 2005, Qiu *et al.* 2006, Stojilkovic 2006). In addition, the regulation of ion transport through plasma membrane or intracellular organelles is critical for exocytosis. The GnRH-R activates the PKA pathway resulting in release of calcium from intracellular stores (Hamid *et al.* 2008). Mobilization of Ca²⁺ is mediated through PKC pathways, including Gq/G₁₁ and phospholipase C β (PLC β) activation. Activation of Gq/G₁₁ and PLC β lead to the production of inositol 1,4,5-trisphosphate and diacylglycerol second messengers, which results in calcium

Figure 4 The effect of three ion channel blockers on LH secretion without GnRH challenge. Primary pituitary cells were incubated with estrogen (1 nM) or vehicle and in the presence of ion channel blockers ω -Agatoxin TK (50 or 200 nM), r-Kurtotoxin (50 or 200 nM), and E-4031 (100 or 1000 nM) to determine whether channel blockers had an effect on basal LH secretion (non-GnRH-induced secretion) into the media. Data were represented by mean $\pm$ S.E.M. ($n=4$).

ion (Ca^{2+}) mobilization and gonadotropin release (Naor 1990, Clarke 1995b, Shacham *et al.* 2001). The biphasic LH secretion is initially dependent on intracellular calcium, while the subsequent plateau phase relies on extracellular calcium influx (Ortmann *et al.* 1994, 1995). These events may also be pertinent to the secretion of PRL from neighboring lactotrophs and thus the regulation of PRL on LH secretion. In this study, we identified three channel components as ER α -dependent estrogen-inducible genes in the proestrus pituitary. *Cacna1a* encodes a subunit for $\text{Ca}_v2.1$, a P/Q type Ca^{2+} channel, which is known to mediate neurotransmitter release via Ca^{2+} -dependent excitation secretion coupling at many central synapses and at the peripheral neuromuscular junction (Uchitel *et al.* 1992). In the pituitary, GnRH triggers action potentials. In this process, the induction of $\text{Ca}_v2.1$ via estrogen may contribute to subsequent Ca^{2+} -mediated LH secretion. In order to maintain the pulsatility of LH secretion during the surge, a quick recovery of membrane potential through repolarization is needed, which can be manifested by increasing K^+ currents or by increasing other types of Ca^{2+} channels such as transient (T)-type calcium channel (Costantin & Charles 2001). Thus, the identification of *Cacna1g*, a component for the T-type Ca^{2+} channel $\text{Ca}_v3.1$, and *Kcne2*, a component of K^+ current, as ER α -dependent estrogen-inducible genes in the pituitary is quite relevant. In particular, the recent finding of Ca^{2+} -activated K^+ channels as potential mediators of estrogen action in priming pituitary gonadotroph in preparation for the LH surge (Waring & Turgeon 2009) substantiates the identification of

these channel proteins as estrogen/ER α -inducible genes in the pituitary. Recent studies have also alluded to the role of estrogen in the regulation of T-type calcium channel subunits in the pituitary via an ER α predominant pathway (Bosch *et al.* 2009). To see whether these three ion channels could increase the responsiveness of estrogen-primed gonadotrophs to GnRH, individual channel function was blocked after estrogen priming and GnRH stimulus *in vitro*. All three channel blockers significantly impaired E₂ enhancement of GnRH-stimulated LH release (Fig. 5). Interestingly, in the case of r-Kurtotoxin, treatment with the lower concentration of the $\text{Ca}_v3.1$ -associated channel blocker provided the reduction in the priming effect, while the higher concentration did not show this effect. However, it is often seen in studies that with increasing concentrations of the administered molecule, the observed effects plateau off and sometimes reverse at higher concentrations as the binding sites or receptors become saturated and oversaturated (Trotta *et al.* 1980, Hyvelin *et al.* 2000). Although more detailed experiments will be required to verify their supposed electro-physiological roles in regulation of LH secretion by GnRH, these initial findings lay the groundwork for the involvement of all three channels in regulating LH secretion.

In addition, the known functions of AEBP1, Opn3, and Cited1 could be directly and/or indirectly related to the role of estrogen in priming the pituitary for GnRH stimulus. AEBP1, verified here as an estrogen/ER α -dependent gene in

Figure 5 The effect of three ion channel blockers on LH secretion with GnRH challenge. Primary pituitary cells were incubated with or without estrogen (1 nM) and in the presence of ion channel blockers ω -Agatoxin TK (50 or 200 nM), r-Kurtotoxin (50 or 200 nM), and E-4031 (100 or 1000 nM) to determine whether channel blockers had an effect on LH secretion (GnRH-induced secretion) into the media. Data were represented by mean $\pm$ S.E.M. ($n=4$). Statistics were calculated for treatments with the three channel blockers compared with E₂ + GnRH by ANOVA and Student–Newman–Keuls method (* $P<0.05$; ** $P<0.01$).

the proestrus pituitary (Fig. 2), and as previously shown in the white adipose tissue (Zhang *et al.* 2005), maintains the activation of MAPK by protecting it from the effects of a MAPK-specific phosphatase (Kim *et al.* 2001, Navratil *et al.* 2010). The signal pathways activated by GnRH-R include major members of the MAPK family (Yang *et al.* 2005, Dobkin-Bekman *et al.* 2006). Opn3 has been shown to be involved in light sensation and circadian rhythms, with its implicated function being light reception for light-induced exocytosis in the PC12 cells and primary embryonic telencephalon cells (Henkel *et al.* 2006). It is well established that the LH surge is closely related to photoperiod in experimental animals (Legan & Karsch 1975, Legan *et al.* 1975), suggesting a possibly similar role for this gene in the regulation of LH secretion. Cited1 has been shown to function as a transcriptional coregulator of estrogen in a manner dependent on ER ligand binding and its interaction with CBP/p300 transcriptional coactivator (Shioda *et al.* 1996, 1998, Yahata *et al.* 2000, 2001, Nair *et al.* 2001). While its role in the pituitary has yet to be studied in depth, the evidence presented in this study suggests that Cited1 may be a coregulator of ER α in this tissue as well. Based on the known functions of these three verified estrogen-inducible/ER α -dependent genes, at least two molecular events would be suggested as mechanisms of estrogen priming in the proestrus pituitary: the reinforcement of the signal transduction pathways from GnRH-R and the regulation of circadian homeostasis related to light–dark cycle. These events might be eventually responsible for increasing sensitivity, capacity, and synchronicity of the proestrous pituitary for the surge level of LH secretion. To this end, estrogen may regulate various genes, but the regulation is expected to have a temporal and spatial specificity (Coser *et al.* 2003), which could be manifested by a pituitary-specific ER α coregulator such as Cited1.

While we show here for the first time genome-wide information consisting of the genes regulated by E₂/ER α in the pituitary and suggest a few molecular mechanisms of estrogen priming of the pituitary for the LH surge, there is much work to be done to elucidate the estrogen priming mechanism. It must be taken into account that estrogen has been shown to cause cellular responses through both rapid, non-genomic action (involving the activation of growth factor receptors and G-protein-coupled receptors, initiating multiple downstream pathways) and the ‘classical’ genomic responses (involving ER acting as a ligand-activated transcription factor). Most hormones, including estrogen, are capable of simultaneously activating both of these mechanisms (Prossnitz & Maggiolini 2009). Therefore, further studies are necessary to elucidate the precise mechanisms of the regulation of these genes by estrogen. In addition, as demonstrated by cholecystokinin-type A receptor, recently reported as a mediator of estrogen priming (Kim *et al.* 2007), the identified genes in this study have various functions. To further verify these mechanisms, it will

be necessary to use various approaches including pituitary-specific gene knockout animal models for each candidate.

While not elaborated in this report, our microarray data showed that the expression of most of the genes that constitute the basic components of secretory machinery (e.g. microtubules, t-SNARE complex) are not influenced by E₂/ER α (data not shown). Whereas, we found that some intracellular transport molecules, such as ankyrin, are under E₂/ER α regulation in the mouse pituitary (Table 1). Interestingly, the microarray analysis found that mRNA expression of GnRH-R, a well-established E₂-regulated gene in the pituitary (Leung & Peng 1996), was significantly higher in the OVX E₂ pituitaries compared with the OVX veh. However, no such significant increase was shown between metestrous and proestrous mouse pituitaries. This finding indicates that while some genes are under E₂/ER α regulation, their expressions are also subject to regulation by other factors, which may render tighter temporal and spatial regulations of the genes under changing physiological conditions.

In conclusion, findings from this study suggest that preovulatory estrogen priming of pituitary is achieved at least in part by regulating the expression of critical components that potentiate gonadotrophs, in addition to other pituitary cells, to be fully responsive to GnRH stimulation for LH surge stimulation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by National Institutes of Health grants: 11R01HD052694 (C K) and P20 RR15592 (C K) and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0006200).

References

- Barral J, Poblette F, Mendoza E, Pineda JC, Galarraga E & Bargas J 2001 High-affinity inhibition of glutamate release from corticostriatal synapses by omega-agatoxin TK. *European Journal of Pharmacology* **430** 167–173. (doi:10.1016/S0014-2999(01)01388-7)
- Becker JB, Arnold AP, Berkley KJ, Blaustein JD, Eckel LA, Hampson E, Herman JP, Marts S, Sadée W, Steiner M *et al.* 2005 Strategies and methods for research on sex differences in brain and behavior. *Endocrinology* **146** 1650–1673. (doi:10.1210/en.2004-1142)
- Bonnefont X, Lacampagne A, Sanchez-Hormigo A, Fino E, Creff A, Mathieu MN, Smallwood S, Carmignac D, Fontanaud P, Travo P *et al.* 2005 Revealing the large-scale network organization of growth hormone-secreting cells. *PNAS* **102** 16880–16885. (doi:10.1073/pnas.0508202102)

- Bosch MA, Hou J, Fang Y, Kelly MJ & Ronnekleiv OK 2009 17Beta-estradiol regulation of the mRNA expression of T-type calcium channel subunits: role of estrogen receptor alpha and estrogen receptor beta. *Journal of Comparative Neurology* **512** 347–358. (doi:10.1002/cne.21901)
- Cheung CY 1983 Prolactin suppresses luteinizing hormone secretion and pituitary responsiveness to luteinizing hormone-releasing hormone by a direct action at the anterior pituitary. *Endocrinology* **113** 632–638. (doi:10.1210/endo-113-2-632)
- Christian CA, Mobley JL & Moenter SM 2005 Diurnal and estradiol-dependent changes in gonadotropin-releasing hormone neuron firing activity. *PNAS* **102** 15682–15687. (doi:10.1073/pnas.0504270102)
- Chuang RS, Jaffe H, Cribbs L, Perez-Reyes E & Swartz KJ 1998 Inhibition of T-type voltage-gated calcium channels by a new scorpion toxin. *Nature Neuroscience* **1** 668–674. (doi:10.1038/3669)
- Clarke IJ 1995a Evidence that the switch from negative to positive feedback at the level of the pituitary gland is an important timing event for the onset of the preovulatory surge in LH in the ewe. *Journal of Endocrinology* **145** 271–282. (doi:10.1677/joe.0.1450271)
- Clarke IJ 1995b The preovulatory LH surge: A case of a neuroendocrine switch. *Trends in Endocrinology and Metabolism* **6** 241–247. (doi:10.1016/1043-2760(95)95218-T)
- Clarke IJ 2002 Multifarious effects of estrogen on the pituitary gonadotrope with special emphasis on studies in the ovine species. *Archives of Physiology and Biochemistry* **110** 62–73. (doi:10.1076/apab.110.1.62.898)
- Clarke IJ & Cummins JT 1984 Direct pituitary effects of estrogen and progesterone on gonadotropin secretion in the ovariectomized ewe. *Neuroendocrinology* **39** 267–274. (doi:10.1159/000123990)
- Coser KR, Chesnes J, Hur J, Ray S, Isselbacher KJ & Shioda T 2003 Global analysis of ligand sensitivity of estrogen inducible and suppressible genes in MCF7/BUS breast cancer cells by DNA microarray. *PNAS* **100** 13994–13999. (doi:10.1073/pnas.2235866100)
- Costantin JL & Charles AC 2001 Modulation of Ca(2+) signaling by K(+) channels in a hypothalamic neuronal cell line (GT1-1). *Journal of Neurophysiology* **85** 295–304.
- DePasquale JA 1999 Rearrangement of the F-actin cytoskeleton in estradiol-treated MCF-7 breast carcinoma cells. *Histochemistry and Cell Biology* **112** 341–350. (doi:10.1007/s004180050415)
- van Dieten JA & de Koning J 1995 From basal luteinizing hormone (LH) concentrations to the pre-ovulatory LH surge: titration of the physiological effect of gonadotrophin surge-inhibiting/attenuating factor. *Human Reproduction* **10** 3110–3116.
- Dobkin-Bekman M, Naidich M, Pawson AJ, Millar RP, Seger R & Naor Z 2006 Activation of mitogen-activated protein kinase (MAPK) by GnRH is cell-context dependent. *Molecular and Cellular Endocrinology* **252** 184–190. (doi:10.1016/j.mce.2006.03.035)
- Douglas BW & Rubin RP 1961 Mechanism of nicotinic action at the adrenal medulla: calcium as a link in stimulus-secretion coupling. *Nature* **192** 1087–1089. (doi:10.1038/1921087b0)
- Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P & Mark M 2000 Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* **127** 4277–4291.
- Fauquier T, Lacampagne A, Travo P, Bauer K & Mollard P 2002 Hidden face of the anterior pituitary. *Trends in Endocrinology and Metabolism* **13** 304–309. (doi:10.1016/S1043-2760(02)00616-1)
- Gallo RV 1981 Pulsatile LH release during the ovulatory LH surge on proestrus in the rat. *Biology of Reproduction* **24** 100–104. (doi:10.1095/biolreprod24.1.100)
- Ghosh BR, Wu JC & Miller WL 1996 Gonadotropin-releasing hormone-stimulated calcium mobilization is altered in pituitary cultures from anestrus ewes. *Biology of Reproduction* **54** 753–760. (doi:10.1095/biolreprod54.4.753)
- Gieske MC, Kim HJ, Legan SJ, Koo Y, Krust A, Chambon P & Ko C 2008 Pituitary gonadotroph estrogen receptor-alpha is necessary for fertility in females. *Endocrinology* **149** 20–27. (doi:10.1210/en.2007-1084)
- Hamid T, Malik MT, Millar RP & Kakar SS 2008 Protein kinase A serves as a primary pathway in activation of Nur77 expression by gonadotropin-releasing hormone in the LbetaT2 mouse pituitary gonadotroph tumor cell line. *International Journal of Oncology* **33** 1055–1064. (doi:10.3892/ijo.00000094)
- Henderson HL, Townsend J & Tortones DJ 2008 Direct effects of prolactin and dopamine on the gonadotroph response to GnRH. *Journal of Endocrinology* **197** 343–350. (doi:10.1677/JOE-07-0536)
- Henkel AW, Upmann I, Bartl CR, Bonsch D, Reichardt C, Maler JM, Nurnberger M, Umstatter R, Reulbach U, Kornhuber J *et al.* 2006 Light-induced exocytosis in cell development and differentiation. *Journal of Cellular Biochemistry* **97** 1393–1406. (doi:10.1002/jcb.20751)
- Hewitt SC & Korach KS 2003 Oestrogen receptor knockout mice: roles for oestrogen receptors alpha and beta in reproductive tissues. *Reproduction* **125** 143–149. (doi:10.1530/rep.0.1250143)
- Hodson DJ, Townsend J, Gregory SJ, Walters C & Tortones DJ 2010a Role of prolactin in the gonadotroph responsiveness to gonadotrophin-releasing hormone during the equine annual reproductive cycle. *Journal of Neuroendocrinology* **22** 509–517. (doi:10.1111/j.1365-2826.2010.01986.x)
- Hodson DJ, Townsend J & Tortones DJ 2010b Characterization of the effects of prolactin in gonadotroph target cells. *Biology of Reproduction* **83** 1046–1055. (doi:10.1095/biolreprod.110.084947)
- Hoeger KM, Kolp LA, Strobl FJ & Veldhuis JD 1999 Evaluation of LH secretory dynamics during the rat proestrous LH surge. *American Journal of Physiology* **276** R219–R225.
- Horvath E, Kovacs K & Ezrin C 1977 Functional contacts between lactotrophs and gonadotrophs in the rat pituitary. *IRCS Medical Science* **5**.
- Howlin J, McBryan J, Napoletano S, Lambie T, McArdle E, Shioda T & Martin F 2006 CITED1 homozygous null mice display aberrant pubertal mammary ductal morphogenesis. *Oncogene* **25** 1532–1542. (doi:10.1038/sj.onc.1209183)
- Hyvelin JM, Martin C, Roux E, Marthan R & Savineau JP 2000 Human isolated bronchial smooth muscle contains functional ryanodine/cafeine-sensitive Ca-release channels. *American Journal of Respiratory and Critical Care Medicine* **162** 687–694.
- Jeong YJ, Choi HW, Shin HS, Cui XS, Kim NH, Gerton GL & Jun JH 2005 Optimization of real time RT-PCR methods for the analysis of gene expression in mouse eggs and preimplantation embryos. *Molecular Reproduction and Development* **71** 284–289. (doi:10.1002/mrd.20269)
- Jones ME, Boon WC, Proietto J & Simpson ER 2006 Of mice and men: the evolving phenotype of aromatase deficiency. *Trends in Endocrinology and Metabolism* **17** 55–64. (doi:10.1016/j.tem.2006.01.004)
- Kadish I, Thibault O, Blalock EM, Chen KC, Gant JC, Porter NM & Landfield PW 2009 Hippocampal and cognitive aging across the lifespan: a bioenergetic shift precedes and increased cholesterol trafficking parallels memory impairment. *Journal of Neuroscience* **29** 1805–1816. (doi:10.1523/JNEUROSCI.4599-08.2009)
- Kim SW, Muise AM, Lyons PJ & Ro HS 2001 Regulation of adipogenesis by a transcriptional repressor that modulates MAPK activation. *Journal of Biological Chemistry* **276** 10199–10206. (doi:10.1074/jbc.M010640200)
- Kim HJ, Sohn HJ, Ha M, Han JY, Kang SS, Choi WS & Cho GJ 2005 Prepubertal chronic ethanol administration alters TTF-1 and Oct-2 expression in the hypothalamus of female rats. *Brain Research. Molecular Brain Research* **136** 262–266. (doi:10.1016/j.molbrainres.2004.12.021)
- Kim HJ, Gieske MC, Hudgins S, Kim BG, Krust A, Chambon P & Ko C 2007 Estrogen receptor alpha-induced cholecystokinin type A receptor expression in the female mouse pituitary. *Journal of Endocrinology* **195** 393–405. (doi:10.1677/JOE-07-0358)
- Legan SJ & Karsch FJ 1975 A daily signal for the LH surge in the rat. *Endocrinology* **96** 57–62. (doi:10.1210/endo-96-1-57)
- Legan SJ, Coon GA & Karsch FJ 1975 Role of estrogen as initiator of daily LH surges in the ovariectomized rat. *Endocrinology* **96** 50–56. (doi:10.1210/endo-96-1-50)
- Leung PC & Peng C 1996 Gonadotropin-releasing hormone receptor: gene structure, expression and regulation. *Biological Signals* **5** 63–69. (doi:10.1159/000109175)
- Liu JH & Yen SS 1983 Induction of midcycle gonadotropin surge by ovarian steroids in women: a critical evaluation. *Journal of Clinical Endocrinology and Metabolism* **57** 797–802. (doi:10.1210/jcem-57-4-797)

- Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25** 402–408. (doi:10.1006/meth.2001.1262)
- Lyles D, Tien JH, McCobb DP & Zeeman ML 2010 Pituitary network connectivity as a mechanism for the luteinizing hormone surge. *Journal of Neuroendocrinology* **22** 1267–1278. (doi:10.1111/j.1365-2826.2010.02084.x)
- Mitchner NA, Garlick C & Ben-Jonathan N 1998 Cellular distribution and gene regulation of estrogen receptors alpha and beta in the rat pituitary gland. *Endocrinology* **139** 3976–3983. (doi:10.1210/en.139.9.3976)
- Morand I, Fonlupt P, Guerrier A, Trouillas J, Calle A, Remy C, Rousset B & Munari-Silem Y 1996 Cell-to-cell communication in the anterior pituitary: evidence for gap junction-mediated exchanges between endocrine cells and folliculostellate cells. *Endocrinology* **137** 3356–3367. (doi:10.1210/en.137.8.3356)
- Nair SS, Chaubal VA, Shioda T, Coser KR & Mojamdar M 2001 Over-expression of MSG1 transcriptional co-activator increases melanin in B16 melanoma cells: a possible role for MSG1 in melanogenesis. *Pigment Cell Research* **14** 206–209. (doi:10.1034/j.1600-0749.2001.140311.x)
- Naor Z 1990 Signal transduction mechanisms of Ca²⁺ mobilizing hormones: the case of gonadotropin-releasing hormone. *Endocrine Reviews* **11** 326–353. (doi:10.1210/edrv-11-2-326)
- Navratil AM, Bliss SP & Roberson MS 2010 Membrane rafts and GnRH receptor signaling. *Brain Research* **1364** 53–61. (doi:10.1016/j.brainres.2010.09.008)
- Nordskog BK, Hammarback JA & Godwin DW 2006 Diurnal gene expression patterns of T-type calcium channels and their modulation by ethanol. *Neuroscience* **141** 1365–1373. (doi:10.1016/j.neuroscience.2006.04.031)
- Ortmann O, Merelli F, Stojilkovic SS, Schulz KD, Emons G & Catt KJ 1994 Modulation of calcium signaling and LH secretion by progesterone in pituitary gonadotrophs and clonal pituitary cells. *Journal of Steroid Biochemistry and Molecular Biology* **48** 47–54. (doi:10.1016/0960-0760(94)90249-6)
- Ortmann O, Bakhit M, Bloh P, Schulz KD & Emons G 1995 Ovarian steroids modulate gonadotropin-releasing hormone-induced biphasic luteinizing hormone secretory responses and inositol phosphate accumulation in rat anterior pituitary cells and alpha T3-1 gonadotrophs. *Journal of Steroid Biochemistry and Molecular Biology* **54** 101–109. (doi:10.1016/0960-0760(95)00121-F)
- Powers CA 1986 Anterior pituitary glandular kallikrein: trypsin activation and estrogen regulation. *Molecular and Cellular Endocrinology* **46** 163–174. (doi:10.1016/0303-7207(86)90095-X)
- Prossnitz ER & Maggiolini M 2009 Mechanisms of estrogen signaling and gene expression via GPR30. *Molecular and Cellular Endocrinology* **308** 32–38. (doi:10.1016/j.mce.2009.03.026)
- Qiu J, Bosch MA, Jamali K, Xue C, Kelly MJ & Ronnekleiv OK 2006 Estrogen upregulates T-type calcium channels in the hypothalamus and pituitary. *Journal of Neuroscience* **26** 11072–11082. (doi:10.1523/JNEUROSCI.3229-06.2006)
- Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M *et al.* 2003 TM4: a free, open-source system for microarray data management and analysis. *BioTechniques* **34** 374–378.
- Sanchez-Criado JE, Martin De Las Mulas J, Bellido C, Tena-Sempere M, Aguilar R & Blanco A 2004 Biological role of pituitary estrogen receptors ERalpha and ERbeta on progesterone receptor expression and action and on gonadotropin and prolactin secretion in the rat. *Neuroendocrinology* **79** 247–258. (doi:10.1159/000079100)
- Sanchez-Criado JE, de Las Mulas JM, Bellido C, Aguilar R & Garrido-Gracia JC 2005 Gonadotrope oestrogen receptor-alpha and -beta and progesterone receptor immunoreactivity after ovariectomy and exposure to oestradiol benzoate, tamoxifen or raloxifene in the rat: correlation with LH secretion. *Journal of Endocrinology* **184** 59–68. (doi:10.1677/joe.1.05748)
- Sanchez-Criado JE, de Las Mulas JM, Bellido C, Navarro VM, Aguilar R, Garrido-Gracia JC, Malagon MM, Tena-Sempere M & Blanco A 2006 Gonadotropin-secreting cells in ovariectomized rats treated with different oestrogen receptor ligands: a modulatory role for ERbeta in the gonadotrope? *Journal of Endocrinology* **188** 167–177. (doi:10.1677/joe.1.06377)
- Sapino A, Pietribiasi F, Bussolati G & Marchisio PC 1986 Estrogen- and tamoxifen-induced rearrangement of cytoskeletal and adhesion structures in breast cancer MCF-7 cells. *Cancer Research* **46** 2526–2531.
- Shacham S, Harris D, Ben-Shlomo H, Cohen I, Bonfil D, Przedecki F, Lewy H, Ashkenazi IE, Seger R & Naor Z 2001 Mechanism of GnRH receptor signaling on gonadotropin release and gene expression in pituitary gonadotrophs. *Vitamins and Hormones* **63** 63–90.
- Shioda T, Fenner MH & Isselbacher KJ 1996 Msg1, a novel melanocyte-specific gene, encodes a nuclear protein and is associated with pigmentation. *PNAS* **93** 12298–12303. (doi:10.1073/pnas.93.22.12298)
- Shioda T, Lechleider RJ, Dunwoodie SL, Li H, Yahata T, de Caestecker MP, Fenner MH, Roberts AB & Isselbacher KJ 1998 Transcriptional activating activity of Smad4: roles of SMAD hetero-oligomerization and enhancement by an associating transactivator. *PNAS* **95** 9785–9790. (doi:10.1073/pnas.95.17.9785)
- Simpson E, Jones M, Misso M, Hewitt K, Hill R, Maffei L, Carani C & Boon WC 2005 Estrogen, a fundamental player in energy homeostasis. *Journal of Steroid Biochemistry and Molecular Biology* **95** 3–8. (doi:10.1016/j.jsmb.2005.04.018)
- Singh SP, Wolfe A, Ng Y, DiVall SA, Buggs C, Levine JE, Wondisford FE & Radovick S 2009 Impaired estrogen feedback and infertility in female mice with pituitary-specific deletion of estrogen receptor alpha (ESR1). *Biology of Reproduction* **81** 488–496. (doi:10.1095/biolreprod.108.075259)
- Smith PF, Frawley LS & Neill JD 1984 Detection of LH release from individual pituitary cells by the reverse hemolytic plaque assay: estrogen increases the fraction of gonadotropes responding to GnRH. *Endocrinology* **115** 2484–2486. (doi:10.1210/endo-115-6-2484)
- Spector PS, Curran ME, Keating MT & Sanguinetti MC 1996 Class III antiarrhythmic drugs block HERG, a human cardiac delayed rectifier K⁺ channel. Open-channel block by methanesulfonanilides. *Circulation Research* **78** 499–503.
- Stojilkovic SS 2006 Pituitary cell type-specific electrical activity, calcium signaling and secretion. *Biological Research* **39** 403–423. (doi:10.4067/S0716-97602006000300004)
- Stojilkovic SS, Zemkova H & Van Goor F 2005 Biophysical basis of pituitary cell type-specific Ca²⁺ signaling-secretion coupling. *Trends in Endocrinology and Metabolism* **16** 152–159. (doi:10.1016/j.tem.2005.03.003)
- Strauss J & Barbieri R 2009. *Neuroendocrine Control of Menstrual Cycle in Jaffe's Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management*, pp 139–154. Philadelphia: Saunders.
- Teramoto T, Kuwada M, Niidome T, Sawada K, Nishizawa Y & Katayama K 1993 A novel peptide from funnel web spider venom, omega-Aga-TK, selectively blocks, P-type calcium channels. *Biochemical and Biophysical Research Communications* **196** 134–140. (doi:10.1006/bbrc.1993.2225)
- Thomas SG & Clarke IJ 1997 The positive feedback action of estrogen mobilizes LH-containing, but not FSH-containing secretory granules in ovine gonadotrophs. *Endocrinology* **138** 1347–1350. (doi:10.1210/en.138.3.1347)
- Thomas SG, Takahashi M, Neill JD & Clarke IJ 1998 Components of the neuronal exocytotic machinery in the anterior pituitary of the ovariectomised ewe and the effects of oestrogen in gonadotropes as studied with confocal microscopy. *Neuroendocrinology* **67** 244–259. (doi:10.1159/000054320)
- Tilbrook AJ, Clarke IJ & de Kretser DM 1995 Human recombinant follistatin-288 suppresses plasma concentrations of follicle-stimulating hormone but is not a significant regulator of luteinizing hormone in castrated rams. *Biology of Reproduction* **53** 1353–1358. (doi:10.1095/biolreprod53.6.1353)
- Trotta EE, Freire GL & Godinho CS 1980 The mode of action of local anesthetics on the calcium pump of brain. *Journal of Pharmacology and Experimental Therapeutics* **214** 670–674.
- Turgeon JL & Waring DW 2001 Luteinizing hormone secretion from wild-type and progesterone receptor knockout mouse anterior pituitary cells. *Endocrinology* **142** 3108–3115. (doi:10.1210/en.142.7.3108)

- Uchitel OD, Protti DA, Sanchez V, Cherksey BD, Sugimori M & Llinas R 1992 P-type voltage-dependent calcium channel mediates presynaptic calcium influx and transmitter release in mammalian synapses. *PNAS* **89** 3330–3333. (doi:10.1073/pnas.89.8.3330)
- Waring DW & Turgeon JL 2009 Ca²⁺-activated K⁺ channels in gonadotropin-releasing hormone-stimulated mouse gonadotrophs. *Endocrinology* **150** 2264–2272. (doi:10.1210/en.2008-1442)
- Xu JH, Long L, Tang YC, Hu HT & Tang FR 2007 Ca(v)1.2, Ca(v)1.3, and Ca(v)2.1 in the mouse hippocampus during and after pilocarpine-induced status epilepticus. *Hippocampus* **17** 235–251. (doi:10.1002/hipo.20263)
- Yahata T, de Caestecker MP, Lechleider RJ, Andriole S, Roberts AB, Isselbacher KJ & Shioda T 2000 The MSG1 non-DNA-binding transactivator binds to the p300/CBP coactivators, enhancing their functional link to the Smad transcription factors. *Journal of Biological Chemistry* **275** 8825–8834. (doi:10.1074/jbc.275.12.8825)
- Yahata T, Shao W, Endoh H, Hur J, Coser KR, Sun H, Ueda Y, Kato S, Isselbacher KJ, Brown M *et al.* 2001 Selective coactivation of estrogen-dependent transcription by CITED1 CBP/p300-binding protein. *Genes and Development* **15** 2598–2612. (doi:10.1101/gad.906301)
- Yang D, Caraty A & Dupont J 2005 Molecular mechanisms involved in LH release by the ovine pituitary cells. *Domestic Animal Endocrinology* **29** 488–507. (doi:10.1016/j.domaniend.2005.02.015)
- Zhang L, Reidy SP, Nicholson TE, Lee HJ, Majdalawieh A, Webber C, Stewart BR, Dolphin P & Ro HS 2005 The role of AEBP1 in sex-specific diet-induced obesity. *Molecular Medicine* **11** 39–47. (doi:10.2119/2006-00021.Ro)

Received in final form 2 June 2011

Accepted 22 June 2011

Made available online as an Accepted Preprint

23 June 2011