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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

Asha Shahed and Kelly A. Young

Department of Biological Sciences, California State University Long Beach, Long Beach, CA 90840

Abstract

Exposure of Siberian hamsters to short photoperiod (SD) inhibits ovarian function, including folliculogenesis, whereas function is restored with transfer to long photoperiods (LD). To investigate the mechanism of photostimulated recrudescence, we assessed key folliculogenic factors: anti-Müllerian hormone (AMH), inhibin- α , growth differentiation factor-9 (GDF-9), and bone morphogenic protein-15 (BMP-15) across the estrus cycle and in photoregressed and recrudescing ovaries. Adult hamsters were exposed to either LD or SD for 14 weeks representing functional and regressed ovaries, respectively. Select regressed hamsters were transferred back to LD for two (post-transfer week 2; PTw2) or eight weeks (PTw8). Ovaries were collected and fixed in formalin for immunohistochemistry or frozen in liquid nitrogen for real-time PCR. AMH, inhibin- α , GDF-9 and BMP-15 mRNA and protein were detected in all stages of the estrus cycle. Fourteen weeks of SD exposure increased ($p < 0.05$) ovarian AMH, GDF-9 and BMP-15, but not inhibin- α mRNA levels as compared to LD. Transfer of regressed hamsters to stimulatory long photoperiod for 8 weeks returned AMH and GDF-9 mRNA levels to LD levels levels, and further increased mRNA levels for inhibin- α and BMP-15. Immunostaining of AMH, inhibin- α , GDF-9 and BMP-15 proteins was most intense in preantral/antral follicles and oocytes. The overall immunostaining extent for AMH and inhibin- α generally mirrored mRNA data, though no changes were observed in GDF-9 or BMP-15 immunostaining extent. Shifts in mRNA and protein levels across photoperiod conditions suggest possible synergistic roles for these folliculogenic factors in photostimulated recrudescence via potential regulation of follicle recruitment, preservation and development.

Keywords

seasonal reproduction; folliculogenesis; photostimulation

Introduction

Siberian hamsters (*Phodopus sungorus*) have adapted to harsh environmental conditions with reduction of reproductive activity during winter months when the days are short and foraging is difficult. Reproductive activity is fully resumed in spring and summer when

*Address Correspondence To: Kelly A. Young, Reproductive Biology Group, Department of Biological Sciences, California State University Long Beach, 1250 Bellflower Blvd, Long Beach, CA 90840, kelly.young@csulb.edu.

environmental conditions improve (Reiter, 1980; Glass 1986, Buchanan and Yellon, 1991; Porka-Heiskanen, 1997). Our laboratory, like others (e.g., Schlatt et al. 1993), has reported that adult Siberian hamsters exposed to short days (SD, 8 hours of light per day: 16 hours of dark per day) for sustained periods (14 weeks) reduce reproductive activity, with significant reductions in ovary and uterine mass, antral follicle and corpora lutea numbers, and serum estradiol concentration as compared to those maintained in long photoperiods (LD, 16L:8D) (Moffatt-Blue et al. 2006). Subsequent transfer of photoinhibited Siberian hamsters to LD for two to eight weeks promotes systemic gonadotropin release leading to ovarian recrudescence: resumption of estrus cyclicity, serum estradiol levels, folliculogenesis and ovulation (Salverson et al. 2008). Return of ovarian function is characterized by resumption of folliculogenesis, a complex process that involves the interaction of pituitary and local gonadotropins with follicle and oocyte-specific factors to guide follicular recruitment and development (reviewed by Trombly et al. 2009). Importantly, the absence of gonadotropins alone does not produce full ovarian regression, nor does the replacement of FSH to SD exposed females fully return the LD ovarian phenotype, suggesting that local ovarian factors beyond gonadotropins play a key role in recrudescence (Zysling et al. 2012).

Members of the TGF- β superfamily have long been implicated as key players in the recruitment, selection, and development of primordial follicles, and the development of primary follicles through ovulation and luteinization (reviewed by Knight and Glister, 2006; Trombly et al. 2009; Myers and Pangas, 2010). Anti-Müllerian hormone (AMH), a homodimeric glycoprotein, is produced in ovarian follicles by granulosa cells of the primary, pre-antral, and small antral, but not primordial follicles (Durlinger et al. 2002a; Durlinger et al. 1999). AMH can constrain follicular development by inhibiting primordial follicle recruitment (preventing their depletion) (Nilsson et al. 2011) and by reducing of FSH sensitivity in growing follicles (reviewed by Knight and Glister 2006; Trombly et al. 2009). Intriguingly, AMH levels also respond to changes in photoperiod; ovarian, but not serum AMH levels are elevated in Siberian hamsters raised in short photoperiods (10L:14D) since birth (Kabitha and Place, 2008; Place and Cruickshank, 2009). Since AMH is critical for regulating follicular development, and is photoresponsive, it is likely to be involved with the return of ovarian function; however, the pattern of AMH mRNA and protein levels during photoperiod induced ovarian recrudescence had not been previously investigated.

Inhibin and activin are gonadal hormones synthesized by granulosa cells of the growing follicles and have opposing functions: activins stimulate pituitary FSH secretion whereas inhibins down-regulate FSH secretion, and both are expressed differentially throughout the estrus cycle (Woodruff et al. 1996; reviewed by Knight and Glister 2006). Inhibin A is a heterodimer of the common α and β_A subunits, inhibin B is a dimer of α and β_B subunits, and activins are homodimers of the β subunits. FSH stimulates granulosa cell production of all three subunits, and inhibins feed back to inhibit FSH release (reviewed by Bernard et al. 2001). Like AMH, short photoperiod exposure reduces inhibin- β subunit mRNA, inhibin dimeric protein, and all three subunits proteins as compared to long day exposed Siberian hamsters (Kenny et al. 2002a, 2002b). Although these reports infer photoperiod regulation of inhibin secretion, its role in photostimulated recrudescence of regressed ovaries had not been examined.

Growth differentiation factor-9 (GDF-9) and most bone morphogenetic proteins (e.g., BMP-15) are oocyte derived homologous growth factor proteins, and members of the TGF- β superfamily that play important roles in different stages of follicular development and oocyte maturation. GDF-9B was identified as a GDF-9 homolog and named BMP-15 in rat ovaries (Jaitinen et al. 1999). Both GDF-9 and BMP-15 are found in ovaries of mice (McGrath et al. 1995), rats (Dube et al. 1998), humans (Fritzpatrick et al. 1998) and hamsters (Wang and Roy, 2004), and are considered oocyte derived factors that facilitate the growth

and maturation of oocytes and inhibit cumulus cell apoptosis (Otsuka et al. 2011; Gilchrist et al. 2008; Sue et al. 2009). While GDF-9 and BMP-15 play crucial roles in oocyte growth and maturation, the effect of photoperiod on GDF-9 and BMP-15 had not been explored.

In Siberian hamsters, short photoperiods severely inhibit reproductive function, which gradually resumes with transfer to photostimulating conditions. We hypothesized that alterations in photoperiod stimulation would influence both mRNA and protein levels of AMH, inhibin- and oocyte derived growth factors GDF-9 and BMP-15, and that these changes may play a role in ovarian recrudescence in Siberian hamsters.

Results

Effect of photoperiod on ovary weight, serum estradiol levels and follicle count

Consistent with previous studies, exposure to 14 weeks of short day (SD; 8L:16D) reduced both ovarian weight and serum estradiol levels as compared to females exposed to LD photoperiod for the same duration ($p < 0.05$ for both measures; Table 1). In contrast, photostimulation (16L: 8D) of regressed females for eight weeks (PTw8) restores both ovarian mass and estradiol concentrations to levels no different than LD (Table 1). Follicle development and ovulation occurs regularly in LD stimulated females; however, is also severely inhibited by SD exposure: no antral follicles or corpora lutea are noted in SD females (Table 2). In addition, a significant increase in hypertrophied 'luteinized' atretic follicles, characteristic of regressed Siberian hamster ovaries, was noted in SD as compared to LD controls ($p < 0.05$; Table 2). Eight weeks of photostimulation restored folliculogenesis, with numbers of antral follicles and CL no different from LD controls ($p > 0.05$; Table 2). To provide background and clarity for the present results, data in Tables 1 and 2 (other than PTw2 and luteinized atretic follicles) have been partially published before in a sister study (Shahed and Young, 2011). All other data represent novel findings.

AMH mRNA levels and protein immunostaining

Estrus cycle—AMH mRNA was detectible in all stages of the estrous cycle, but was highest in proestrus (P) compared to the estrus (E), diestrus I (DI), and diestrus II (DII) stages ($p < 0.05$; Figure 1a). AMH immunostaining was also detected in all stages of the estrus cycle, with high levels of immunodetection noted predominantly in preantral and antral follicles (Table 3). AMH immunostaining intensity peaked in antral follicles during P as compared to the DII stage ($p < 0.05$; Table 3). No differences in immunostaining intensity were noted for other ovarian structures, and overall relative extent of immunostaining did not change significantly across estrus cycle stages ($p > 0.05$; Table 3).

Photoperiod induced regression/recrudescence—AMH mRNA levels were 4-fold higher in SD ovaries as compared to levels in LD control hamsters ($p < 0.05$; Figure 1b). Photostimulation of regressed hamsters for two weeks (PTw2) maintained high AMH mRNA levels as compared to LD ($p < 0.05$). In contrast, AMH mRNA levels in the PT hamsters were not different from LD controls after eight weeks ($p > 0.05$; Figure 1b).

AMH immunostaining intensity in LD ovaries was most prominent in preantral (PA) and early antral (A) follicles, with lower levels of staining detected in corpora lutea (CL), stromal connective tissue, and steroidogenic stroma in LD controls ($p < 0.05$; Table 4). While staining was most intense in preantral follicles, AMH immunodetection was also robust in luteinized granulosa cells of atretic follicles that are characteristic of SD ovaries (Figure 1c), leading to the peak of intensity noted in steroidogenic stroma among SD and PT ovaries as compared to LD ($p < 0.05$; Table 4). In antral follicles, AMH staining intensity peaked in PTw2 as compared to LD and PTw8 ($p < 0.05$); similarly, the relative overall extent of AMH

immunostaining across the ovarian sections also peaked in PTw2 as compared to LD controls ($p < 0.05$; Table 4).

Inhibin- α mRNA levels and protein immunostaining

Estrus cycle—Inhibin- mRNA was present in all stages of the estrous cycle; however, relative levels were significantly higher during E, DI, and DII as compared to P (Figure 2a). Inhibin- protein immunoreactivity was also observed throughout the estrous cycle. While immunostaining intensity did not change across estrus cycle stages in preantral follicles, CL, and stromal tissues (all $p > 0.05$; Table 3), inhibin- intensity did peak in antral follicles in P and E as compared to DI ($p < 0.05$; Table 3). The average overall extent of inhibin- immunolocalization across ovarian cross sections peaked in E as compared DI and DII ($p < 0.05$; Table 3).

Inhibin- α during regression/recrudescence—Inhibin- mRNA was present in all groups: LD, SD, PTw2 and PTw8 (Figure 2b). Fourteen weeks of SD exposure reduced inhibin- mRNA levels in SD as compared to LD controls ($p < 0.05$; Figure 2b). Subsequent transfer of regressed hamsters to LD increased intra-ovarian inhibin- mRNA, reaching higher relative levels in PTw8 as compared to both SD and LD ($p < 0.05$; Figure 2b).

Immunostaining for the inhibin- protein was primarily detected in preantral and antral follicles, along with very reduced amounts in CL tissue, and no detection in the stroma (Figure 2c). The intensity of preantral follicle immunostaining dropped significantly in SD as compared to the LD and PT groups ($p < 0.05$; Table 4), whereas no changes were noted across the LD and PT ovaries for intensity of immunostaining in antral follicles ($p > 0.05$; Table 4). The average overall extent measurements of inhibin- immunostaining, like the mRNA, were significantly lower in SD compared to LD controls, but returned to LD levels with photostimulation in the PTw2 and PTw8 groups ($p < 0.05$; Table 4).

Growth and differentiation factor-9 (GDF-9) and bone morphogenic protein 15 (BMP-15) mRNA levels and protein immunostaining

GDF-9 and BMP-15 in the estrus cycle—GDF-9 and BMP-15 mRNA was present throughout the estrous cycle. GDF-9 mRNA levels were lowest in P as compared to the E, DI, and DII stages ($p < 0.05$; Figure 3a). Showing a slightly different pattern, BMP-15 mRNA levels peaked in E as compared to in P ($p < 0.05$) but were not different than DI and DII (Figure 4a). Like mRNA, immunostaining for GDF-9 and BMP-15 proteins was detected in all stages of the estrus cycle, although no differences were noted in average overall extent across stages for either protein ($p > 0.05$; Table 3). Immunostaining intensity for both GDF-9 and BMP-15 showed peaks in DI, with GDF-9 intensity higher in DI than DII in corpora lutea, and BMP-15 intensity higher in DI as compared to DII in preantral follicles ($p < 0.05$; Table 3).

GDF-9 and BMP-15 during regression/recrudescence—GDF-9 mRNA was present throughout the photoperiod exposure groups. GDF-9 mRNA levels were higher in SD as compared to LD ($p < 0.05$), an increase subsided once females were transferred to photostimulating conditions; mRNA returned to LD control levels in both PTw2 and PTw8 ovaries (Figure 3b). BMP-15 mRNA levels responded slightly differently; while they increased with SD compared to LD exposure, they remained high in PTw2 and PTw8 ovaries as compared to LD ($p < 0.05$; Figure 4b).

GDF-9 and BMP-15 immunostaining showed a similar pattern and was detected in all ovaries regardless of photoperiod exposure (Figures 3c and 4c). Both GDF-9 and BMP-15 overall immunostaining intensity was more intense in PA and A follicles and CL tissue, as

compared to stromal tissues ($p < 0.05$; Figure 3d, 4d), with particularly high immunostaining intensity observed in oocytes and granulosa cells for both proteins.

GDF-9 immunostaining was detected in the ovaries of all photoperiod groups, although it showed no significant differences in overall extent between groups ($p > 0.05$; Table 4). Immunostaining of GDF-9 was predominantly localized to pre-antral follicles, antral follicles, and corpora lutea, with a moderate amount in steroidogenic stroma, and no staining noted in connective tissue-based stroma (Figure 3c). The intensity of GDF-9 immunostaining did differ between groups in preantral follicles and corpora lutea, with preantral follicles in LD ovaries showing higher intensity staining than preantral follicles in SD and PTw8 ($p < 0.05$; Table 4), and corpora lutea staining intensity higher in LD and PTw2 as compared to PTw8 ($p < 0.05$; Table 4). BMP-15 protein was also detected in all photoperiod groups, although no differences were noted in individual intensities or average overall extent of staining across the ovarian sections ($p > 0.05$; Table 4).

Discussion

Because members of the TGF- β superfamily play crucial roles in folliculogenesis, this study investigated the mRNA and protein levels of follicular growth factors (AMH, inhibin- β , GDF-9 and BMP-15) during photostimulated ovarian recrudescence. These factors act at different stages of follicular growth and appear to be differentially regulated during the return to ovarian function. We show, for the first time, that exposing adult Siberian hamsters to 14 weeks of short photoperiod significantly increases ovarian AMH, GDF-9 and BMP-15, and decreases inhibin- β mRNA as compared to LD. Additionally, immunostaining of AMH, inhibin- β , GDF-9, and BMP-15 proteins show differential staining during the estrous cycle and photoperiod induced regression/ recrudescence in Siberian hamsters.

In the present study, ovarian AMH mRNA and protein peaked in the proestrus stage of the estrous cycle compared to other stages (Figure 1a). This differs from what has been reported in postnatal rats, with no changes noted in the *in situ* detection of AMH and AMHIR mRNA, except a reduction in preantral follicles during estrus, as compared to other stages (Baarends et al. 1995). However, since levels were highest in small preantral follicles, it follows that AMH mRNA might peak when these follicles are plentiful in the ovary, such as during the proestrus and estrus stages, where the number of preantral follicles is highest in Siberian hamsters (Vrooman and Young, 2011). In the present study, AMH immunostaining was highest in granulosa cells of primary, preantral and antral follicles with very little in CLs (Figure 1c). This pattern is consistent with previous reports in Siberian hamster (Kabithe and Place 2008), mouse (Baarends et al. 1995), human, and rat ovaries (Weenen et al. 2004).

Ovarian AMH mRNA and protein levels were also altered with exposure to different photoperiods in Siberian hamsters. While short photoperiod exposure reduces ovarian function, it increases AMH mRNA levels as compared to LD ovaries (Figure 1b). These results are similar to a previous report, where Siberian hamsters raised under SD conditions (10L:14D) from birth showed increased levels of AMH protein as compared to those raised in LD (16L:8D) (Kabithe and Place, 2008). In contrast, these results differ from a recent report showing no changes in ovarian AMH mRNA in Siberian hamsters raised in SD conditions for 7.5 weeks postnatally (Zysling et al. 2012). This difference may, in part, be related to experimental design, as the current study used adult hamsters instead of neonates and the SD exposure time was longer (14 weeks). An increase in AMH in SD ovaries is consistent with its role as an inhibitor of both follicle growth and primordial follicle recruitment (McGee and Hseuh, 2000; Durlinger et al. 2002). While the lack of gonadotropin support provides a central signal to initiate gonadal regression, a local increase

in AMH in the ovary may assist in maintaining gonadal quiescence. When AMH is knocked out in mice, the ovaries respond with more preantral and small antral follicles (Durlinger et al. 1999); such recruitment and growth is in direct opposition of the cessation of folliculogenesis observed in ovarian atrophy. Interestingly, the levels of both AMH mRNA and protein remain high during the first weeks of recrudescence, not returning to LD levels until 8 weeks of photostimulation, when ovarian steroidogenesis and folliculogenesis fully recover (Figure 1b; Table 4). The persistence in increased AMH may be related to its inhibitory effect on FSH stimulated follicle growth and in maintaining primordial pool numbers (reviewed by Durlinger et al. 2002); as it would be critical to restrict the number of growing follicles during the rapid resurgence of FSH during recrudescence. Taken together, it is likely that the SD rise in AMH is involved with maintaining the primordial pool and limiting photostimulated follicular growth in Siberian hamsters.

Inhibins, also members of TGF β superfamily, are critical players in folliculogenesis that are both stimulated by and negatively regulate FSH (Bernard et al. 2001). In Siberian hamsters, levels of both inhibin- α subunit mRNA and protein immunostaining were constant through most of the estrus cycle, declining only in proestrus (Figure 2a), and were higher in growing follicles as compared to CL (Figure 2c, Tables 3, 4). In rats, inhibin- α subunit mRNA also varies across the estrus cycle, declining at times when FSH concentrations increase, and peaking on the evening of proestrus (Meunier et al. 1988; Murata et al. 1997; Woodruff et al. 1996). Timing of sample collection and species difference may account for this difference in results, as ovaries in the present study were collected after vaginal cytology was assessed, but timing of estrus stages was not synchronized.

Inhibin- β has been used as a marker of follicular growth (reviewed by Knight and Glistler, 2006), which is consistent with the results of the present study where inhibin- β levels were high in functional or recrudescing ovaries and declined with SD induced ovarian atrophy (Figure 2b, Table 4). These results concur with the decreases in inhibin- β , but not inhibin- α and - γ subunit mRNA levels in that occur in SD exposed Siberian hamster ovaries (Kenny et al. 2002a), and with the decline in inhibin immunostaining observed during SD induced testicular atrophy in male Siberian hamsters (Culler et al. 1991). In contrast, testicular inhibin- β increases in male Siberian hamsters raised in SD (Rao et al. 1995), as does inhibin-like activity in Sertoli cell cultures from regressed (optically enucleated) golden hamsters (Berkowitz and Heindel, 1987). While the lower inhibin- β levels in SD regressed ovaries may simply reflect the lack of follicles beyond the preantral stage (Table 2), it may be that the reduced levels observed in SD preantral follicles may serve to limit initial feedback to FSH secretion as the ovary resumes function during recrudescence (Table 4). During photostimulated recrudescence, inhibin- α subunit mRNA and extent of protein immunoreactivity increased significantly as compared to both LD and SD groups (Figure 2b, Table 4). These data correspond to increases observed in inhibin- α mRNA levels following a gonadotropin challenge in LD exposed female Siberian hamsters (Kenny et al. 2002b), as recrudescence follows the return of gonadotropin secretion. Interestingly, the response to gonadotropin stimulation was blunted in SD exposed females as compared to LD (Kenny et al. 2002b), which may explain the staggered increase in ovarian inhibin- α observed in recrudescing groups in the current study (Figure 2b).

Growth differentiation factor-9 (GDF-9) and bone morphogenic protein-15 (BMP-15) are members of the TGF β superfamily produced by oocytes that play species-specific cooperative roles in functional ovaries (Yan et al. 2001). GDF-9 and BMP-15 are present during all stages of follicular growth, and are likely released into the follicular fluid to act on granulosa cells (Silva et al. 2005; Jayawardana et al. 2006; Sun et al. 2010; Otsuka et al. 2011; Motterhead et al. 2012). Both GDF-9 and BMP-15 receptor II are expressed in Siberian hamster ovaries, with GDF-9 mRNA critical for primordial follicle growth (Wang

and Roy 2006, 2009). We investigated changes of these oocyte-derived factors during the estrus cycle in Siberian hamsters, finding that both GDF-9 and BMP-15 mRNA and protein were present in all stages of the cycle, and that their mRNA, but not protein levels (Figure 3, 4, Table 3), declined in proestrus as compared to all other stages. While BMP-15 mRNA levels change with the stage of follicular development in rats, no apparent changes were noted across the stages of the estrous cycle (Ericson and Shimasaki, 2003), although levels of both GDF-9 and BMP-15 mRNA decline as follicles reach the antral stage in mice (Monti and Redi, 2009). Intense GDF-9 and BMP-15 immunostaining was localized primarily to the oocytes of preantral and antral follicles in the present study; however, some staining was also detected in granulosa cells (Figure 3c, 4c). These data correlate with localization patterns observed in mice, pigs, and goats, where GDF-9 and BMP-15 protein is localized to oocytes and granulosa cells, while other studies demonstrate immunoreactivity in CL of humans and goats (Sun et al. 2010; Silva et al. 2005). In contrast, in 15-day old Siberian hamsters, GDF-9 immunostaining localizes exclusively to oocytes (Wang and Roy, 2006). This apparent difference may be due to the fact that hamsters used in the present study were adults more than 20 weeks old, and it is likely that developmental changes alter patterns of GDF-9 localization.

While GDF-9 and BMP-15 mRNA were expressed in Siberian hamster ovaries regardless of photoperiod exposure, mRNA levels peaked with 14 weeks of SD exposure (Figures 3b and 4b). These increased levels were similar to results observed with AMH, and it suggests that these genes may be critical to maintain follicles within the photoregressed ovary. Both GDF-9 and BMP-15 promote follicular survival by suppressing granulosa cell apoptosis and thus reducing follicular atresia (Hussein et al. 2005; Orisaka et al. 2006). Transferring reproductively regressed females to LD photoperiods returned GDF-9 mRNA to levels observed in the LD controls (Figure 3b); however, this was not the case for BMP-15 mRNA which remained at high levels despite weeks of photostimulation (Figure 4b). The decrease in GDF-9 may be related to its role in granulosa cell stimulation; limiting proliferation and follicle growth during initial return to ovarian function might be necessary to prevent an excessive response to initial resumption of FSH stimulation. Examination of the protein levels during photoperiod changes revealed decreases in the intensity of GDF-9 immunoreactivity within preantral follicles during SD exposure, which reverses with photostimulation (Table 4). In contrast, no changes were noted in staining intensity in other ovarian structures or in the extent of GDF-9 immunostaining across photoperiod exposure groups, and no changes in protein levels were noted in any structure for BMP-15 (Table 4). The discrepancy between these results and mRNA levels may be in the quantification method: scoring of immunohistochemistry patterns provides relative and semi-quantitative data, and while they give additional insight into protein changes, do not reflect the more quantitative whole-ovary mRNA analysis afforded by real time PCR.

Folliculogenesis is a complex process in which endocrine, ovarian, follicle, and oocyte derived growth factors play key and cooperative roles (Yan et al. 2001). Adding to the complexity of folliculogenesis is the process of photoperiod induced ovarian regression and recrudescence. While this process is centrally regulated by gonadotropins, inhibition of GnRH and replacement of FSH alone do not fully mimic photoperiod mediated ovarian regression and recrudescence (Zysling et al. 2012), suggesting that local intra-ovarian factors are critical to these processes. Our results show that AMH, inhibin- β , GDF-9 and BMP-15 are altered differentially during photoperiod induced regression and recrudescence. In photoregressed ovaries, AMH, GDF-9 and BMP-15, but not inhibin- β , mRNA levels were higher than ovaries from LD exposed females. Since SD exposure reduces plasma FSH concentrations that promote inhibin synthesis, the lower inhibin- β mRNA levels in SD ovaries follow the expected pattern. Although GDF-9 and BMP-15 promote development of normal ovarian follicles, an increase in the levels of these factors along with increases in

AMH in SD ovaries is interesting and consistent with roles in follicular preservation via their anti-apoptotic properties (GDF-9 and BMP-15), and limitation of primordial follicle recruitment (AMH). Photostimulated recrudescence of the ovary appears to be a process that differs from typical LD ovarian function and from gonadal development/maturation. While AMH and GDF-9 mRNA return to LD levels with post transfer photostimulation, both inhibin- and BMP-15 levels increased. These results stress to the need for continued study of the complex process of photostimulated recrudescence, as the return to ovarian function appears to occur via pathways that may differ from those that regulate normal ovarian function.

Methods and Materials

Animals

Female Siberian hamsters (*Phodopus sungorus*, > 60 days of age) were obtained from the breeding colony of Dr. Katherine Wynne-Edwards, Queen's University (Kingston, Ontario, Canada) and were housed individually at $20 \pm 2^\circ\text{C}$, in our AAALAC-approved facilities and received food and water *ad libitum*. All experiments were conducted as per California State University Long Beach and NRC guidelines for use of laboratory animals, and with specific project approval from our Institutional Animal Care and Use Committee.

Estrous cycle experiment

To stimulate and help synchronize the estrous cycle, four male hamsters were placed in the same room as the females and soiled male bedding was placed into individual female cages prior to tissue collection (Moffatt-Blue et al., 2006, Dodge et al., 2002). Ovaries were initially staged in proestrus (P), by estrus (E), diestrus I (DI), and diestrus II (DII) by vaginal cytology as described previously (Moffatt-Blue et al., 2006) and stages later confirmed with ovarian morphology. Hamsters (n=4–5/group) were anesthetized (ketamine [200 mg/kg] plus xylazine [20 mg/kg]) and euthanized via cervical dislocation. Ovaries were removed, and one was frozen in liquid nitrogen (qPCR analysis) and the contralateral gonad was fixed in 4% buffered formalin (follicle counts and immunohistochemistry).

Regression/recrudescence experiment

A portion of these hamsters represent a subset from a previously published study examining different ovarian factors (Shahed and Young, 2011). Briefly, four groups of hamsters were exposed to long days (LD, 16L:8D) or short days (SD, 8L:16D) as follows: LD group (n=5) 14 weeks in LD; SD group (n=6) 14 weeks in SD; post transfer week 2 (PTw2, n=6) group: 14 weeks in SD, then transferred to LD for two weeks; post transfer week 8 (PTw8, n=6) 14 weeks in SD, then transferred to LD for eight weeks. Hamsters were anesthetized and euthanized and ovaries were removed as above.

Total RNA extraction and cDNA synthesis

Ovarian RNA was extracted using Trizol LS reagent (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer directions. One μg of total RNA (260/280 ratio >1.6) was used for cDNA synthesis using ImProm Reverse Transcription System (Promega, Madison, WI) according to the manufacturers' directions in a total volume of 20 μl as described (Shahed and Young 2010). cDNA was diluted 1:5 with water (RNAase DNase free) and used for qPCR analysis.

Relative real time PCR

The relative real time PCR was done on Mx3000 thermocycler using Absolute QPCR SYBR green mix (Thermo Fisher Scientific ABgene, Surrey, UK) as described before (Shahed and

Young, 2011). Briefly, the qPCR reaction mix contained 1 μ l cDNA (1:5 dilution of cDNA transcribed using 1 μ g total RNA) + 1 μ l each of forward and reverse primers (80nM concentration) + 6 μ l SYBRgreen mix + 3 μ l water (Promega DNase, RNase, Protease free) to a total volume of 12 μ l. PCR cycles consisted of 15 min hold at 95°C (1 cycle), then 40 amplification cycles at appropriate T_m (Table 5), extension (1 min at 72°C) followed by dissociation. Non-template negative controls and standards were included in the same plate as unknown samples. In addition, qPCR products were analyzed on agarose gels to confirm that the correct size (base pairs, Table 4) product was obtained and also to visualize potential secondary and nonspecific amplification. A four point standard curve (efficiency 80 to 100%, correlation coefficient > 0.96) was done by pooling equal aliquots of cDNAs from all samples and diluted fivefold and amplified as above using the specific primers for gene of interest and housekeeping genes (Shahed and Young 2011). The relative amounts of mRNA were calculated using standard curves of each gene of interest and house keeping genes GAPDH and HPRT1 (Tan et al., 2012). Subsequently the ratios of gene of interest to both house keeping genes were calculated. Since ratios between normalizations to both housekeeping genes; therefore for simplicity, only mean $\pm$ SEM of the HPRT1-normalized ratios are presented.

Immunohistochemical detection of AMH, Inhibin, BMP-15 and GDF-9 proteins

Paraffin embedded ovary tissue was sectioned (6 μ) and deparaffinized in xylene, rehydrated through a graded series of ethanol, washed in phosphate buffer (PBS), and heated in Antigen Unmasking Solution Citra (Vector Laboratories Burlingame, CA) for 8 min at high power in microwave and then cooled in water and washed in PBS (Shahed and Young 2011). Sections were then placed in 0.3% hydrogen peroxide/methanol solution, blocked with species matched normal horse serum/Tween-20 (0.1%) for 40 min, washed twice in PBS incubated with either: anti MIS goat polyclonal (1: 1000; Santa Cruz Biotechnology Inc. Santa Cruz, CA), anti-GDF9 polyclonal antibody (1:50) inhibin- antibody (1:20–40; Thermo Fisher Scientific Rockford, IL.) or BMP-15 antibody (1:50; Thermo Fisher Scientific Rockford, IL.) for one hour at room temperature followed by overnight at 4°C in humidified chamber. Sections were then processed using a biotinylated pan-specific secondary antibody from the Ready to Use Vectastain Universal Quick Kit and NovaRed Substrate (Vector Laboratories, Burlingame, CA) as per manufacturer's protocol, counterstained with hematoxylin, dehydrated, and mounted. Negative controls were processed without primary antibody.

Quantification of immunostaining intensity was separately recorded for each protein: preantral follicles (PA), antral follicles (A), corpora lutea (CL), stroma between follicles and CL (containing mostly connective/endothelial tissue, Stroma CT), and stroma containing potentially steroidogenic interstitial cells not incorporated into a defined follicle (Stroma Ste). A staining intensity index was developed following methods described by Vrooman and Young 2010. Briefly, a numerical value of 0–4 was assigned to each structure where: a score of 0 indicated no staining; a score of 1 meant some faint staining in the structures of the subtype being scored, a score of 2 indicated light staining in the structures of the subtype being scored, a score of 3 specified medium-intense staining in the structures of the subtype being scored, and a score of 4 specified intense staining in the structures of the subtype being scored. Because a minimum of three ovarian cross sections per hamster that showed each structure type was required for that individual to be included in the group analysis of that structure type, all structures were not scored for all groups. For example, regressed ovaries of hamsters responding to SD photoperiod lack both antral follicles and corpora lutea, and were therefore not scored for these structures. For all counts, scores for three-six ovarian cross sections (60–100 microns apart at minimum) per animal were averaged, and included in the group mean (n = 4–6 animals per group) used in the ANOVA analysis.

Statistical analysis

Results are presented as mean $\pm$ SEM and analyzed by one-way ANOVA with Newman Keuls post hoc tests using Prism 4 software (GraphPad Software, Inc, San Diego, CA). p 0.05 was considered statistically significant.

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Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AMH	anti-Müllerian hormone
BMP-15	bone morphogenic protein 15
cDNA	copy of deoxyribonucleic acid
CL	corpus luteum or corpora lutea
DI	diestrus I
DII	diestrus II
E	estrus
FSH	follicle stimulating hormone
GDF-9	growth differentiation factor 9
HPRT	hypoxanthine phosphoribosyl transferase 1
inh-	inhibin-
LD	long day photoperiod exposure 16 hours of light 8 hours of dark
LH	luteinizing hormone
MIS	Müllerian inhibitory substance
mRNA	messenger ribonucleic acid
P	proestrus
PBS	phosphate buffered saline solution
PTw2	post-transfer from short to long photoperiod for 2 weeks
PTw8	post-transfer from short to long photoperiod for 8 weeks
qPCR	real time polymerase chain reaction
RNA	messenger ribonucleic acid
SD	short day photoperiod exposure 8 hours of light 16 hours of dark
TGF-	transforming growth factor beta

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Figure 1. Ovarian AMH

1a) Ovarian AMH mRNA levels during the estrus cycle. P (proestrus), E (estrus), DI (diestrus I) and DII (diestrus II). *1b*) Ovarian AMH mRNA levels during photoperiod induced regression/recrudescence. LD= long day exposure/control, SD= inhibitory short day exposure, PTw2= post transfer from SD to LD for 2 weeks, PTw8= post transfer from SD to LD for 8 weeks. *1c*) AMH immunostaining during photoperiod induced regression / recrudescence. Representative immunostaining histographs of ovarian sections from each photoperiod group are shown. Inset depicts negative control processed without primary antibody. All graphical results are presented as mean ± SEM, and groups with different letters are significantly different (p<0.05).

Figure 2. Ovarian Inhibin-

2a) Ovarian Inhibin- mRNA levels during the estrus cycle. P (proestrus), E (estrus), DI (diestrus I) and DII (diestrus II). **2b)** Ovarian Inhibin- mRNA levels during photoperiod induced regression/recrudescence. LD= long day exposure/control, SD= inhibitory short day exposure, PTw2= post transfer from SD to LD for 2 weeks, PTw8= post transfer from SD to LD for 8 weeks. **2c)** Inhibin- immunostaining during photoperiod induced regression / recrudescence. Representative immunostaining histographs of ovarian sections from each photoperiod group are shown. Inset depicts negative control processed without primary antibody. All graphical results are presented as mean $\pm$ SEM, and groups with different letters are significantly different ($p < 0.05$).

Figure 3. Ovarian GDF-9

3a) Ovarian GDF-9 mRNA levels during the estrus cycle. P (proestrus), E (estrus), DI (diestrus I) and DII (diestrus II). 3b) Ovarian GDF-9 mRNA levels during photoperiod induced regression/recrudescence. LD= long day exposure/control, SD= inhibitory short day exposure, PTw2= post transfer from SD to LD for 2 weeks, PTw8= post transfer from SD to LD for 8 weeks. 3c) GDF-9 immunostaining during photoperiod induced regression / recrudescence. Representative immunostaining histographs of ovarian sections from each photoperiod group are shown. Inset depicts negative control processed without primary antibody. All graphical results are presented as mean ± SEM, and groups with different letters are significantly different (p<0.05).

Figure 4. Ovarian BMP-15

4a) Ovarian BMP-15 mRNA levels during the estrus cycle. P (proestrus), E (estrus), DI (diestrus I) and DII (diestrus II). 4b) Ovarian BMP-15 mRNA levels during photoperiod induced regression/recrudescence. LD= long day exposure/control, SD= inhibitory short day exposure, PTw2= post transfer from SD to LD for 2 weeks, PTw8= post transfer from SD to LD for 8 weeks. 4c) BMP-15 immunostaining during photoperiod induced regression / recrudescence. Representative immunostaining histograms of ovarian sections from each photoperiod group are shown. Inset depicts negative control processed without primary antibody. All graphical results are presented as mean ± SEM, and groups with different letters are significantly different (p<0.05).

Table 1

Ovarian mass and estradiol production are affected by photoperiod exposure.

	LD	SD	PTw2	PTw8
Ovary weight (mg)	13.18 ± 1.8 ^a	6.01 ± 1.0 ^b	11.84 ± 1.6 ^a	12.20 ± 0.7 ^a
Serum estradiol (pg/ml)	85.99 ± 13.5 ^a	27.12 ± 2.8 ^b	42.89 ± 15 ^b	53.27 ± 11 ^a

Results are expressed as mean ± SEM. A subset of these data (LD, SD and PTw8) has been previously published in a companion study (Shahed and Young 2011).

Table 2

Photoperiod exposure affects dominant ovarian structures.

Ovarian Structure	LD	SD	PTw2	PTw8
Preantral	5.6 ± 1.4^a	1.93 ± 0.26^b	5.25 ± 1^a	5.16 ± 0.8^a
Antral Follicles	0.87 ± 0.2^a	0.12 ± 0.06^b	0.50 ± 0.1^a	1.26 ± 0.2^a
Corpora lutea	2.25 ± 0.46^a	0.04 ± 0.04^b	0.17 ± 0.1^b	2.15 ± 0.26^a
Lutenized atretic follicles	0.27 ± 0.12^a	6.05 ± 1.2^b	1.08 ± 0.4^a	0.75 ± 0.2^a

Ovarian sections (n=5-6 hamsters/group, 3 section/slide) were stained with eosin and hemotoxylin and follicles were counted. A subset of these data (PA, A, and CL data for LD, SD and PTw8) has been previously published in a companion study (Shahed and Young 2011).

Table 3

Average relative immunostaining index across ovarian structures, and overall immunostaining extent in the estrous cycle.

	Proestrus	Estrus	Diestrus I	Diestrus II
AMH				
Preantral	3.68 ± .2	3.67 ± 0.2	3.28 ± 0.5	2.67 ± .6
Antral	3.2 ± 0.4 ^a	2.53 ± 0.7 ^{a,b}	2.27 ± 0.5 ^{a,b}	0.93 ± 0.4 ^b
Corpora lutea	---	1.73 ± 0.5	1.07 ± 0.7	0.20 ± 0.1
Stroma CT	0.36 ± 0.1	0.53 ± 0.1	0.45 ± 0.0	0.13 ± 0.1
Stroma Ste	1.20 ± 0.4	1.47 ± 0.7	1.60 ± 0.4	0.40 ± 0.2
Overall Extent	2.70 ± 0.2	3.07 ± 0.6	2.27 ± 0.7	1.80 ± 0.6
Inhibin-				
Preantral	1.60 ± 0.5	2.80 ± 0.5	2.70 ± 0.2	2.10 ± 0.7
Antral	1.90 ± 0.7 ^a	2.30 ± 0.2 ^a	0 ± 0 ^b	1.1 ± 0.4 ^{a,b}
Corpora lutea	---	0.2 ± 0.2	0 ± 0	0 ± 0
Stroma CT	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Stroma Ste	0.42 ± 0.2	0.13 ± 0.1	0 ± 0	0.13 ± 0.1
Overall Extent	1.62 ± 0.32 ^{a,b}	2.66 ± 0.15 ^a	0.89 ± 0.22 ^b	1.24 ± 0.5 ^b
GDF-9				
Preantral	0.90 ± 0.1	0.95 ± 0.2	1.15 ± 0.5	0.43 ± 0.1
Antral	0.66 ± 0.1	0.72 ± 0.1	0.10 ± 0.1	0.23 ± 0.2
Corpora lutea	---	1.14 ± 0.1 ^{a,b}	1.35 ± 0.4 ^a	0.12 ± 0.1 ^b
Stroma CT	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Stroma Ste	0.93 ± 0.3	0.95 ± 0.1	1.11 ± 0.4	0.41 ± 0.2
Overall Extent	1.13 ± .1	1.09 ± 0.3	1.52 ± 0.5	0.28 ± 0.1
BMP-15				
Preantral	2.17 ± 0.4 ^{a,b}	2.67 ± 0.4 ^{a,b}	3.21 ± 0.2 ^a	2.00 ± 0.1 ^b
Antral	2.66 ± 0.4	3.06 ± 0.5	3.00 ± 0.5	2.27 ± 0.2
Corpora lutea	---	1.68 ± 0.9	2.23 ± 0.2	1.45 ± 0.3
Stroma CT	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Stroma Ste	1.50 ± 0.5	1.75 ± 0.2	1.42 ± 0.4	1.06 ± 0.5
Overall Extent	3.00 ± 0.6	3.14 ± 0.3	3.29 ± 0.3	2.57 ± 0.2

Average relative immunostaining intensity by ovarian structure and average overall extent of immunostaining across ovarian cross sections (n=3–6 per animal, 4–6 animals per group) for: AMH, Inhibin , GDF-9 and BMP-15 following during the estrus cycle. Both immunostaining intensity and extent were scored on a scale of 0–4. Stroma CT= stroma primarily consisting of connective tissue, Stroma Ste= stroma including potential steroidogenic interstitial glands. Values represented as mean (SEM). Groups with different letters are significantly different ($p < 0.05$).

Table 4

Average relative immunostaining index across ovarian structures, and overall immunostaining extent with different photoperiod exposure.

	LD	SD	PTw2	PTw8
AMH				
Preantral follicles	3.87 ± 0.1	3.07 ± 0.5	3.52 ± 0.5	2.87 ± 0.6
Antral follicles	1.17 ± 0.4 ^a	---	3.00 ± 0.4 ^b	1.67 ± 0.4 ^a
Corpora lutea	0.57 ± 0.4	---	1.10 ± 0.3	0.87 ± 0.2
Stroma CT	0.19 ± 0.1	0.56 ± 0.3	0.73 ± 0.2	0.33 ± 0.1
Stroma Ste	0.20 ± 0.1 ^a	1.37 ± 0.5 ^b	1.51 ± 0.1 ^b	0.73 ± 0.2 ^{a,b}
Overall Extent	1.39 ± 0.3 ^a	2.56 ± 0.4 ^{a,b}	3.29 ± 0.4 ^a	2.67 ± 0.4 ^{a,b}
Inh				
Preantral follicles	3.11 ± 0.4 ^a	1.70 ± 0.1 ^b	3.00 ± 0.5 ^a	3.90 ± 0.3 ^a
Antral follicles	3.83 ± 0.1	---	3.50 ± 0.3	3.42 ± 0.3
Corpora lutea	0 ± 0	---	0 ± 0	0 ± 0
Stroma CT	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Stroma Ste	0 ± 0	0 ± 0	0.29 ± 0.1	0.04 ± 0
Overall Extent	1.67 ± 0.4 ^a	0.70 ± 0.1 ^b	2.66 ± 0.1 ^c	2.55 ± 0.3 ^c
GDF-9				
Preantral follicles	2.51 ± 0.2 ^a	1.62 ± 0.2 ^b	2.39 ± 0.1 ^{a,b}	1.61 ± 0.2 ^b
Antral follicles	2.75 ± 0.3	---	3.04 ± 0.3	1.94 ± 0.2
Corpora lutea	2.59 ± 0.2 ^a	---	2.99 ± 0.5 ^a	1.43 ± 0.1 ^b
Stroma CT	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Stroma Ste	0.72 ± 0.3	1.63 ± 0.3	1.79 ± 0.6	1.08 ± 0.3
Overall Extent	2.96 ± 0.4	2.81 ± 0.3	3.13 ± 0.3	2.75 ± 0.2
BMP-15				
Preantral follicles	1.12 ± 0.4	1.23 ± 0.3	1.30 ± 0.3	1.22 ± 0.4
Antral follicles	1.33 ± 0.3	---	1.32 ± 0.1	1.30 ± 0.2
Corpora lutea	1.08 ± 0.2	---	1.50 ± 0.2	1.79 ± 0.4
Stroma CT	0.25 ± 0.1	0 ± 0	0 ± 0	0 ± 0
Stroma Ste	0.72 ± 0.3	1.63 ± 0.3	1.79 ± 0.6	1.07 ± 0.4
Overall Extent	2.30 ± 0.3	2.25 ± 0.4	2.19 ± 0.4	2.29 ± 0.4

Average relative immunostaining intensity by ovarian structure and average overall extent of immunostaining across ovarian cross sections (n=3–6 per animal, 4–6 animals per group) for: AMH, Inhibin, GDF-9 and BMP-15 following different photoperiod exposures. Both immunostaining intensity and extent were scored on a scale of 0–4. Stroma CT= stroma primarily consisting of connective tissue, Stroma Ste= stroma including potential steroidogenic interstitial glands. Values represented as mean (SEM). Groups with different letters are significantly different (p < 0.05).

Table 5

Primer Information

Name	Sequence	Tm	bp	References
AMH	F- gca tgg cca act ggt aca ct R- gat gtg tgt gtg agg cct tg	60	153	Primer sequence provided by Dr. Ned Place
Inhibin	F- ctg ccc tca aca tct cct tc R-ctc atg ctg cct ggt aga gc	60	211	Gen bank Acc. NM012590
GDF-9	F-gcg gtc agg cat cgg tat R-aat ggt caa cac gct caa gg	60	100	Wang and Roy (2006)
BMP-15	F- tat ttc ttg ccc cgt ctt tg R-atc ttg caa agc ttg gtg ct	56	111	Gen bank Acc. NM009757
HPRT-1	F- tga tca gtc aac agg gga ca R- ctg gcc gat atc caa cac tt	55	209	Shahed and Young, 2011