Published in final edited form as:

J Exp Zool A Ecol Genet Physiol. 2015 November ; 323(9): 627–636. doi:10.1002/jez.1953.

Rapid changes in ovarian mRNA induced by brief photostimulation in Siberian hamsters (*Phodopus sungorus*)

Asha Shahed¹, Carling F. McMichael¹, and Kelly A. Young^{1,*}

¹Reproductive Biology Group, Department of Biological Sciences, California State University Long Beach, Long Beach, CA 90840

Abstract

This study sought to characterize the rapid intraovarian mRNA response of key folliculogenic factors that may contribute to the restoration of folliculogenesis during 2-10 days of photostimulation in Siberian hamsters. Adult hamsters were exposed to short photoperiod (8L:16D) for 14 weeks (SD). A subset were then transferred to long photoperiod (16L:8D) for 2(PT day-2), 4(PT day-4), or 10 days (PT day-10). Quantitative real-time PCR was used to measure intraovarian mRNA expression of: gonadotropin releasing hormone (GnRH), follicle stimulating hormone β -subunit (FSH β -subunit), luteinizing hormone β -subunit (LH β -subunit), FSH and LH receptors, estrogen receptors α and β (Esr1 and Esr2), matrix metalloproteinase (MMP)-2 and -9, anti-Müllerian hormone (AMH), inhibin- α subunit, fibroblast growth factor-2 (FGF-2) and proliferating cell nuclear antigen (PCNA). Compared to SD, plasma FSH concentrations increased on PT day-4 and the number of antral follicles and corpora lutea increased on PT day-10. FSHR and inhibin- α mRNA expression also increased on PT day-4, whereas LHR and proliferation marker PCNA both increased on PT day-10 as compared to SD. Esr1 mRNA increased on PT day-2 and remained significantly increased as compared to SD, whereas Esr2 mRNA increased only on PT day-2, similar to FGF-2 and MMP-2 results. No differences were observed in mRNA expression in ovarian GnRH, FSH β - and LH β -subunits, AMH, and MMP-9 mRNA with 2-10 days of photostimulation. Rapid increases in intraovarian FSHR and inhibin- α mRNA and antral follicle/corpora lutea numbers suggest that the ovary is primed to react quickly to the FSH released in response to brief periods of photostimulation.

Introduction

Most temperate vertebrates reproduce seasonally, with maximal reproductive function concomitant with times of the year when resources are abundant. Exposure to short day lengths (8 hours of light: 16 hours of dark per 24h period, SD) severely compromises reproductive function in Siberian hamsters (*Phodopus sungorus*) via reductions in ovarian mass, number of antral follicles, and number of corpora lutea (Schlatt et al., 93; Moffatt-Blue et al., 2006). Subsequent transfer to long day lengths (16L:8D, LD) initiates recrudescence of regressed ovaries; exposure to 2-8 weeks of long photoperiod restores ovarian mass and folliculogenesis (Salverson et al., 2008). While evidence of

*Correspondence to: Kelly A. Young, Ph.D., Department of Biological Sciences, California State University, Long Beach, Long Beach, California 90840-3702, Telephone: 562.985.4859, Telefax: 562.985.8878, kelly.young@csulb.edu.

ovulation is absent in photoregressed ovaries, corpora lutea appear at two weeks of photostimulation (Salverson et al., 2008), suggesting that intraovarian factors must be responding rapidly to the change in day length.

Recrudescence of photoregressed ovaries is a complex and progressive process that is stimulated centrally by reactivation of the hypothalamic pituitary gonadal (HPG) axis. The resurgence of follicle stimulating hormone (FSH) and luteinizing hormone (LH) promotes resumption of both folliculogenesis and steroidogenesis. In addition to this central regulation, low levels of GnRH-1 and gonadotropin (FSH β - and LH β -subunit) mRNA and proteins are present in Siberian hamster ovaries, and respond to changes in photoperiod length, although their local role in recrudescence is not known (Shahed and Young 2011). One result of restored gonadotropin stimulation is an increase in steroidogenesis; plasma concentrations of estradiol, which are reduced with SD exposure, increase with photostimulation (Salverson et al., 2008). Because both gonadotropins and estrogen are necessary for ovarian function (Uilenbroek and Richards, '97; Byers et al., '97), and FSH and estrogen receptors are present in golden hamster ovaries (Yang et al., 2002; Oxberry and Greenwald, '82; Yang et al., 2004; Roy et al., '87), early up-regulation of these receptors may be a critical factor in the photostimulated recrudescence process in Siberian hamsters.

Hormonal stimulation can induce a cascade of local ovarian events during recrudescence, including altering expression of members of the transforming growth factor β (TGF- β) superfamily, a group of proteins that are key in the recruitment, development and luteinization of follicles (reviewed by: Knight and Glister, 2006; Trombly et al., 2009; Myers and Pangas, 2010). Anti-Müllerian hormone (AMH), a member of the TGF- β Super family, regulates key aspects of the recruitment of primordial follicles, and AMH limitation of the transition into primary follicles is critical to maintaining the primordial follicle pool (Nilsson et al., 2011). AMH mRNA and protein levels are elevated in adult Siberian hamsters exposed to SD (Shahed and Young, 2013) or raised in short photoperiods since birth (Kabithe and Place, 2008; Place and Cruickshank, 2009). Because AMH mRNA expression remains elevated at 2 weeks post-transfer to LD photostimulation (Shahed and Young, 2013), AMH may be an early intraovarian target during recrudescence due to its influence on primordial activation. Inhibin, another member of the TGF- β superfamily, down regulates FSH secretion and inhibits progesterone production and is essential for normal ovarian cyclicity (reviewed by Knight and Glister, 2006). Short photoperiod exposure reduces inhibin- α subunit RNA as compared to cycling hamsters, a decrease that is reversed with two weeks after transfer to LD photostimulation (Shahed and Young 2013; Kenny et al., 2002). Because inhibin is critical for FSH regulation, it is possible that changes in inhibin- α may be occurring prior to the 2 weeks of photostimulation time point.

Regulators of folliculogenesis, including the members of the TGF- β superfamily, often target processes involving cellular proliferation and follicle expansion. Both proliferating cell nuclear antigen (PCNA) and fibroblast growth factor 2 (FGF-2) are used as markers for the onset of follicular growth in many species, including rodents (Oktay et al., '95). FGF-2, localized in growing follicles, is involved with stimulating granulosa cell mitosis and serves as a primordial follicle inducing factor (Nilsson et al., 2001). Indices of growth should return rapidly to photostimulated ovaries as folliculogenesis resumes. Follicular growth is often

coupled with expression of matrix metalloproteinases (MMPs), a family of Zn^{2+} -dependent endopeptidases that mediate remodeling of ovarian tissue (Curry and Osteen, 2003). MMP turnover of extracellular matrix is essential for follicle growth, ovulation, and corpus luteum formation, particularly by the gelatinases (MMP-2 and MMP-9) (reviewed by Smith et al., 2002). Because gelatinases are present in Siberian hamster ovaries, are influenced by weeks of photoperiod exposure (Salverson et al., 2008; Vrooman and Young, 2010), and have multifaceted roles in ovarian function, MMP-2 and MMP-9 may be among the intraovarian factors that are rapidly stimulated following brief photostimulation.

Photostimulated recrudescence of regressed ovaries is a complex process triggered centrally by resumption of gonadotropin release that induces coordinated changes in ovarian and follicular factors. However, the ovarian response to short periods (< 2 weeks) of photostimulation had not been investigated. Therefore, this study was designed as an initial examination of the changes that may occur within Siberian hamster ovaries following 2-10 days of photostimulation. We specifically hypothesized that mRNA expression of a variety of key ovarian factors (GnRH and gonadotropin β -subunits, gonadotropin and estrogen receptors, AMH, inhibin- α , FGF-2, MMP-2 and MMP-9, and proliferation marker PCNA) would change within days of photostimulation as compared to mRNA levels observed in photoregressed ovaries.

Methods

Animals

Adult, female Siberian hamsters obtained from our breeding colony were treated in compliance with California State University Long Beach and NRC guidelines for the use of laboratory animals, and under the requirements of approved CSULB IACUC protocol #237. All animals were housed in individual polypropylene cages prepared with bedding and tap water, and were given *ad libitum* access to food (a mixture of lab rodent diet 5001 and Mazuri Hamster and Gerbil Diet, both from Purina, Brentwood, MO). After two weeks of acclimation, hamsters (n=40; 10 per group) were exposed to short photoperiods (8L:16D) for 14 weeks. One group of hamsters was euthanized at the 14 weeks of SD exposure time point to serve as the control (SD group, regressed ovaries). The remaining hamsters were transferred to long days (16L:8D) for 2, 4, or 10 days, and were euthanized on the assigned day post-transfer (PT) to photostimulation (PT day 2-10, experimental groups of early recrudescing ovaries). At each collection time point, blood and ovaries were collected as previously described (Moffatt-Blue et al., 2006; Salverson et al., 2008). Briefly, following ketamine/xylazine intra-peritoneal anesthesia/analgesia, a retro-orbital blood sample was obtained, hamsters were euthanized via cervical dislocation, and ovaries were dissected during necropsy following euthanasia. One ovary per animal was frozen in liquid nitrogen and the contralateral ovary was fixed in 10% buffered formalin.

Histological Staining and Follicle Counts

Fixed ovaries were dehydrated, embedded in paraffin wax and cut into 6 μ m serial sections. Ovarian cross-sections were mounted onto Super frost plus slides, stained with hematoxylin, and counterstained with eosin (Vector Laboratories, Burlingame, CA) using a standard

protocol (Moffatt-Blue et al., 2006). Preantral follicles (including primordial, primary, and early secondary follicles), antral follicles (with developed antrum), and corpora lutea were counted across six cross sections per animal (each 60µm apart) by a researcher blind to group assignment, and counts were averaged per cross section (Moffatt-Blue et al., 2006; Salverson et al., 2008; Vrooman and Young, 2010). Only those follicles with visible oocytes were counted.

Radioimmunoassay

Plasma estradiol concentrations were measured using the Ultra-Sensitive Estradiol RIA ¹²⁵I double antibody kit (Diagnostic Systems Laboratories, Inc., Webster, TX), using the protocol provided by the manufacturer (Moffatt-Blue et al., 2006; Vrooman and Young, 2010). All samples were assayed in duplicate and their radioactivity was measured using a Perkin-Elmer Cobra II gamma counter (Packard Instruments Co., Boston MA) and values were calculated using Sigma Plot software (SPSS Inc., Chicago IL). The lower limit of estradiol detection was set at 5pg/ml, and low cross-reactions to other steroids (0.64-2.40%) are indicated by this kit. Calculated intra-assay % coefficient of variation averaged 4.44 %CV, comparable to the intra-assay range of 6.5-8.9 % CV, and inter-assay range of 7.5-12.2 % CV provided by the manufacturer.

Enzyme Immunoassay

Plasma follicle stimulating hormone concentrations were determined by enzyme immunoassay using an FSH immunometric EIA kit (Caymen Chemical, MI). All plasma samples (50 µl) and standards curve were run in duplicate and the assay was conducted as per manufacturers' instructions. A 6-point standard curve was simultaneously run on the same plate and absorbance for all wells was measured at 450 nm. Concentration in mIU was calculated from the standard curve. Calculated intra-assay % coefficient of variation averaged 9.04 %CV, comparable to the intra-assay range of 8.6-9.4 % CV, and inter-assay range of 9.4-11.8 % CV provided by the manufacturer.

Extraction of total RNA, cDNA synthesis and qPCR analysis

Trizol LS reagent (Invitrogen Life Technologies, Carlsbad, CA) was used to extract total RNA from the ovaries as per manufacturer's protocol. One µg of total RNA was used for cDNA synthesis using ImProm Reverse Transcription System (Promega, Madison, WI). For qPCR analysis cDNA was diluted 1:5 with DNAase/RNAase free water. The relative real-time PCR was done on an Mx3000 thermocycler using Absolute QPCR SYBR green mix (Thermo Fisher Scientific ABgene, Surrey, UK), as described previously (Shahed and Young, 2011). Briefly, the qPCR reaction mix contained 1 µl cDNA and 1 µl each of specific forward and reverse primers (80nM concentration) and 6 µl SYBR green mix in a total volume of 12µl. Primer sequences and T_m are listed in Table 1. A four point standard curve using pooled cDNA from all analyzed samples was included with each run. The relative amounts of mRNA were calculated using standard curves of each gene of interest and the reference gene, hypoxanthine phosphoribosyl transferase 1 (HPRT-1) (Tan et al., 2012). Expression of HPRT-1 did not change across groups (p= 0.48, data not shown), and mRNA expression data are presented as ratios of gene of interest to HPRT-1 mRNA (after Shahed and Young, 2013).

Statistical analysis

Data were analyzed using Prism 4 statistical software 240 package (GraphPad Software, Inc., San Diego, CA). One-way ANOVAs were performed on all groups with normalized data with the significance level set at $p < 0.05$. If results were significant with a 95% confidence interval, a Newman-Keuls post-hoc test was used to compare experimental groups. Paired ovarian mass and relative *Esr1*, *FSH β* -subunit, and *LHR* mRNA values were square root-transformed to reduce variance. Antral follicle and corpora lutea counts were analyzed via Kruskal Wallis with the planned Dunn's Multiple Comparison test, due to unequal variances.

Results

Masses, follicle counts, and plasma hormone concentrations

Body mass did not change over the 10 day experimental period ($p = 0.08$). Paired ovarian mass was low in SD and remained low in post transfer to stimulatory photoperiod day 2 (PT day 2) and PT day 4. By PT day 10, ovarian mass had increased in the d10 group compared to all other groups ($p < 0.05$; Table 2). Plasma estradiol concentrations were low with SD exposure, and did not significantly increase within the ten days of photostimulation ($p = 0.14$; Table 2). Ovaries from females exposed to 14 weeks of SD had low numbers of mature follicles, and exhibited the characteristic eosinophilic hypertrophied granulosa cell clusters common in SD females (Schlatter et al., '93; Moffatt-Blue et al., 2006; Kabitha and Place, 2008) (Fig 1A). PT day 2 ovaries looked very similar to SD ovaries, with greater development observed in the PT day 4 and especially PT day 10 ovaries (Fig. 1B-D). When quantified, the average number of preantral follicles with oocytes did not change across groups ($p = 0.61$), although numbers of both antral follicles and corpora lutea increased on PT day 10 as compared to SD ($p < 0.05$; Table 2). Plasma FSH concentrations were noted in each group, with increases observed at PT day 4 and PT day 10 as compared to SD ($p < 0.05$; Fig. 2).

mRNA expression of HPG axis hormone subunits and receptors

GnRH, *FSH β* - and *LH β* -subunit mRNA was expressed in ovarian tissue from each group, with higher relative levels of both GnRH and *FSH β* -subunit mRNA as compared to *LH β* -subunit levels; however normalized values showed no significant differences with photostimulation (GnRH, $p = 0.99$; *FSH β* , $p = 0.21$; *LH β* , $p = 0.27$; Table 3). In contrast, *FSHR* mRNA levels increased significantly on PT day 4 and PT day 10 as compared to both SD and PT day 2 values (Fig. 3A), and *LHR* mRNA levels were significantly elevated at PT day 10 as compared to SD values (Fig. 3B). A similar pattern of increased expression with photostimulation was observed with the mRNA for estrogen receptor α , where low levels of *Esr1* increased significantly in all photostimulated groups as compared to SD (Fig. 3C). Expression of *Esr2* mRNA was also stimulated with photostimulation, but the significant increase over SD values was only observed in PT day 2, not in PT day 4 and PT day 10 groups (Fig. 3D).

Matrix metalloproteinase -2 and -9 mRNA expression

MMP-2 mRNA levels were low in the SD group, and increased 3.3-fold on PT day 2 as compared to SD ($p<0.05$; Fig. 4A). This increase was transient, as levels in PT day 4 and PT day 10 did not differ from SD values ($p>0.05$; Fig. 4A). In contrast, no changes were noted between the SD and photostimulation groups for MMP-9 mRNA levels ($p=0.52$; Fig. 4B).

Anti-Müllerian hormone and inhibin- α mRNA expression

AMH mRNA was present in relatively high levels in ovaries in all groups, with no observed difference with photostimulation as compared to SD ($p=0.97$; Fig. 5A). In contrast, inhibin- α mRNA levels, although detectable in SD and PT day 2 ovaries, increased significantly on PT day 4 and PT day 10 (Fig. 5B).

Growth factor FGF-2 and proliferation marker PCNA mRNA expression

PCNA mRNA levels were present, but relatively low in SD ovaries, and increased significantly with 10 days of photostimulation as compared to SD values (Fig. 6A). While photostimulation also increased FGF-2 mRNA levels, this increase only was observed on PT day 2 as compared to PT day 10, when levels were once again no different from SD values (Fig. 6B).

Discussion

This study is the first to demonstrate alterations occurring at the follicular, endocrine, and mRNA level in Siberian hamster ovaries with short-term photostimulation. We have previously reported that recrudescence of photoregressed ovaries is a progressive process, with the ovary becoming fully functional within 4-8 weeks of photostimulation (Salverson et al., 2008). Results from the present study suggest for the first time that photostimulation also has rapid effects on central FSH release in females and can quickly alter the expression of genes involved in key ovarian functions.

Although some evidence of renewed follicle growth appears within four days of photostimulation, it takes ten days following transfer to LD to stimulate significant increases in ovarian mass, number of antral follicles and corpora lutea in adult Siberian hamsters (Table 2, Fig. 1). Presumably, this ovarian activity reflects a rapid response of the HPG axis to photoperiod change. This idea is supported by significant increases in both plasma FSH and ovarian FSH receptor mRNA after 4 days of photostimulation as compared to regressed hamsters (Figs. 2 and 3), with tangible increases in follicle development noted six days later (Table 2). These data parallel the early pituitary response noted in male Siberian hamsters transferred from SD to photostimulated conditions. In males, increases in both serum FSH concentrations and pituitary FSH β -subunit mRNA peaked at post transfer day 5 (Bernard et al., '99). This rapid FSH response has been linked to an increase in GnRH reactive neurons (Porkka-Heiskanen et al., '97) and GnRH mRNA in the pituitary (Bernard et al., '99; Bernard et al., 2000). In experiments examining the resumption of ovarian function in Siberian hamsters during spontaneous, as opposed to photostimulated recrudescence, increases in both pituitary and serum FSH correspond to increases in the number of medium and antral follicles (Schlatt et al., '93). It is possible that the up-regulation of ovarian FSHR mRNA by

PT day 4 in the present study stems from the observed increase in plasma FSH in response to photostimulation. Interestingly, no increases in local GnRH or FSH β -subunit mRNA levels were noted in the ovary itself. Intraovarian GnRH, gonadotropin α -subunit, and FSH β -subunit mRNA decreases after 2 weeks of photo stimulation compared to SD (Shahed and Young, 2011), suggesting that transcription of these genes is photoperiod-sensitive. However, because GnRH and FSH β -subunit mRNA did not demonstrate changes within the first 10 days of photostimulation as compared to SD controls in the present study, the increase in ovarian FSHR may be driven centrally by release of pituitary FSH (Figs 2 and 3). The concomitant rise in inhibin- α mRNA on PT day 4 and 10 (Fig. 5B) also supports the rising plasma FSH concentration data, because FSH upregulates inhibin synthesis (Kenny et al., 2002). On the basis of these results it may be that early photostimulated recrudescence in Siberian hamsters is primarily stimulated by observed increases in FSH, similar to the process of spontaneous ovarian recrudescence in this species. Furthermore, the presence of local GnRH and gonadotropin FSH β - and LH β -subunit mRNA in the ovary (Table 3), although unchanging, may contribute to early recrudescence by keeping the follicles primed to respond quickly upon activation of the HPG axis.

Acting through its receptors, estrogen is essential for cyclic ovarian function; it promotes follicle development via stimulating proliferation and inhibiting apoptosis of granulosa cells (Richards '80; Billig et al., '93). In Siberian hamsters, photoperiod affects plasma estradiol concentrations, with reductions noted with 9-14 weeks of SD exposure as compared to cycling ovaries (Moffatt-Blue et al., 2006; Salverson et al., 2008). Photostimulation gradually increases plasma estradiol, which can take multiple weeks to increase significantly as compared to SD concentrations (Salverson et al., 2008; Shahed and Young, 2011; Shahed and Young 2013). This mismatch in the resumption of folliculogenesis, which shows significant increases at ten days of photostimulation, as compared to steroidogenesis, which takes weeks to show a significant increase, may reflect the slower increase in ovarian LHR (PT day 10) as compared to FSHR (PT day 4) (Fig. 3). However, this mismatch is likely attributed to our measurement of estradiol in plasma as opposed to extracting estradiol from the ovary itself (Jamnongjit et al., 2005). Indeed, in male Siberian hamsters, evidence of testis and seminal vesicle growth is noted six weeks prior to increases in plasma testosterone during spontaneous recrudescence (Schlatt et al., '95), and a similar gap of 4 weeks between recovery of spermatogenesis and plasma testosterone is noted in recrudescing male white footed mice (*Peromyscus leucopus*) (Young et al., 2001). An alternative measure of local ovarian needs for estradiol is to examine responses of the estrogen receptors. These receptors are necessary for normal folliculogenesis (Dupont et al., 2000), and act independently in the ovary; ER β (Esr2) directly promotes antral formation and maturation of preovulatory follicles, and ER α (Esr1) is involved predominantly in ovulation (Emmen et al., 2005). While uterine Esr1 and Esr2 mRNA levels do not differ between 4-week old Siberian hamsters raised in SD or LD (Phalen et al., 2009), in the present study expression of estrogen receptor mRNA did show a response to photostimulation. Both Esr1 and Esr2 increased within 2 days of transfer to stimulatory LD photoperiod as compared to SD, a time period that actually precedes the initial increases in both plasma FSH and ovarian FSHR at PT day 4 (Figs. 2 and 3).

Members of the TGF- β superfamily including AMH and inhibin play significant roles in folliculogenesis (Knight and Glister, 2006; Trombly et al., 2009; Myers and Pangas 2010; Nilsson et al., 2011), and are differentially expressed in photoregressed and recrudescing Siberian hamster ovaries (Shahed and Young, 2013). The increase in inhibin- α mRNA on PT day 4 and PT day 10, concomitant with changes observed in follicle counts, supports the role of inhibin- α as a key mediator for the initial resumption of folliculogenesis during recrudescence (Fig. 5B). In contrast, AMH inhibits the recruitment of primordial follicles in adult cycling ovaries, thus restraining follicular development (Nilsson et al., 2011; Durlinger et al., '99). In the present study AMH mRNA expression remained at SD levels during 10 days of photostimulation (Fig. 5A). Previously we have reported that SD exposure increases AMH mRNA levels as compared to cycling adult Siberian hamster ovaries, and that AMH mRNA levels remain high until 4 weeks of photostimulation (Shahed and Young, 2013). The current data follow these previous results, with no changes between SD and up to 10 days of photostimulation. Because AMH levels remain constant across SD and early photostimulation, and because no changes were observed in pre-antral follicles across photoperiod groups, it may be that initial increases observed in antral follicles at PT day 10 stem from growth of existing primary and secondary follicles suddenly supported by the restoration of serum FSH, as opposed to rapid onset of primary follicle recruitment from the primordial pool.

Matrix metalloproteinases play an important role in ovarian recrudescence via their involvement in extracellular matrix reorganization, an essential process for ovarian function. Indeed, *in vivo* inhibition of MMPs by daily intra-peritoneal injection of the MMP inhibitor GM6001 impedes ovarian recrudescence in SD exposed Siberian hamsters upon photostimulation (Whited et al., 2010). While MMP-9 mRNA levels change across the Siberian hamster estrus cycle (Vrooman and Young, 2010), ovarian MMP-9 mRNA does not change with photoperiod in Siberian hamster adults (Salverson et al., 2008; Vrooman and Young, 2010). Data in the present study follow that trend, with no change within the first 10 days of photostimulation (Fig. 5B), suggesting that MMP-9 is present in the ovary regardless of photoperiod. In contrast to MMP-9, mRNA levels of MMP-2 increase with two days of photostimulation as compared to SD (Fig. 5A), suggesting a potential direct contribution of this gelatinase to the tissue remodeling of extracellular matrix required for resumption of photostimulated follicle development. This rapid PT day 2 peak of MMP-2 mRNA expression is similar to the increase in growth factor FGF-2 observed at PT day 2 as compared to PT day 10 (Fig. 6B). The early increase in both factors could be linked, as MMP cleavage of extracellular matrix can release local growth factors. An early and transient increase in FGF-2 stimulation of endothelial cell proliferation and ovarian angiogenesis (Redmer and Reynolds, '97) may serve to boost initial follicle development as folliculogenesis resumes, and these transient and early peaks are similar to the pattern of both serum FSH and pituitary expression of the FSH β -subunit mRNA observed in photostimulated male Siberian hamsters at post-transfer day 5 (Bernard et al., 2000).

While previous studies have shown the ability of the pituitary to change rapidly with photostimulation, results of the present experiment demonstrate that the ovary itself is quick to respond to change in photoperiod. We show for the first time that 2-10 days of photostimulation result in increases in plasma FSH concentrations in female hamsters, rapid

follicular growth, and alterations in mRNA expression of multiple genes related to ovarian function. Importantly, photostimulation increases *Esr1*, *Esr2*, and *MMP-2* mRNA within two days, plasma FSH, FSHR and inhibin- α mRNA within four days, and significant increases in proliferation, antral follicles and corpora lutea within ten days. In conclusion, photostimulated ovarian recrudescence, while a gradual process stimulated centrally by gonadotropin restoration, includes rapid initial changes at the level of the ovary. In particular, early increases in mRNA levels of FSHR and inhibin- α , and increases in antral follicles and corpora lutea suggest that the ovary is primed to react quickly to the pituitary FSH released in response to photostimulation. Future studies examining in what way these rapid changes in ovarian mRNA translate to functional alterations in proteins, and how different follicle types react to photoperiod-driven changes in gonadotropins will further elucidate the mechanism of how photoregressed mammalian ovaries return to function.

Acknowledgments

The authors thank undergraduates Lisa Vrooman, Daoud Majid, and Greer McMichael for their assistance in tissue collection and histological processing, and Master's students Jesus Reyes and Kerri Loke-Smith for their help with the radioimmunoassay. We are also grateful for the helpful and constructive suggestions from peer review, which strengthened this manuscript. This project was supported by the Howell/CSUPERB Undergraduate Research Fellowship (CFM), the Provost's Undergraduate Student Summer Stipend Program for Research, Scholarly and Creative Activity (CFM), and NIH SCORE grant 1SC3GM089611-01(KAY).

Research supported by: NIH SCORE Grant 1SC3GM089611-01 (KAY).

Literature Cited

- Bernard DJ, Abuav-Nussbaum R, Horton TH, Turek FW. Photoperiodic Effects on Gonadotropin-Releasing Hormone (GnRH) Content and the GnRH-Immunoreactive Neuronal System of Male Siberian Hamsters. *Biol Reprod.* 1999; 60:272–276. [PubMed: 9915990]
- Bernard DJ, Merzlyak I, Horton TH, Turek FW. Differential regulation of Pituitary Gonadotropin Subunit Messenger Ribonucleic Acid Levels in Photostimulated Siberian Hamsters. *Biol Reprod.* 2000; 62:155–161. [PubMed: 10611080]
- Billig H, Furuta I, Hsueh AJ. Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. *Endocrinology.* 1993; 133:2204–2012. [PubMed: 8404672]
- Byers M, Kuiper GG, Gustafsson JA, Park-Sarge OK. Estrogen receptor β expression in rat ovary: downregulation by gonadotropins. *Mol Endocrinol.* 1997; 11:172–182. [PubMed: 9013764]
- Curry TE Jr, Osteen KG. The matrix metalloproteinase system: changes, regulation and impact throughout the ovarian and uterine reproductive cycle. *Endocr Rev.* 2003; 24:428–465. [PubMed: 12920150]
- Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. Effect of single and compound knockouts of estrogen receptors (ER) and (ER) on mouse reproductive phenotypes. *Development.* 2000; 127:4277–4291. [PubMed: 10976058]
- Durlinger AL, Kramer P, Karels B, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN. Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. *Endocrinology.* 1999; 140:5789–5796. [PubMed: 10579345]
- Emmen JM, Couse JF, Elmore SA, Yates MM, Kissling GE, Korach KS. In vitro growth and ovulation of follicles from ovaries of estrogen receptor (ER) α and ER β null mice indicate a role for ER β in follicular maturation. *Endocrinology.* 2005; 146:2817–2826. [PubMed: 15731357]
- Jamnongjit M, Gill A, Hammes SR. Epidermal growth factor receptor signaling is required for normal ovarian steroidogenesis and oocyte maturation. *Proc Natl Acad Sci.* 2005; 102:16257–16262. [PubMed: 16260720]
- Kabitha EW, Place NJ. Photoperiod-dependent modulation of anti-Müllerian hormone in female Siberian hamsters, *Phodopus sungorus*. *Reproduction.* 2008; 135:335–342. [PubMed: 18299426]

- Kanimozhi V, Palanivel VK, Kadalmani B, Krikun G, Taylor HS. Ap E Induction in Syrian Hamster Testis Following Tributyltin Exposure: A Potential Mechanism of Male Infertility. *Reprod Sci*. 2014; 21:1006–1014. [PubMed: 24516040]
- Kenny HA, Bernard DJ, Horton TH, Woodruff TK. Photoperiod-dependent regulation of inhibin in Siberian hamsters: II. Regulation of inhibin production and secretion by pregnant mare serum gonadotropin. *J Endocrinol*. 2002; 174:85–94. [PubMed: 12098666]
- Knight PG, Glistler C. TGF- β superfamily and ovarian follicle development. *Reproduction*. 2006; 132:191–206. [PubMed: 16885529]
- Moffatt-Blue CS, Sury JJ, Young KA. Short photoperiod-induced ovarian regression is mediated by apoptosis in Siberian hamsters (*Phodopus sungorus*). *Reproduction*. 2006; 131:771–782. [PubMed: 16595728]
- Myers M, Pangas SA. Regulatory roles of transforming growth factor beta super family in folliculogenesis. *Wiley Interdiscip. Rev Syst Biol Med*. 2010; 2:117–125.
- Nilsson EE, Parrott JA, Skinner MK. Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. *Mol Cell Endocrinol*. 2001; 175:123–130. [PubMed: 11325522]
- Nilsson EE, Schindler R, Savenkova MI, Skinner MK. Inhibitory effect of anti-Müllerian hormone (AMH) on ovarian primordial assembly. *PloS One*. 2011; 6:e20087. [PubMed: 21637711]
- Oktyay K, Schenken RS, Nelson JF. Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat. *Biol Reprod*. 1995; 53:295–301. [PubMed: 7492681]
- Oxberry BA, Greenwald GS. An autoradiography study of binding of 125I-labeled follicle -stimulating hormone, human chorionic gonadotropin and prolactin to the hamster ovary throughout the estrous cycle. *Biol Reprod*. 1982; 27:505–516. [PubMed: 6812657]
- Phalen AN, Wexler R, Cruickshank J, Park SU, Place NJ. Photoperiod- induced differences in uterine growth in *Phodopus Sungorus* are evident at an early age when serum estradiol and uterine receptor levels are not different. *Comp Biochem Physiol*. 2009; 155:115–121.
- Place NJ, Cruickshank J. Graded response to short photoperiod during development and early adulthood in Siberian hamsters and the effects on reproduction as females age. *Horm Behav*. 2009; 55:390–397. [PubMed: 19470367]
- Porkka-Heiskanen A, Khoshaba N, Scabrough K, Urban J, Vitaerana MH, Levine J, Turek FW, Horton TH. Rapid Photoperiod induced increase in detectable GnRH mRNA-containing cells in Siberian hamsters. *Am J Physiol*. 1997; 42:R2032–R2039.
- Redmer DA, Reynolds LP. Angiogenesis in the ovary. *Rev Reprod*. 1997; 182–192.
- Richards JS. Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. *Physiol Rev*. 1980; 60:51–89. [PubMed: 6243782]
- Roy SK, Wang SC, Greenwald GS. Radioreceptor and autoradiographic analysis of FSH, hCG and prolactin binding sites in primary to antral hamster follicles during the periovulatory period. *J Reprod Fertil*. 1987; 79:307–13. [PubMed: 3820181]
- Salverson TJ, McMichael GE, Sury JJ, Shahed A, Young KA. Differential expression of matrix metalloproteinases during stimulated ovarian recrudescence in Siberian hamsters (*Phodopus sungorus*). *Gen Comp Endocrinol*. 2008; 155:749–761. [PubMed: 17980368]
- Schlatt S, Geyter MD, Kliesch S, Nieschlag E, Bergmann M. Spontaneous recrudescence of spermatogenesis in the photoinhibited male Djungarian hamsters, *Phodopus sungorus*. *Biol Reprod*. 1995; 53:1169–1177. [PubMed: 8527522]
- Schlatt S, Niklowitz P, Hoffmann K, Nieschlag E. Influence of short photoperiods on reproductive organs and estrous cycle of normal and pinealectomized female Djungarian hamsters. *Biol Reprod*. 1993; 49:243–250. [PubMed: 8373948]
- Shahed A, Young KA. Intraovarian expression of GnRH-1 and gonadotropin mRNA and protein levels in Siberian hamsters during the estrous cycle and photoperiod induced regression/recrudescence. *Gen Comp Endocrinol*. 2011; 170:356–64.
- Shahed A, Young KA. Anti-müllerian hormone (AMH), inhibin- α , growth differentiation factor-9 (GDF-9) and bone morphogenic protein-15 (BMP-15) mRNA and protein are influenced by photoperiod-induced ovarian regression and recrudescence in Siberian hamster ovaries. *Mol Repro Develop*. 2013; 80:895–907.

- Smith MF, Ricke WA, Baake LJ, Dow MPD, Smith GW. Ovarian tissue remodeling: role of matrix metalloproteinases and their inhibitors. *Mol Cell Endocrinol.* 2002; 191:45–65. [PubMed: 12044918]
- Tan SC, Carr CA, Yeoh KK, Schofield CJ, Davies KE, Calrke K. Identification of valid housekeeping genes for quantitative RT-PCR analysis of cardiosphere -derived cells preconditioned under hypoxia or with propyl-4- hydroxylase inhibitors. *Mol Biol Rep.* 2012; 39:4857–4867. [PubMed: 22065248]
- Trombly D, Woodruff TK, Mayo K. Roles of transforming growth factor beta super family in folliculogenesis. *Semin Repro Med.* 2009; 27:14–23.
- Uilenbroek JT, Richards JS. Ovarian follicular development during the rat estrous cycle: gonadotropin receptors and responsiveness. *Biol Reprod.* 1997; 20:1159–1165.
- Vrooman LA, Young, KA. Ovarian matrix metalloproteinases are differentially regulated during the estrous cycle but not during short photoperiod induced regression in Siberian hamsters (*Phodopus sungorus*). *Reproductive Biology and Endocrinology.* 2010; 8:79. [PubMed: 20579366]
- Wang C, Roy SK. Expression of growth differentiation factor 9 in the oocytes is essential for the development of primordial follicles in the hamster ovary. *Endocrinology.* 2006; 147:1725–34. [PubMed: 16384866]
- Whited J, Shahed A, McMichael CF, Young KA. Inhibition of matrix metalloproteinases in Siberian hamsters impedes photostimulated recrudescence of ovaries. *Reproduction.* 2010; 140:875–883. [PubMed: 20881024]
- Yang P, Kriatchko A, Roy SK. Expression of ER-alpha and ER-beta in the hamster ovary: differential regulation by gonadotropins and ovarian steroid hormones. *Endocrinology.* 2002; 143:2385–98. [PubMed: 12021204]
- Yang P, Wang J, Shen Y, Roy SK. Developmental expression of estrogen receptor (ER) alpha and ER Beta in the hamster ovary: regulation by follicle-stimulating hormone. *Endocrinol.* 2004; 145:5757–66.
- Young KA, Zirkin BR, Nelson RJ. Testicular apoptosis is down-regulated during spontaneous recrudescence in white-footed mice (*Peromyscus leucopus*). *J Biol Rhythms.* 2001; 16:479–88. [PubMed: 11669421]

Figure 1. Representative cross sections of ovaries from Siberian hamsters stained with hematoxylin and eosin

1A) Short day (SD) regressed ovaries. *1B)* Post-transfer from SD to LD for 2 days (PT day 2) recrudescent ovaries. *1C)* Post-transfer from SD to LD for 4 days (PT day 4) recrudescent ovaries. *1D)* Post-transfer from SD to LD for 10 days (PT day 10) recrudescent ovaries. Antral follicles (A); corpus luteum (CL); preantral follicles (PA); hypertrophied granulosa cells (H).

Figure 2. Plasma FSH Concentrations (mIU/ml) across early recrudescence
Mean + SEM plasma FSH concentrations (mIU/ml) in Siberian hamsters exposed to 14 weeks of short photoperiod (SD) as compared to hamsters transferred into photostimulatory conditions for 2-10 days (PT day 2-10). Groups with different letters are significantly different ($p < 0.05$).

Figure 3. Ovarian mRNA expression of gonadotropin and estrogen receptor genes
3A) Follicle stimulating hormone receptor (FSHR), *3B*) Luteinizing hormone receptor (LHR), *3C*) Estrogen receptor- α Esr1, and *3D*) Estrogen receptor- β Esr2 mRNA levels in photoregressed (SD) ovaries as compared to 2-10 days of photostimulation (PT day 2-10). All graphical results are presented as mean + SEM, relative to HPRT-1, and groups with different letters are significantly different ($p < 0.05$).

Figure 4. Ovarian mRNA expression of gelatinases

4A) Matrix metalloproteinase-2 (MMP-2, gelatinase A) and 4B) Matrix metalloproteinase-9 (MMP-9, gelatinase B) mRNA levels in photoregressed (SD) ovaries as compared to 2-10 days of photostimulation (PT day 2-10). Details of data presentation are as in Figure 3.

Figure 5. Ovarian mRNA expression of AMH and inhibin- α .

5A) Anti-Müllerian hormone (AMH) and 5B) inhibin subunit- α mRNA levels in photoregressed (SD) ovaries as compared to 2-10 days of photostimulation (PT day 2-10). Details of data presentation are as in Figure 3.

Figure 6. Ovarian mRNA expression of PCNA and FGF-2

6A) PCNA proliferation factor and 6B) fibroblast growth factor-2 mRNA levels in photoregressed (SD) ovaries as compared to 2-10 days of photostimulation (PT day 2-10). Details of data presentation are as in Figure 3.

Table 1**Primer sets**

Gene	Forward primer	Reverse primer	Tm
AMH	GCATGGCCAACTGGTACACT	GATGTGTGTGTGAGGCCTTG	60
Esr1	CAGGTGCCCTACTACCTGGA	CAGTCTCTCTCGGCCACTCT	60
Esr2	TGCAGAACCTCAAAAGAGTCC	AGCATCCCTCTTTGAACTCG	60
FGF-2	GCTGCTGGCTTCTAAGTGTGT	CCAACTGGAGTATTTCCGTGA	60
FSH β	TGCATCCTATTCTGGTGCTG	TTTCTGGGTATTGGGTCTGG	60
FSHR	TTTACTTGCCTGGAAGCGACTAA	CCCAGGCTCCTCCACACA	57
GnRH	TCTGGTCATGTTGTCCGTGT	CTTGCTGGTGTGTGGTATGC	61
HPRT-1	TGATCAGTCAACAGGGGACA	CTGGCCGATATCCAACACTT	55
Inhibin- α	CTGCCCTCAACATCTCCTTC	CTCATGTCCCTGGTAGAGC	60
LH β	CTGCTATGGCTGTTGCTGAG	AACACTCGGACCATGCTAGG	60
LHR	CATTCAATGGGACGACTCTA	GCCTGCAATTGGTGGA	60
MMP-2	ATGATGTCAGCTTCCCCATC	ACCTGCACCCTGAAACAGTG	55
MMP-9	ACTTTGAAAACGCAAATGGT	AGTCTCTCACTGGGGCAGAA	61
PCNA	AGCACTCGTATTTGAAGCACCA	TCACCAGAAGGCATCTTTACCA	62

Primer sequence for Esr1, Esr1 and Cyp19a1 (Phalen et al., 2009); AMH primer sequence provided by Dr. Ned Place; GDF-9 (Wang and Roy, 2006); FSHR (Kanimozhi et al., 2014); GnRH, LH β and FSH β primers (Shahed and Young, 2010).

Table 2
Changes in reproductive parameters with brief photostimulation

	SD	PT Day 2	PT Day 4	PT Day 10
Body mass (g)	30.10 ± 1.3	26.20 ± 0.9	28.55 ± 1.0	32.08 ± 2.0
Ovary mass (mg)	7.40 ± 0.3 ^a	6.04 ± 0.0 ^a	7.32 ± 0.0 ^{a,b}	14.50 ± 2.1 ^b
Plasma estradiol (pg/ml)	9.51 ± 3.0	9.40 ± 2.8	5.90 ± 3.1	18.21 ± 4.4
Preantral follicles	1.43 ± 0.1	1.10 ± 0.2	1.35 ± 0.3	1.67 ± 0.4
Antral follicles	0.00 ± 0.0 ^a	0.06 ± 0.1 ^{a,b}	0.15 ± 0.1 ^{a,b}	0.30 ± 0.1 ^b
Corpora lutea	0.00 ± 0.0 ^a	0.08 ± 0.1 ^{a,b}	0.32 ± 0.2 ^{a,b}	0.67 ± 0.2 ^b

Data are presented as mean ± SEM, columns with different letters differ significantly ($p < 0.05$). Follicle counts are presented as average number per section containing oocytes across the serially sectioned ovary, for 14 weeks of short photoperiod exposure (SD), or 2, 4, or 8 days post transfer to long photoperiod after 14 weeks of SD exposure (PT Day 2, 4, 8).

Table 3
Relative expression of GnRH and gonadotropin mRNA with brief photostimulation

	SD	PT Day 2	PT Day 4	PT Day 10
GnRH	3.46 ± 1.1	3.93 ± 1.3	3.38 ± 0.8	3.62 ± 1.3
FSHβ	3.96 ± 1.6	2.65 ± 0.8	1.55 ± 0.3	4.00 ± 0.8
LHβ	0.23 ± 0.1	0.31 ± 0.1	0.35 ± 0.1	0.15 ± 0.0

Relative real time PCR expression data of gene of interest normalized to HPRT-1 reference gene expression in Siberian hamster ovaries for 14 weeks of short photoperiod exposure (SD), or 2, 4, or 8 days post transfer to LD after 14 weeks of SD exposure (PT Day 2, 4, 8). Data are presented as mean ± SEM; no significant differences were noted between any groups.