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# Assessing Recrudescence of Photoregressed Siberian Hamster Ovaries Using *In Vitro* Whole Ovary Culture

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## Abstract

*In vitro* culture has been used to study different aspects of ovarian function; however, this technique has not been applied to study recrudescence, or the return of ovarian function in seasonally breeding species. In Siberian hamsters, exposure to inhibitory photoperiods induces declines in ovarian function which are restored with photostimulation. Because these changes are mediated by changes in systemic gonadotropin (GT) secretion, we hypothesized that culturing photoregressed ovaries with GT would restore aspects of function and induce expression of key folliculogenic factors. Adult female Siberian hamsters were exposed to either long (LD; 16L:8D) or short day (SD, 8L:16D) photoperiods for 14 weeks to maintain *in vivo* cyclicity or induce gonadal regression, respectively. Isolated ovaries were then cultured for 10 days with or without GT. Ovarian mass and mRNA expression of mitotic marker *Pcna* were increased in cultured SD ovaries (cSD) ovaries with GT as compared to without GT, with no changes noted among cLD ovaries. Media estradiol and progesterone concentrations increased in both cLD and cSD ovaries cultured with GT as compared to without GT. No differences in follicle numbers or incidence of apoptosis were noted across groups. Additionally, differential mRNA expression of folliculogenic growth factors (*Bmp-4*, *Ntf-3*, *Inh*-α, *Gdf-9, Igf-1, Has-2 and Cox-2*) was observed in cSD treated with or without GT. Together, these results suggest that this *in vitro* model could be a useful tool to 1) study the return of function in photoregressed ovaries, and 2) to identify the specific roles folliculogenic factors play in ovarian recrudescence.

## Introduction

*In vitro* studies, including whole ovary organ culture, are useful tools to study ovarian function (Smitz et al. 2010). Fetal and neonatal ovaries (Baker and Neal, 1973, Eppig and O'Brien, 1996, Challoner, 1975, Salehnia et al. 2016) and isolated follicles (Cain et al. 1995, Hayashi et al. 1999) have been cultured for 2–14 days showing the necessity of gonadotropins for follicular growth. *In vitro* exposure to gonadotropins promotes both folliculogenesis and steroidogenesis in addition to growth factor release in cultured ovaries (reviewed in: Devine et al. 2002). Neonatal ovarian culture has also been used to investigate

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both primordial follicle recruitment and assembly (Durlinger et al. 2002; Chen et al. 2007), and the effect of various growth factors and hormones on primordial and primary follicle development (Nilsson et al. 2001, Kezele et al. 2002, Nilsson and Skinner, 2003, 2004; Chen et al. 2013). While the factors regulating the initiation of folliculogenesis have been studied broadly in neonatal and pubertal models, less is known about restoring function in reproductively quiescent adult ovaries. We sought to develop an *in vitro* whole ovarian culture system to identify the factors involved in the restoration of adult ovarian function in photoregressed ovaries, using a natural model of photostimulated recrudescence.

Seasonally breeding animals reduce their reproductive function following exposure to inhibitory photoperiod regimes, whereas exposure to stimulatory photoperiod promotes recrudescence of gonadal function (Buchanan and Yellon 1991, Glass, 1986). In Siberian hamsters (*Phodopus sungorus*), exposure to less than 12 hours of light per day inhibits the hypothalamic pituitary gonadal (HPG) axis, ultimately reducing circulating follicle stimulating hormone (FSH) and luteinizing hormone (LH) concentrations (van den hurk et al., 2002, Glass, 1986, Buchanan and Yellon, 1991, Salomon et al., 2018). As a result, short photoperiod exposure induces declines in folliculogenesis, estradiol production, and ovarian mass (Moffatt-Blue et al. 2006, Salverson et al. 2008, Schlatt et al. 1993). These regressed ovaries are substantially different both functionally and morphologically from ovaries in reproductively active Siberian hamsters; folliculogenesis does not progress past the secondary follicle stage, no tertiary follicles or corpora lutea are present, and clusters of hypertrophied granulosa cells (hgc, aka luteinized atretic follicles) which may represent atretic secondary follicles are noted (Moffat-Blue et al. 2006; van den Hurk et al., 2002; Zysling et. al., 2012; Salomon et al., 2018). Subsequent transfer of reproductively regressed Siberian hamsters to photoperiods longer than 12 hours of light restores the HPG axis, with increases in plasma FSH and estradiol, ovarian mass, tertiary follicle development and corpora luteal formation noted 1–8 weeks post transfer to stimulatory long photoperiod (Salverson et al., 2008, Shahed et al., 2015a, Salomon et al., 2018). These *in vivo* studies suggest that FSH is a key mediator in photostimulated resumption of ovarian function; indeed, *in vivo* FSH injections to photoregressed Siberian hamsters induce increases in ovarian mass (Zysling et al., 2012), further conveying the importance of gonadotropin stimulation to ovarian recrudescence.

While gonadotropins are vital to the process of recrudescence, intraovarian growth factors also play an essential role. These factors are essential in the overall function of the ovary, and are particularly important in follicle recruitment, selection, and maturation (Fortune, 2003, Atwood and Vadakkadath Meethal, 2016, Richards, 2005). Both gonadotropins and intraovarian factors respond quickly *in vivo* when female photoregressed hamsters are transferred to stimulatory long days; plasma follicle stimulating hormone (FSH) concentrations, ovarian FSH receptor (*Fshr*) and inhibin α (*Inh-α*) mRNA expression increases significantly following four days of long photoperiod stimulation, suggesting the importance of these factors in the recrudescence process (Shahed and Young, 2015a). Additionally, *in vivo* mRNA expression of intraovarian growth factors such as anti-Müllerian hormone (*Amh*), bone morphogenic protein (*Bmp-15*), growth differentiation factor-9 (*Gdf-9*), vascular endothelial growth factor (*Vegfa*), angiopoietin-2 (*Ang-2*),

epidermal growth factor receptor (*Egfr*), and amphiregulin (Shahed and Young, 2013, 2015b) change with photoperiod in Siberian hamsters.

*In vitro* culture has been used to establish the role of several growth factors such as bone morphogenic protein −4 and −7 (*Bmp-4, Bmp-7*) (Nilsson and Skinner 2003; Lee et al. 2001), fibroblast growth factor 2 (*Fgf-2,* aka basic fibroblast growth factor *bFgf*), and kit ligand (*Kitl*) (Nilsson and Skinner, 2004) in the initiation of folliculogenesis, primordial follicle development, or the transition to primary follicles. Growth differentiation factor −9 (*Gdf-9*), an oocyte derived growth factor, also promotes early follicle growth, transition, and differentiation, in addition to oocyte maturation (Cook-Andersen 2016; reviewed in: Fortune, 2003, Hayashi et al. 1999). Later follicular growth is regulated by FSH, either directly with stimulating mitotic granulosa cell growth, or via the production of inhibin by the granulosa cells. Inhibin can be used as a marker of follicular growth and plays a role in preovulatory follicular selection (Knight and Glister, 2006). The final phase of folliculogenesis is ovulation, a complex multistep process, promoted by the LH surge. As part of the ovulatory cascade, LH induces the expression of matrix deposition proteins, hyaluronic acid synthase-2 (*Has-2*) and tumor necrosis factor gene-6 (*Tsg-6*), along with cyclooxygenase-2 (*Cox-2*), a key factor in follicle rupture (Richards, 2005). These *in vitro* studies have established the importance of specific folliculogenic factors in cycling ovaries and during the prepubertal initiation of the folliculogenic process (Fortune, 2003, Atwood and Vadakkadath Meethal 2016).

Although *in vitro* culture of whole ovaries, isolated follicles, and isolated ovarian cells have been previously reported, culture has not been used to study the return of ovarian function in photoregressed ovaries. Because photoregressed ovaries are both functionally and morphologically different than normal cycling ovaries, neonatal, or embryonic ovaries, they may respond differently in a culture system. To better tease apart the contribution of intraovarian and other factors, we sought to develop an *in vitro* whole ovary culture model using photoregressed ovaries from adult hamsters provided with gonadotropin stimulation. In the present study, photoregressed ovaries along with cycling ovaries as controls were cultured for 10 days, a time empirically determined by preliminary culture and *in vivo* results (Shahed et al., 2015a) in the presence and absence of gonadotropins. Ovary weight, media estrogen and progesterone concentrations, and mRNA expression of proliferating cell nuclear antigen (*Pcna*) were measured as markers for ovarian growth and function. Expression of intraovarian growth factors known to participate in key steps in folliculogenesis were assessed.

## Results

### Ovarian mass declined with *in vivo* SD exposure and increases with *in vitro* culture with gonadotropins

Exposure of Siberian hamsters to 14 weeks to short days (SD; 8 hours of light per day: 16 hours of dark) resulted in a 52.9% *in vivo* reduction in ovarian mass (3.00 ± 0.2 mg) as compared to hamsters exposed to long days (LD; 16L:8D; 6.37 ± 0.7 mg), ensuring that photoregression occurred prior to culture. Following 10 days of culture, there was no significant difference between cultured long day exposed ovaries (cLD) with (+GT) or

without gonadotropin (-GT) treatment (Figure 1A); however, the difference between photoperiod exposure remained, with cLD ovaries weighing significantly more than cSD ovaries, regardless of *in vitro* GT treatment (Figure 1A). While GT treatment did not restore cSD ovary mass to cLD levels, culture with GT did induce a significant increase (50.8%) in ovarian mass in cSD+GT as compared to cSD-GT ovaries (Figure 1A). As an additional measure to monitor *in vitro* ovarian growth, mRNA for the mitotic marker *Pcna* was measured in ovaries after 10 days in culture. GT treatment did not alter *Pcna* mRNA expression between cLD groups (Figure 1B); however, *Pcna* mRNA increased significantly in cSD+GT group as compared to the cSD-GT ovaries (Figure 1B).

## No changes in follicle type or apoptosis were noted in LD and SD ovaries cultured for 10 days with and without gonadotropins

Histological assessment of hematoxylin and eosin stained cultured ovarian cross sections revealed primordial, primary, and secondary follicles. Large antral follicles were not observed in either the cLD or cSD ovaries, regardless of gonadotropin treatment, and corpora lutea were observed only rarely among LD animals with gonadotropin treatment, but not in SD animals regardless of treatment (Figure 2). Clusters of hypertrophied granulosa cells, the unique, potentially atretic secondary follicles that characterize photoregressed Siberian hamster ovaries (van den Hurk et al., 2002; Moffatt-Blue et al., 2006, Park et al., 2014) were also noted among the SD, but not LD ovaries (Figure 2). When follicles were quantified, no significant differences were observed in the number of either primordial, primary, or secondary follicles across photoperiod or gonadotropin treatment groups (Figure 3). No differences in number of hypertrophied granulosa cell clusters were observed among cultured SD ovaries with and without gonadotropin treatment (Figure 3). While atretic/irregular follicles were noted, no differences in number were observed across photoperiod or gonadotropin treatment (Figure 3). TUNEL labeling denoting early-to-mid stages of apoptosis, was predominantly observed in granulosa or stromal cells, although staining levels across all groups were low, despite abundant staining obtained with the positive controls (Figure 4). Immunostaining for germ cell marker MSY2 revealed oocytes in all culture groups (Figure 5).

## Estrogen and progesterone concentrations increased in media from LD and SD ovaries cultured with gonadotropins

During the 10 days of ovarian culture, media was changed every 48h resulting in four sets of media for each of the four groups. While media estradiol concentrations in the cLD-GT and cSD-GT groups were low, and remained unchanged across all time points, culture with FSH induced a gradual increase in media estradiol concentrations that peaked on culture day 8 (prior to LH addition; data not shown). At culture day 10, media estradiol concentrations did not differ between cLD-GT and cSD-GT groups; however, media estradiol increased significantly 5- to 6-fold with gonadotropin treatment in the cLD+GT and cSD+GT groups (Figure 6A). Media progesterone concentrations did not differ between cLD-GT and cSD-GT; however, progesterone concentrations increased in both cLD+GT and cSD +GT groups on day 8 (data not shown) and day 10 of culture. On culture day 10, no significant difference was observed in media progesterone concentration between the cLD-GT and cSD-GT

groups, although gonadotropin treatment significantly increased media progesterone 8-fold in the cLD+GT group and 4-fold in the cSD+GT group (Figure 6B).

### Differential expression of *Bmp4*, *Ntf-3*, *Kitl*, and *Fgf-2* mRNA was observed in LD and SD ovaries cultured with and without gonadotropins

After 10 days of culture, mRNA expression of genes involved in primordial to primary follicle transition and/or growth of primary follicles were measured by real time PCR. *Bmp-4* mRNA was expressed in both cLD-GT and cLD+GT groups, but no significant difference among these cLD groups was observed with the addition of gonadotropins (Figure 7A). *Bmp-4* mRNA was expressed at significantly lower levels in cSD-GT as compared to cLD-GT ovaries; however, was elevated more than 5-fold with gonadotropin stimulation in cSD+GT ovaries when compared to cSD-GT (Figure 7A). While gonadotropin treatment did not alter expression of neurotrophin 3 (*Ntf-3)* mRNA between the cLD-GT and cLD+GT groups; *Ntf-3* mRNA expression was increased in the cSD+GT as compared to cSD-GT ovaries (Figure 7B). Fibroblast growth factor 2 (*Fgf-2)* mRNA was present in all four groups, with significantly lower levels observed with gonadotropin stimulation in the cLD+GT as compared to the cLD-GT group (Figure 7C). In contrast, no difference was observed in *Fgf-2* mRNA expression between cSD-GT and cSD+GT ovaries (Figure 7C). Finally, no significant differences were noted across photoperiod or GT treatment in *Kitl* mRNA expression levels (Fig. 5D).

### Gonadotropin treatment altered expression of *Inh-α*, *Gdf-9* and *Igf-1* mRNA in cultured LD and SD ovaries

At 10 days of culture, mRNA expression for genes involved in mid-follicular development was assessed. Expression of *Inh-α* mRNA in both cLD+GT and cSD+GT groups increased significantly as compared to cLD-GT and cSD-GT ovaries, with no differences noted across photoperiods (Figure 8A). *Gdf-9* mRNA expression did not change with gonadotropin treatment in cLD+GT as compared to cLD-GT ovaries, although *Gdf-9* expression was significantly higher in cSD+GT compared to cSD-GT ovaries (Figure 8B). Insulin like growth factor 1 (*Igf-1)* mRNA expression was significantly higher in cLD-GT when compared to cSD-GT ovaries and was significantly higher in both cLD+GT and cSD+GT as compared to cLD-GT and cSD-GT groups, respectively (Figure 8C).

### Gonadotropin treatment during culture differentially affected expression of *Tsg-6, Has-2* and *Cox-2* mRNA in LD and SD ovaries

Expression of factors critical for late aspects of folliculogenesis revealed differences across photoperiod and gonadotropin treatment groups (Figure 9). *Tsg-6* mRNA was expressed in all cultured ovary groups (Figure 9A). At day 10 of culture, *Tsg-6* mRNA expression increased significantly in gonadotropin treated cLD+GT group compared to the untreated cLD-GT group, but no differences between cSD-GT and cSD+GT groups were noted (Figure 9A). *Cox-2* mRNA expression did not change with gonadotropin treatment among cLD ovaries, although cLD *Cox-2* expression was higher in cLD groups as compared to levels observed in cSD-GT ovaries (Figure 9B). In contrast, the lower levels of Cox-2 mRNA expression in the cSD-GT ovaries increased with gonadotropin treatment in the cSD

+GT group (Figure 9B). *Has-2* mRNA expression was significantly higher in both cLD-GT and cSD-GT as compared to cLD+GT and cSD+GT ovaries, respectively (Figure 9C).

## Discussion

This study presents an *in vitro* culture model using photoregressed ovaries to investigate recrudescence of ovarian function. Photoregressed ovaries cultured in the presence of gonadotropins for 10 days (8 days with FSH alone and 2 days with FSH+LH), responded with increases in ovarian mass, estradiol and progesterone production and *Pcna* mRNA expression as compared to photoregressed ovaries cultured without GT. Furthermore, mRNA expression of intra-ovarian growth factors involved in various stages of follicle development; *Bmp-4*, *Fgf-2*, *Ntf-3*, *Inh-α*, *Ggf-9*, *Igf-1*, *Tsg-6, Has-2, Cox-2* but not *Kitl* showed differential expression in ovaries cultured with or without gonadotropins. Two-way analysis of the data shows that the effect of *in vivo* short day exposure persists in cSD ovaries for 10 days without gonadotropins, but is partially ameliorated in gonadotropin treated ovaries when compared to similarly treated cLD ovaries.

Consistent with previous *in vivo* studies, ovaries from adult Siberian hamsters housed for 14 weeks in SD were photoregressed, as demonstrated by reductions in ovarian mass as compared to ovaries from hamsters housed in stimulatory LD (Salverson et al. 2008, Shahed and Young, 2011). *In vivo* transfer of photoregressed Siberian hamsters to LD stimulation increases plasma FSH and estradiol concentrations, ovarian mass, antral follicle development and ovulation (Shahed et al., 2015a, Salomon et al. 2018, Salverson et al. 2008, Zysling et al., 2012), with increases in both plasma FSH concentration and *fshr* mRNA noted after just four days post transfer from SD to LD photoperiods (Shahed et al., 2015a). While the intraovarian mechanism of *in vivo* photostimulated recrudescence has not been fully elucidated, less is known about the survival or growth of cultured photoregressed ovaries. In the current study, atretic/irregular follicles were noted under the present cultured conditions, although there were no differences across groups and both cSD and cLD ovaries showed low numbers of TUNEL positive cells after 10 days. *In vivo* SD exposure does increase TUNEL labeling and caspase-3 in Siberian hamster ovaries; however, apoptosis levels peak after three weeks in SD, and are at low levels in ovaries from hamsters exposed to either LD or SD conditions for 12 weeks (Moffat-Blue et al., 2006). These data suggest that levels of ovarian apoptosis in the current study after 14 weeks of SD *in vivo* photoperiod exposure would be expected to be low.

When photoregressed Siberian hamsters are transferred to stimulatory LD *in vivo*, plasma FSH and intra-ovarian FSH and LH receptor mRNA expression increased within 10 days of photostimulation (Shahed et al. 2015a), suggesting that regaining gonadotropin responsiveness is a key aspect of photostimulated recrudescence. Developing a culture model using photoregressed ovaries is key to better understand the impact of gonadotropins on the photostimulated return of ovarian function. The presence of FSH in the culture media can be protective against ovarian apoptosis (Hsueh et.al., 1994), and may have encouraged growth among the cSD+GT ovaries. In the current *in vitro* study, ovarian mass increased 50.8% when photoregressed cSD ovaries were cultured for 10 days with gonadotropins as compared to those cultured without gonadotropins, while no change was observed in

similarly treated cLD ovaries (Figure 1). This increase may be a result of follicle growth; however, as no differences were observed in the number of follicles of any type, it is likely to include expansion of either current secondary follicles responsive to FSH or stromal tissue, as reflected by the increased mRNA expression of established proliferation marker, *Pcna* (Kubben et al. 1994). Changes in preantral follicle numbers are not noted in Siberian hamsters with *in vivo* exposure to short or long photoperiod, as it appears that the photoperiod-mediated changes in the HPG axis affect tertiary follicle development and ovulation and not early stages of folliculogenesis (Salomon et al., 2018). While similar results for preantral numbers occurred in the current *in vitro* and previous *in vivo* studies, increases in later folliculogenic development beyond the secondary follicle stage were not observed in either cLD or cSD ovaries under the present experimental protocol. Although a precise explanation for this observation is elusive and needs further investigation, we speculate that the length of culture duration in the present protocol with FSH (8 days) alone may not be sufficient, particularly for the cSD ovaries to support growth beyond the secondary follicle stage. Photoregressed ovaries are different than cycling ovaries in many respects; reductions in mass, increases in atresia, a lack of tertiary follicles and corpora lutea, and reduced estradiol production typify ovaries from hamsters exposed to SD. In addition, SD ovaries show altered mRNA expression of key folliculogenic and steroidogenic factors compared to ovaries from LD exposed Siberian hamsters (Salverson et al. 2008, Shahed and Young 2013, Shahed et al. 2015b). In normal mouse ovaries cultured with FSH, antrum formation was observed after three days (Boland et al., 1993), suggesting that our model of Siberian hamster ovaries differs from cultured ovaries from other cycling rodents. The addition of LH along with FSH on culture day 8 could potentially interfere with follicle growth beyond the secondary stage; the addition of hCG or LH along with FSH to culture media induced follicular degeneration within a few days, but not with media with FSH alone (Cain et al. 1995). The addition of LH in the current study also increased production of progesterone among cultured ovaries (Figure 6B) and increases in progesterone can reduce *in vitro* ovarian follicle growth (Peluso, 2006, Ting et al., 2015). For example, injecting golden hamsters (*Mesocricetus auratus*) with progesterone and gonadotropins *in vivo* reduced the number of antral follicles observed (Kim and Greenwald, 1987). Therefore, the presence of LH and the resultant increase in progesterone in the current *in vitro* culture study may have impeded follicular growth beyond secondary follicles.

Production of ovarian sex steroids, estradiol and progesterone, is vital to reproductive function. Estradiol is produced by granulosa cells in conjunction with theca cells, whereas progesterone is produced by the interstitium, theca interna and granulosa cells (Saidapur and Greenwald 1978). Estrogen and progesterone production *in vitro* peaks in the presence of gonadotropins (Roy and Greenwald, 1987; Gutierrez et al. 1997; McNatty et al. 1979) but can also be produced with or without the addition of FSH or LH to the culture media (Funkenstein et al. 1980, Uilenbroek et al., 1983). Our previous *in vivo* studies have shown that plasma estradiol concentrations and mRNA expression of steroidogenic enzymes in Siberian hamster ovaries decline with exposure to SD photoperiod as compared to higher levels in both cycling LD hamsters and those undergoing photostimulated recrudescence (Shahed et al. 2015 a, b). In contrast, changes in photoperiod exposure do not appear to alter plasma progesterone concentrations in hamsters *in vivo* (Moffatt-Blue et al, 2006). Although

low levels of both estradiol and progesterone were noted in the media from ovaries cultured without gonadotropins (cLD-GT and cSD-GT) in the current study, both increased significantly with gonadotropins in both cLD and cSD ovaries (Figure 3). The significant increases in media sex steroids in both cLD and cSD ovaries cultured with gonadotropins suggests that our model of whole ovarian culture recapitulates some aspects of natural ovarian function, and the significant difference in progesterone concentrations between cLD +GT and cSD+GT also demonstrates that photoregressed ovaries may react differently than cycling cLD ovaries to gonadotropin stimulation.

The recruitment and transition of primordial to primary follicles is a critical part of the folliculogenic process. There is ample *in vivo* and *in vitro* evidence suggesting a prominent role of intraovarian growth factors *Bmp-4, Ntf-3*, *Kitl*, and *Fgf-2* in early folliculogenesis in functional ovaries (Adhikari and Liu, 2009, 2015; Nilsson and Skinner, 2003, 2004; Lee et al., 2001); however, little is known about the role of these factors in photoregressed ovaries. The addition of exogenous *Bmp-4* in 4-day old rat ovaries in culture for 2 weeks increases the primordial to primary follicle transition and survival (Nilsson and Skinner, 2003), and in the present study, *in vitro* gonadotropin stimulation promoted both *Bmp-4* mRNA expression (Figure 7A) and early follicle development in cSD photoregressed ovaries (Figure 3), suggesting that this intraovarian factor may be important for *in vivo* recrudescence. Several neurotropins (NGF, BDNF, and NTF-3, −4, −5) and their tyrosine kinase receptors are expressed in the ovary and also function in the transition of primordial to primary follicles (Dissen et al., 2009, Adihikari and Liu, 2009). Similarly, in cultured Siberian hamster ovaries, *Ntf-3* mRNA expression increased in photoregressed ovaries cultured with gonadotropin, as opposed to cSD-GT ovaries; and again, no changes were observed in *Ntf-3* mRNA among cLD ovaries (Figure 7B). *Kitl* and *Fgf-2* promote the primordial to primary follicle transition in cultured 4-day old mouse ovaries (Parrott and Skinner, 1999; Nilsson et al., 2001; Nilsson and Skinner 2004); however, in the current study, the addition of gonadotropins did not affect *Fgf-2* mRNA expression in photoregressed cSD ovaries and reduced the level of *Fgf-2* mRNA expression among cLD ovaries (Figure 7C). Similarly, ovarian *Fgf-2* mRNA expression decreases *in vivo* as FSH increases in Siberian hamsters are transferred from inhibitory to stimulatory photoperiods for 2 to 10 days (Shahed et al., 2015). While the cause of this decline is unknown, it may be that the timing of collection following gonadotropin stimulation influences *Fgf-2* mRNA expression, as *in vivo* GnRH treatment increased *Fgf-2* mRNA in bovine preovulatory follicles at 4 hours but that increase had declined by 10 hours (Berisha et al., 2006). In the present study, *Fgf-2* mRNA was assessed after 10 days of culture with the final gonadotropin treatment occurring on day 8. Although *Kitl* mRNA was expressed in the cultured cLD and cSD ovaries in the presence or absence of gonadotropins, no significant changes in expression were noted across photoperiod or gonadotropin treatments (Figure 7D). These results mirror *in vivo* observations in Siberian hamsters where photoperiod changes have no effect on the mRNA expression of multiple early folliculogenic factors, including *Kitl* and its receptor *cKit* (Salomon et al., 2018). Additional studies using whole ovarian culture of photoregressed ovaries may be able to tease apart the individual contributions of intraovarian factors and gonadotropins to the process of recrudescence.

Members of the TGF-β superfamily, including inhibin and *Gdf-9*, play a significant role in folliculogenesis (reviewed in: Knight and Glister, 2006). Expression of the *inh-α* subunit and *Gdf-9* mRNA are inversely affected by photoperiod *in vivo*; *Inh-α* mRNA expression is reduced by SD exposure and increases after photostimulation, whereas *Gdf-9* increases in photoinhibited ovaries and declines as the ovary returns to function (Shahed and Young, 2013). The present results show that *Inh-α* mRNA expression increases after 10 days in culture in both cSD+GT and cLD+GT as compared to without gonadotropin treatment (Figure 8A), consistent with the *in vivo* observations. The results are also consistent with the role of these growth factors in stimulating follicle growth and survival. Another granulosa cell produced factor, *Igf-1* enhances the response of granulosa cells to FSH by upregulating *Fshr* expression (Zhou et al. 1997), and, in the mouse ovary, appears to be active predominantly when follicle growth enters the FSH dependent stage (Monget and Bondy, 2000). After ten days of culture with gonadotropins, both cycling cLD and photoregressed cSD ovaries show an increase in *Igf-1* mRNA when compared to groups cultured without FSH and LH (Figure 8), although the amount of intraovarian *Ifg-1* mRNA was significantly higher among cLD ovaries. The effects of *Igf-1*on folliculogenesis are species dependent (Monget and Bondy, 2000), and although the effect of *Igf-1*on recrudescence of photoregressed Siberian hamster ovaries is unknown, it may act to increase the responsiveness of granulosa cells to the increase in both local (Shahed and Young, 2011) and systemic FSH (Shahed, et al., 2015) that occurs as part of the resumption of ovarian function.

The LH surge upregulates a number of genes as part of the ovulatory process including, *Cox-2*, *Has-2*, and *Tsg-6*; these three genes are involved in matrix formation and cumulus cell expansion critical to ovulation (reviewed in Richards, 2005). Interestingly, culture with gonadotropins increased *Tsg-6* mRNA expression only among cLD ovaries, increased *Cox-2* mRNA expression only among photoregressed cSD ovaries and decreased *Has-2* mRNA expression in both cLD and cSD ovaries (Figure 9). These observations are partially similar to those reported for rat preovulatory follicle culture; where *Tsg-6*, *Cox-2*, and *Has-2* mRNA expression all increased 6 h after the addition of LH in culture; however, only *Tsg-6* and *Cox-2* mRNA expression remained elevated at 9 h (Ashkenazi et al. 2005). Because ovarian recrudescence is a process that takes weeks (Salverson et al., 2008), the culture period in the current study is protracted. These genes are typically involved in the relatively short-term process of ovulation, and therefore chronic gonadotropin stimulation may not affect normal cycling cLD ovaries after 10 days in culture. In contrast, the addition of FSH and LH to the photoregressed ovaries may have a greater impact on some genes to allow for rapid return to function with the onset of the gonadotropin signal. Changing the time-frame used in the current culture model could potentially distinguish the roles of these periovulatory genes during recrudescence.

In conclusion, the results of this investigation suggest that a whole ovary *in vitro* culture model is a viable tool to study extra- and intra-ovarian factors that may mediate recrudescence of adult photoregressed Siberian hamster ovaries. Our results corroborate and expand the conclusions from prior *in vivo* work that suggest that photoregressed ovaries are different than cycling ovaries in more ways than anovulation and reduced estradiol production. Expression of many intraovarian factors was enhanced with gonadotropin

treatment only in photoregressed ovaries for multiple genes, highlighting potentially enhanced sensitivity to FSH and LH in SD ovaries. Further studies involving longer culture durations with FSH alone and ablation/replacement of ovarian growth factors are needed to enhance follicle growth beyond the secondary follicles observed under the conditions of this study. Finally, because the mRNA expression of many of the folliculogenic factors studied in the present paper is similar to that reported in *in vivo* studies in Siberian hamsters, our *in vitro* model could be a useful tool to study ovarian recrudescence and could serve to potentially identify specific roles that intraovarian growth factors play in the photostimulated return to ovarian function.

## Methods and Materials

### 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 protocols #316 and #357. All animals were housed in individual polypropylene cages prepared with bedding and tap water and were given *ad libitum* access to food (Laboratory Rodent Diet 5001 Purina, Brentwood, MO). After two weeks of acclimation, hamsters were divided in two groups: the long day (LD) group (n=16) was exposed to 16 hours of light per day: 8 hours of dark, which maintains ovarian function, and the short day (SD) group (n=16) was exposed to 8L:16D for 14 weeks, which induces reproductive regression.

### Whole ovary culture

Hamsters from both LD and SD groups were euthanized at 14 weeks of photoperiod exposure following ketamine/xylazine intra-peritoneal anesthesia/analgesia (Moffatt-Blue et al., 2006; Salverson et al., 2008) and ovaries were removed at necropsy under clean, decontaminated conditions. Extracted ovaries were cleaned, weighed, and placed in sterile complete media containing Hyclone DME/F-12 1:1 (1X), 0.1% Albumax (cell culture grade, Gibco Life Technologies, Waltham, MA), 0.05mg /ml ascorbic acid (ACROS Organics, ThermoFisher, Waltham, MA), 0.1% Bovine serum albumin (Sigma, St. Louis, MO), ITS complex (insulin, transferrin, selenium 100×) and penicillin-streptomycin. Ovaries were cultured in 24-well plates on 3.0μM 24-well cell culture inserts (BD Falcon, Corning, NY). Inserts were placed in each well containing 0.8ml of culture media and equilibrated in a humidity-controlled incubator (37°C, 95% $O_2$ and 5% $CO_2$) between the two surfaces. Because *in vivo* transfer of photoregressed Siberian hamsters to stimulatory long days increases recrudescence markers such as ovarian mass, follicle growth, plasma FSH, estradiol concentration after 10 days, ovaries in the present study were cultured for a 10-day duration. Two ovaries from the same hamster were placed on each insert and covered with an additional drop of media. Ovaries from *in vivo* LD or SD exposed hamsters were cultured with or without gonadotropins (GT) in one of four groups: cSD+GT (regressed ovary experimental group), cSD-GT (control for GT treatment), cLD+GT (control for photoperiod exposure) and cLD-GT (control for photoperiod and GT treatment). Groups treated with gonadotropins were cultured in complete media supplemented with 250ng/ml follicle stimulating hormone (FSH, human pituitary, Sigma, St. Louis, MO) alone for 8 days and

then 100ng/ml luteinizing hormone (LH, human pituitary, Sigma, St. Louis, MO) + FSH was added to the media on the 8$^{th}$ day, with ovary collection on the 10$^{th}$ day. Groups without gonadotropins were similarly cultured, but with complete media alone. Media for all groups was replaced every 48 hours, with collected media stored for estradiol and progesterone measurements. On culture day 10, ovaries were removed from the culture inserts, weighed, and one ovary was fixed in Bouin's solution for histology and the contralateral ovary was flash frozen in liquid nitrogen and stored at −80°C for RNA extraction.

### Histological processing

Following fixation in Bouin's solution, ovaries for histological analysis were embedded in paraffin wax (n=32), the entire ovary was serially sectioned at 6μm, and sections were mounted onto Superfrost Plus slides (ThermoFisher Scientific, Waltham, MA) with each section separated by at least by 60μm. For each analysis, sections from across the entire ovary were used. Slides were deparaffinized and stained with hematoxylin and eosin using standard techniques to allow for follicle counts across 5–6 cross sections taken from different locations across each ovary. Primordial (one layer of squamous granulosa cells), primary follicles (one layer of cuboidal granulosa cells) and secondary follicles (two or more layers of cuboidal granulosa cells) were quantified along with hypertrophied granulosa cell clusters and atretic/misshapen/irregular follicles (Uslu et al., 2017); no antral follicles were identified, and corpora lutea were only sporadically noted.

### TUNEL Assay for Apoptosis

To ensure that the culture system was not inducing substantial increases in programmed cell death, the degree of apoptosis was assessed by *in situ* terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) using the TACS•XL Blue Label Kit (Trevigen Inc., Gaithersburg, MD), following manufacturer's instructions. Briefly, cross sections were deparaffinized, hydrated in PBS, and then incubated and washed as specified with proteinase K, TdT label, BrDU antibody, and blue label in succession, followed by counterstaining with nuclear fast red. Both positive (pre-treated with DNA-fragment inducing TACS-Nuclease) and negative (without the BrDU antibody/ without the TdT enzyme) controls were conducted alongside experimental slides.

### MSY2 Immunostaining

To visualize oocytes within the cultured ovary sections, immunohistochemical staining for germ cell marker MSY2 (Y-box-binding protein 2 YBX2; Gu et al; 1998) was conducted across ovarian cross sections. Slides were deparaffinized in xylene, rehydrated through a graded series of ethanol solutions and washed in phosphate buffered saline (PBS). Citra Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) was then used for antigen retrieval. Tissue was washed in PBS, placed in 3% hydrogen peroxide/methanol solution, then blocked using normal horse serum (Vector Laboratories). Sections were then treated with MSY2 polyclonal antibody (1:200, anti-MSY2 NBP2–19422 Novus Biologicals, Littleton, CO) and subsequently developed using the Vectastain Elite ABC Kit (Vector Laboratories) as per manufacture's protocol. The Vector NovaRED Substrate Kit (Vector Laboratories) was used to detect protein immunostaining, followed by counterstaining with hematoxylin.

### Estrogen and progesterone concentrations

Estradiol concentrations were measured in duplicate in the media samples collected every 48h using an Estradiol ELISA kit according to manufacturer's protocol (n=20 animals; Cayman Chemical, Ann Arbor, MI). Progesterone concentrations were measured in duplicate on day 10 of culture using a progesterone ELISA kit from Cayman Chemical (Cayman Chemical, Ann Arbor, MI) as used by others (Ocón-Grove et.al., 2012). Prior to the assay, undiluted and different dilutions of the media were used in the assay to ensure that amounts calculated were reflective of the dilutions and that B/B0 ratios were within the (20–80%) range suggested in the kit. Briefly, all samples were assayed in duplicate using 50µl of the media. Culture media samples from the same collection day were all run on the same plate along with a standard curve. Hormone concentrations were calculated using the analysis tools on the Cayman Chemical website. $R^2$ values from the standard curves were 0.98 and 0.93 for estradiol and progesterone, respectively. Intra-assay coefficient of variation (%CV) values that represent results obtained are listed by the manufacturer as 7.8– 10.8 for estradiol and 6.9– 7.3 for progesterone.

### Total RNA extraction and cDNA synthesis

Total RNA was extracted from cultured ovaries using Trizol LS reagent (n=20; Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's directions and as described previously (Shahed and Young, 2013). Following RNA extraction, 1µg of total RNA (260/280 ratio >1.6) was examined on an RNA agarose gel to ensure lack of degradation, treated with DNase to remove DNA contamination, then processed for cDNA synthesis using ImProm Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's directions in a total volume of 20 µl (Shahed and Young 2011). The resulting cDNA was then diluted 1:5 with RNase DNase free water and used for real time PCR analysis.

### Relative real time PCR

The relative real time PCR analysis was done on an Mx3000P thermocycler (Stratagene/ Agilent, Santa Clara, CA) using Absolute QPCR SYBR green mix (ThermoFisher Scientific, Waltham, MA). Briefly, the PCR reaction mix contained 1µl cDNA (1:5 dilution of cDNA transcribed using 1µg total RNA) + 1 µl each of forward and reverse primers (80nM concentration) + 6µl SYBR green mix + 3 µl DNase, RNase free water to a total volume of 12 µl. PCR cycles consisted of 15 min hold at 95°C (1 cycle), then 40 amplification cycles at an empirically-determined Tm (Table 1), extension (1 min at 72°C) followed by dissociation. Melt curves for all products were produced to identify the presence of non-specific products. Non-template negative controls and a standard curve for each gene were included in the same plate for each run. In addition, real time PCR products were analyzed on agarose gels to confirm that the correct size 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 created by pooling equal aliquots of cDNA from all samples, diluting the pool five-fold. This curve was included on plates for each gene of interest and for the reference gene (Shahed and Young 2011). The relative amounts of mRNA were calculated using standard curves of each gene of interest and the

values from each sample for the reference gene glyceraldehyde 3-phosphate dehydrogenase *(Gapdh)* (Shahed and Young, 2013). The mRNA expression of *Gapdh* mRNA did not change significantly among groups ($p>0.05$). Data are presented as mean ± SEM of the ratio of the mRNA expression of the gene of interest to the mRNA expression of *Gapdh*.

### Statistical analysis

Results were analyzed by Prism software using two-way ANOVA with Newman-Keul's or Holm-Sidak's multiple comparison tests used across the two photoperiod groups (cLD and cSD) and two treatments (-GT and +GT). Media estradiol and progesterone data across culture days were assessed using a two-way repeated measures ANOVA. Hypertrophied granulosa cell clusters are only present among SD ovaries, therefore counts for only the two SD groups were analyzed using a Student's t test. Progesterone data and all mRNA data were log transformed prior to analysis to reduce variability. A $p<0.05$ was considered statistically significant. Results are presented as mean ± SEM.

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## Abbreviations

| | |
|---|---|
| **Ang-2** | Angiopoietin-2 |
| **Amh A** | Anti-Müllerian hormone |
| **Bmp-4** | Bone morphogenic protein-4 |
| **cLD** | cultured long day group |
| **cSD** | cultured short day group |
| **Cox-2** | Cyclooxygenase |
| **Egfr** | Epidermal growth factor receptor |
| **Fgf-2** | Fibroblast growth factor 2 |
| **bFgf** | aka basic fibroblast growth factor |
| **FSH** | Follicle stimulating hormone |
| **Gapdh** | Glyceraldehyde 3-phosphate dehydrogenase |
| **GT** | Gonadotropins |
| **GnRH** | Gonadotropin releasing hormone |

| | |
|---|---|
| **Gdf-9** | Growth differentiation factor 9 |
| **hgc** | Hypertrophied granulosa cells |
| **HPG** | Hypothalamic pituitary gonadal axis |
| **Igf-1** | Insulin-like growth factor 1 |
| **Inh-α** | Inhibin-α |
| **Kitl** | Kit ligand |
| **LH** | Luteinizing hormone |
| **Ntf-3** | neurotrophin 3 |
| **Pcna** | Proliferating cell nuclear antigen |
| **Tsg-6** | Tumor necrosis factor gene-6 |
| **TUNEL** | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| **Vegfa** | Vascular endothelial growth factor a |

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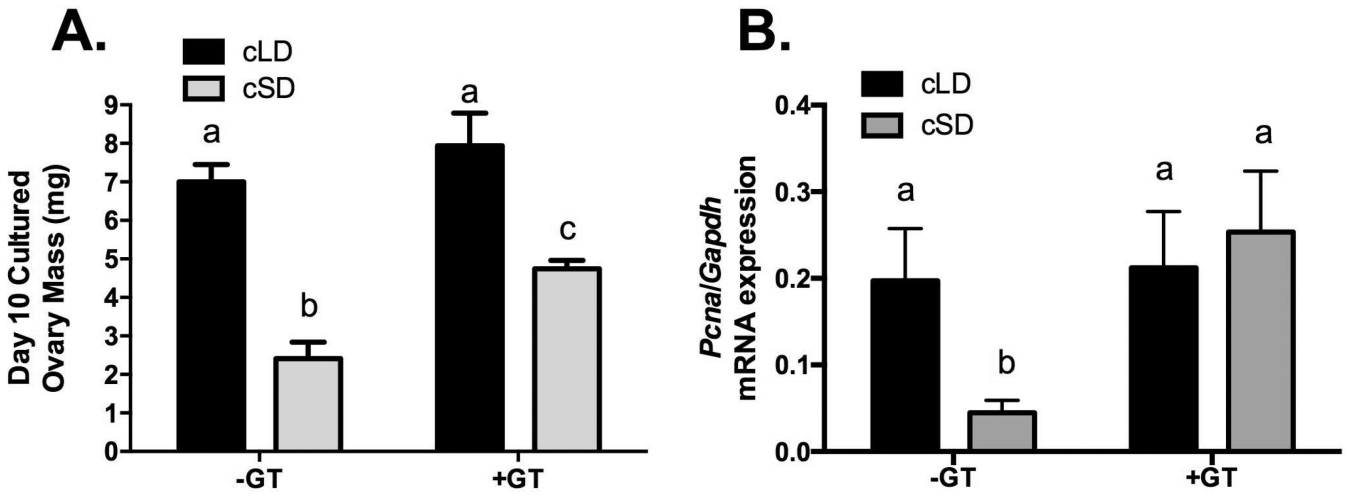


**Figure 1. Ovarian mass PCNA mRNA expression increased in SD ovaries cultured with gonadotropins.**

*A*) mean paired ovarian mass and *B)* mean *Pcna* mRNA expression following 10 days in culture without (-GT) or with (+GT) gonadotropins in ovaries from females who were exposed to *in vivo* long (LD, black bars) or short (SD, gray bars) days for 14 weeks prior to culture. Data are presented as mean ± SEM, columns with different letters indicate significant differences between groups ($p < 0.05$).

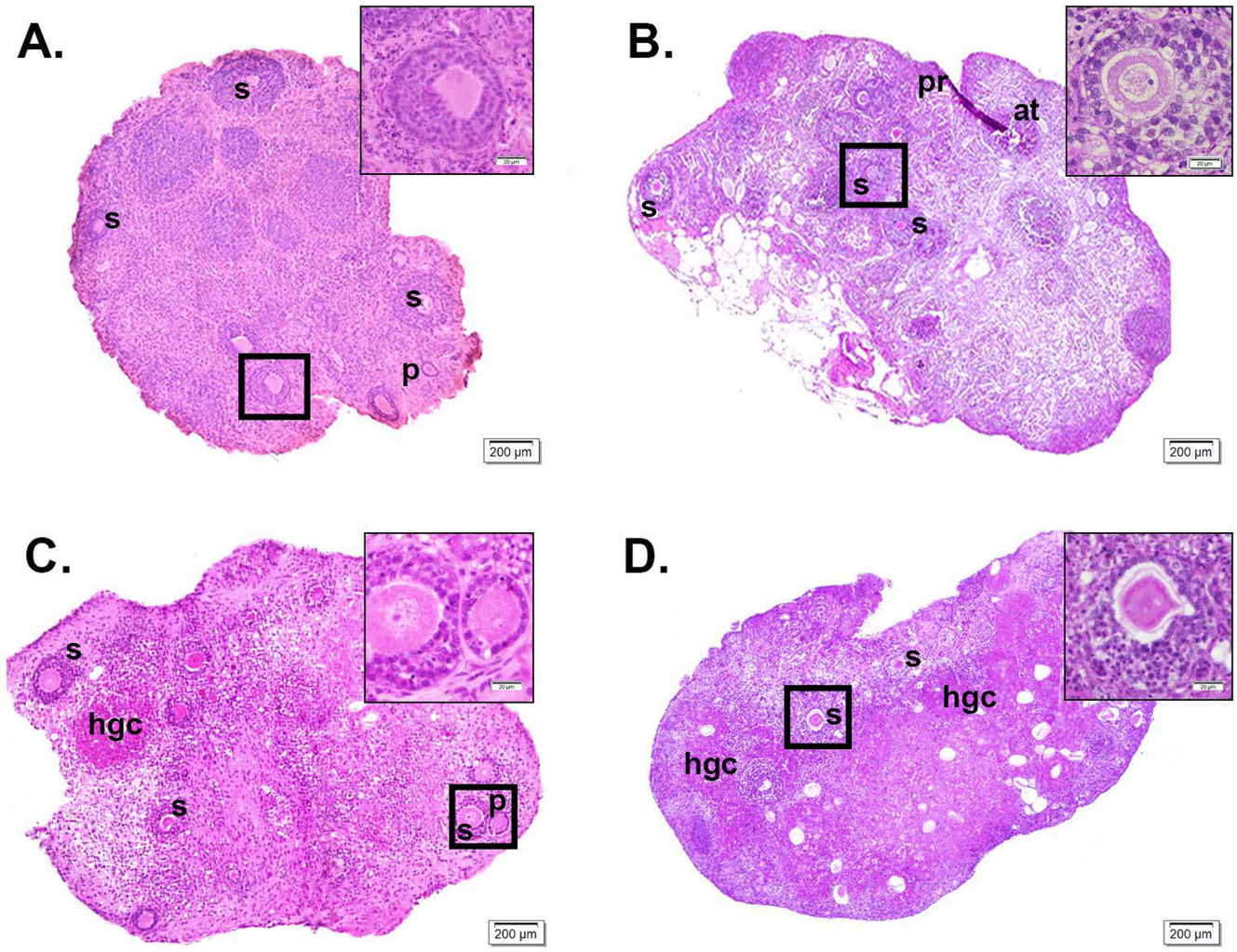


**Figure 2. Hematoxylin and eosin staining of representative ovarian cross sections of LD and SD ovaries cultured for 10 days revealed early follicle development.**

*A)* long day cycling ovaries cultured without gonadotropins (cLD-GT), *B)* long day cycling ovaries cultured with gonadotropins (cLD+GT), *C)* short day regressed ovaries cultured without gonadotropins (cSD-GT), *D)* short day regressed ovaries cultured with gonadotropins (cSD+GT). Insets represent a higher magnification photo of the follicle indicated in the square to show detail. Primordial follicles (pr), primary follicles (p), secondary follicles (s), atretic/irregular follicles (at), hypertrophied granulosa cell clusters (hgc).

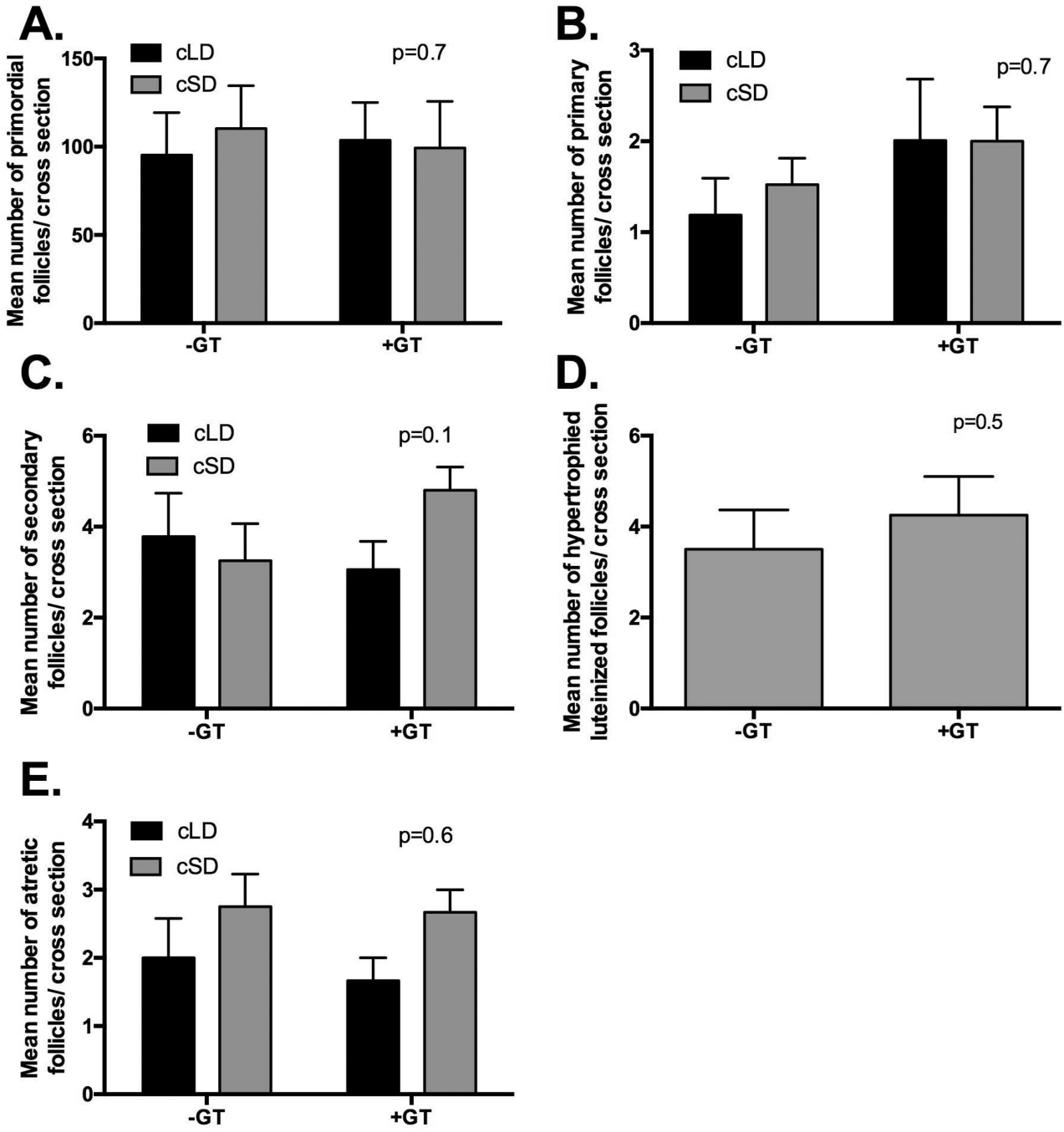


**Figure 3. Follicle counts (average number/section) in either cLD or cSD ovaries did not change significantly after 10 days of culture.**

*A*) primordial follicles, *B)* primary follicles, *C)* secondary follicles, *D)* hypertrophied granulosa cell clusters, and *E)* atretic, misshapen or irregular follicles. Counts were made across serially sectioned ovaries from hamsters exposed to long (LD) or short (SD) days for 14 weeks, then cultured with (+GT) or without (-GT) gonadotropin for 10 days. Hypertrophied granulosa cell clusters were only observed among SD ovaries, therefore only SD data are shown. Data are presented as mean ± SEM, columns with different letters indicate significant differences between groups ($p < 0.05$).

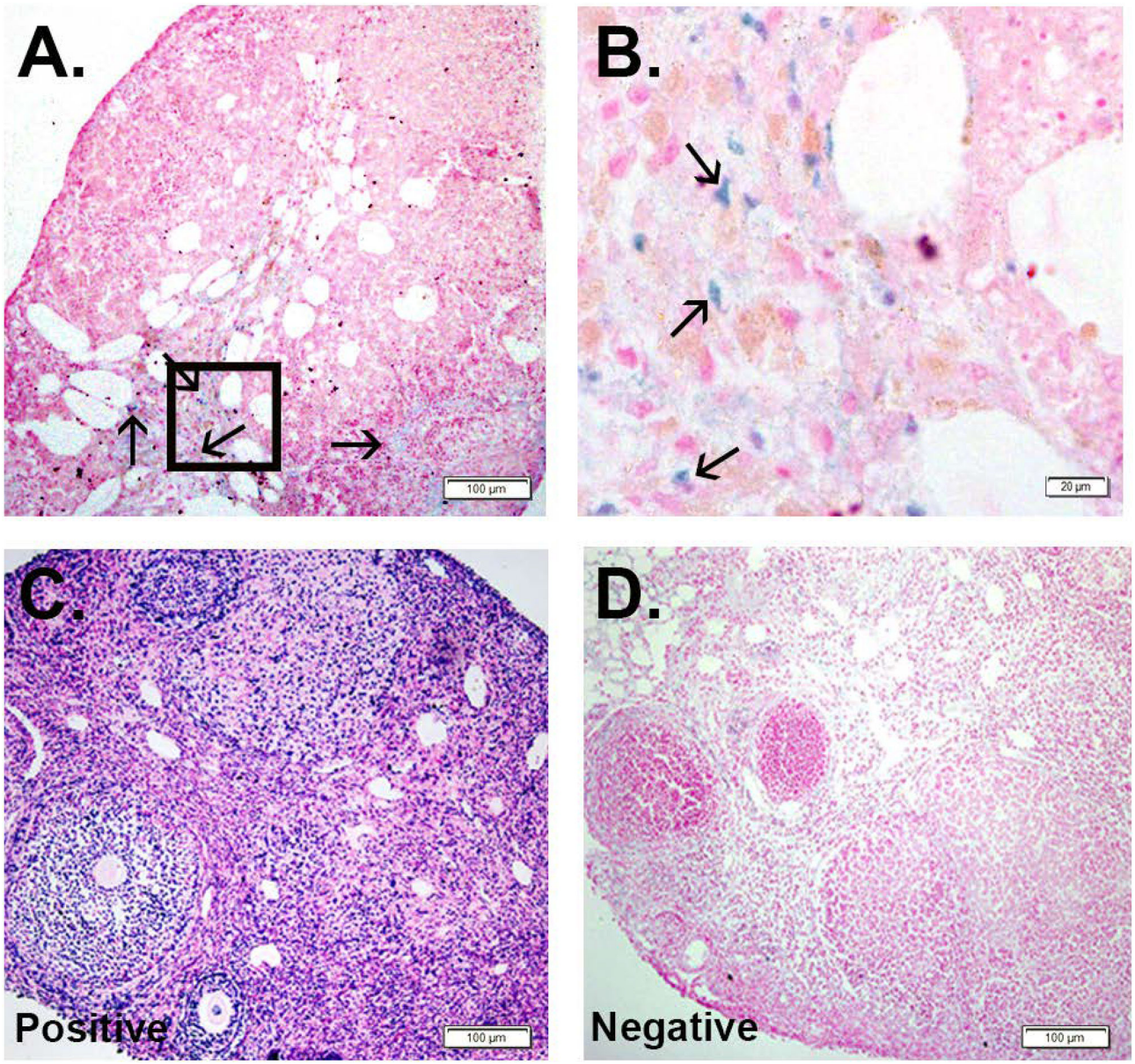


**Figure 4. Ovarian cross sections showed low levels of labeling for apoptotic cell death.**

*A)* a representative cross section from a cSD+GT ovary with TUNEL positive cells *B)* individual TUNEL-positive granulosa cells from square in panel A. *C)* positive control generated with TACS-nuclease *D)* negative control processed without the BrDU antibody. Arrows indicate TUNEL positive granulosa and stromal cells in panels A and B.

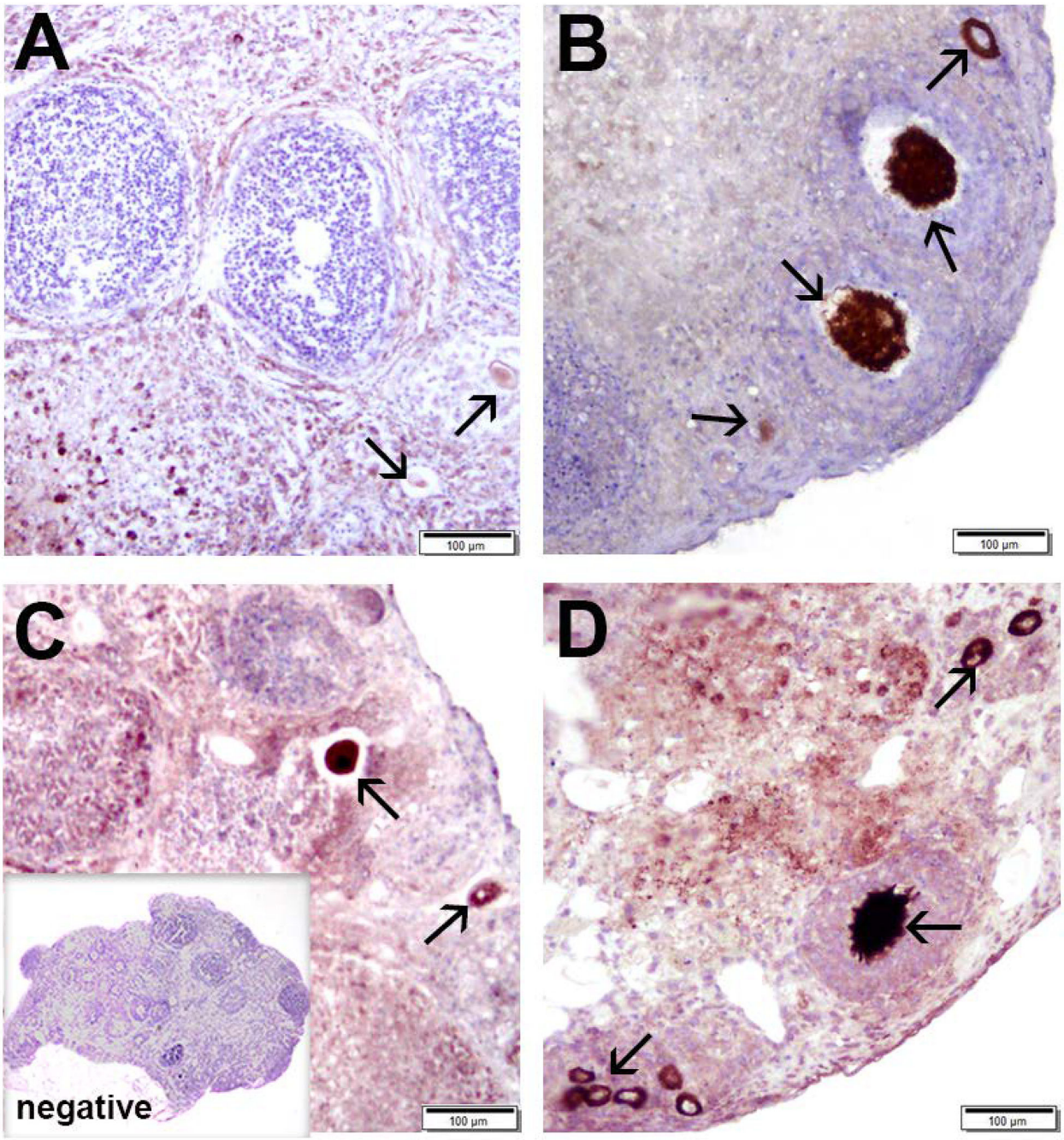


**Figure 5. Immunohistochemical staining of oocyte marker MSY2 was present in cross sections from ovaries after 10 days of culture across four culture groups.**

*A)* long day cycling ovaries cultured without gonadotropins (cLD-GT), *B)* long day cycling ovaries cultured with gonadotropins (cLD+GT), *C)* short day regressed ovaries without gonadotropins (cSD-GT), *D)* short day regressed ovaries cultured with gonadotropins (cSD +GT). Inset depicts negative control processed without primary antibody. Arrows indicate positive MSY2 oocyte staining.

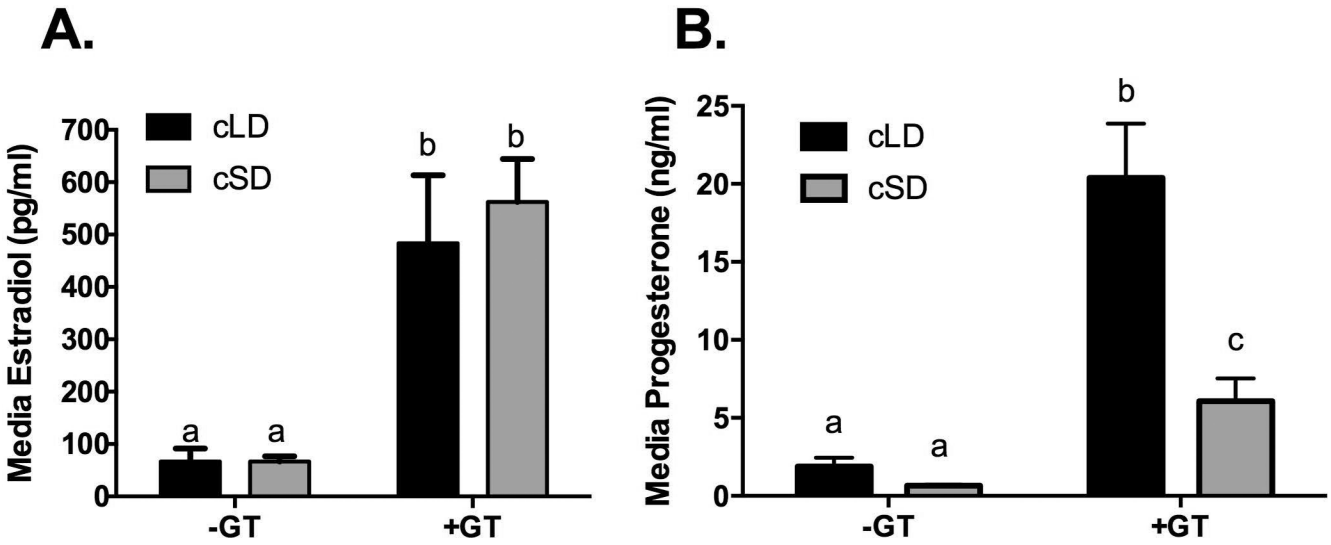


**Figure 6. Estradiol and progesterone concentration increased significantly in the media from cLD+GT and cSD+GT on day 10 of culture.**

Mean concentration of *A)* estradiol (pg/ml) and *B)* progesterone (ng/ml) in media collected on day 10 of culture of ovaries from females exposed to 14 weeks of long (LD) or short (SD) days. Ovaries were cultured with (+GT) or without (-GT) gonadotropins. Data are presented as mean ± SEM, columns with different letters indicate significant differences between groups ($p < 0.05$).

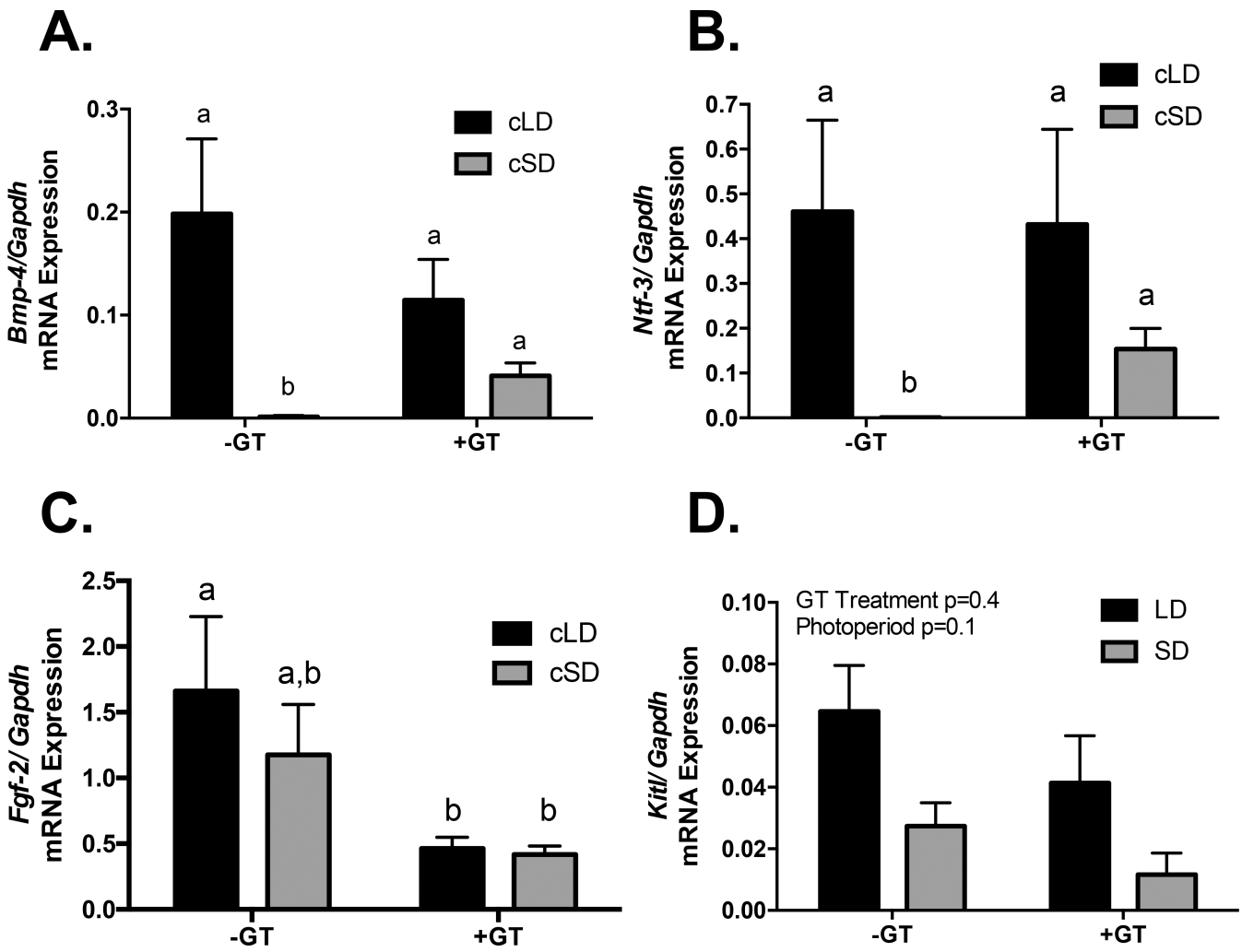


**Figure 7. Ovarian mRNA expression of genes involved in the primordial to primary follicle transition and/or growth of primary follicles differed across long day (LD) and short day (SD) ovaries after 10 days of culture with (+GT) or without (-GT) gonadotropins.**

Mean mRNA expression of *A)* bone morphogenic protein 4 (*Bmp-4*), *B)* neurotrophin 3 (*Ntf-3*), *C)* Fibroblast growth factor 2 (*Fgf-2*), and *D)* kit ligand (*Kitl*). Graphical results are presented as mean ± SEM, relative to *Gapdh*, and columns with different letters are significantly different ($p < 0.05$).

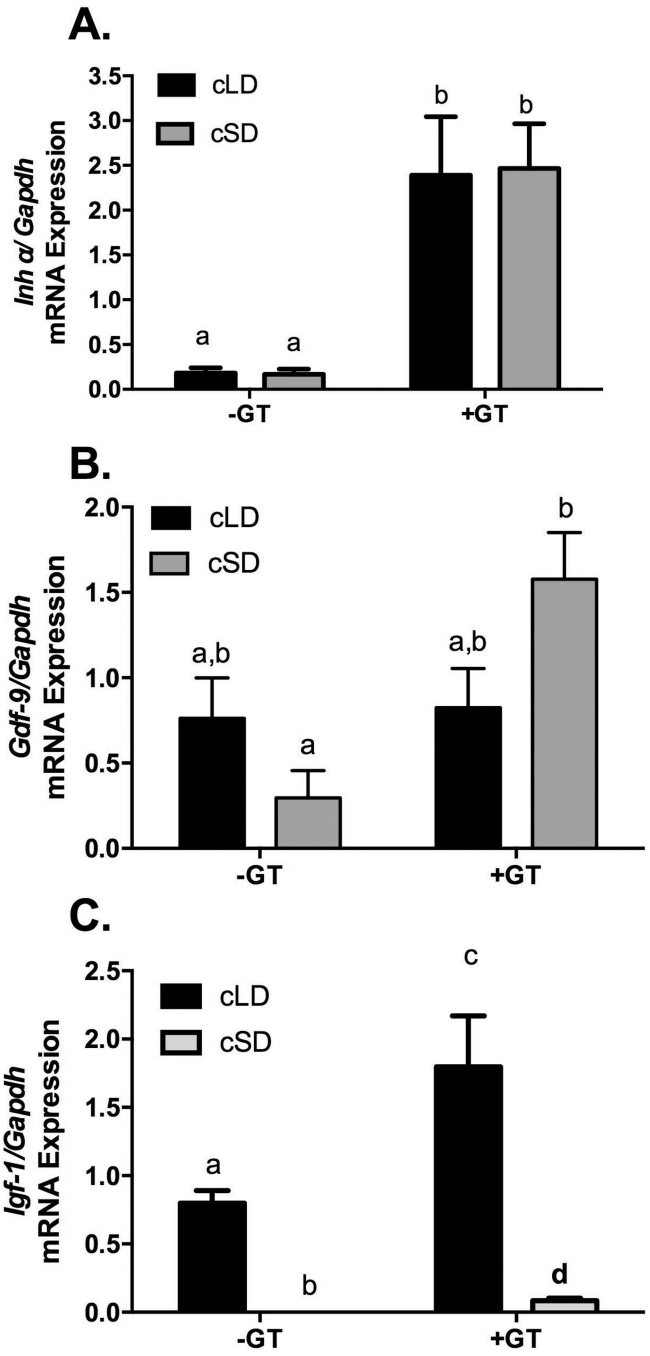


**Figure 8. Ovarian mRNA expression of factors involved in follicle development differed across long day (LD) and short day (SD) ovaries after 10 days in culture with (+GT) or without (-GT) gonadotropins.**

Mean mRNA expression of *A)* inhibin-α (*Inh*-α), *B)* growth differentiation factor 9 (*Gdf-9*), and *C)* insulin like growth factor 1 (*Igf-1*). Graphical results are presented as mean ± SEM, relative to *Gapdh*, and columns with different letters are significantly different ($p < 0.05$).

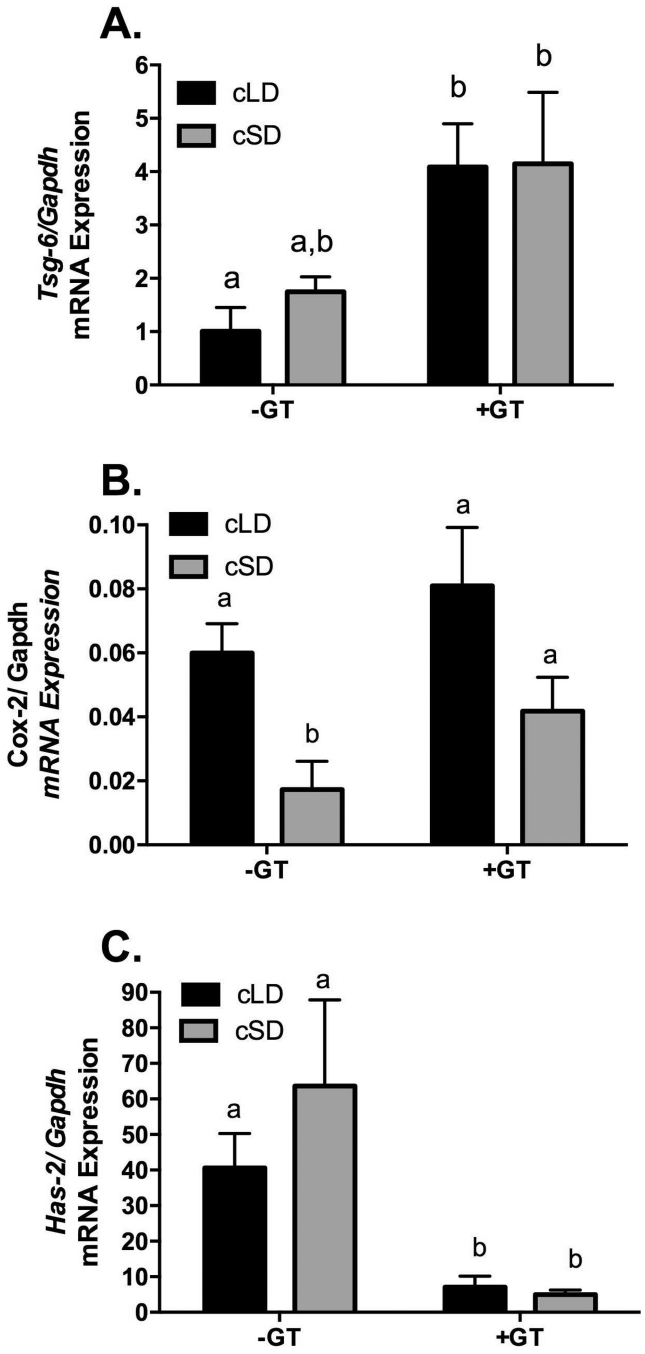


**Figure 9. Divergent patterns of ovarian mRNA expression for factors important in late stages of follicular development were noted between long day (LD) and short day (SD) ovaries after 10 days of culture with (+GT) or without (-GT) gonadotropins.**

Mean mRNA expression of: *A)* tumor necrosis factor gene-6 (*Tsg-6*), *B)* Cyclooxygenase-2 (*Cox2*), and *C)* hyaluronic acid synthase-2 (*Has-2*). Graphical results are presented as mean ± SEM, relative to *Gapdh*, and columns with different letters are significantly different ($p < 0.05$).

**Table 1:**

Real Time PCR Primer Parameters

| Gene | Forward | Reverse | Tm°C | References |
|---|---|---|---|---|
| *bFgf* | GCTGCTGGCTTCTAAGTGTG | CCAACTGGAGTATTTCCGTGA | 60 | Shahed et al. 2015a |
| *Bmp-4* | GCAACCCAGCCTGAGTATCT | TCGCTCCGAATGGCACTAC | 56 | *NM_007554.3* |
| *Cox-2* | CAACTCCCTTGGGTGTGA | TCCTCGTTTCTGATCTGTCT | 56 | Salomon et al., 2018 |
| *Gapdh* | GGAGAAAGC TGCCAAGTA | TGTCAT TGAGAGCGATGC | 55 | *Shahed et al. 2013* |
| *Gdf-9* | GCGGTCAGGCATCGGTAT | AATGGTCAACACGCTCAAGG | 60 | Shahed and Young 2013 |
| *Igf-1* | CTGGTGGATGCTCTTCAGTTC | CCAGTCTCCTCAGATCACAGC | 65 | Salomon et al., 2018 |
| *Inh-α* | CTGCCCTCAACATCTCCTTC | CTCATGCTCCCTGGTAGAGC | 60 | Shahed and Young 2013 |
| *Kitl* | GAATCTCCGAAGAGGCCAGA | GCTGCAACAGGGGGTAACAT | 58 | Salomon et al., 2018 |
| *Ntf-3* | CCCCGTCAGCCAGGATAATG | TCCATGCTGTTGCCTTGGAT | 60 | *NM_008472* |
| *Pcna* | AGCACTCGTATTTGAAGCACCA | TCACCAGAAGGCATCTTTACCA | 62 | Shahed et al. 2015a |
| *Tsg-6* | ATTTGAAGGTGGTCGTCTCG | TCGTACTCATTTGGGAAGCC | 55 | *Yoshino et al. 2006* |