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Matrix metalloproteinase inhibition influences aspects of photoperiod stimulated ovarian recrudescence in Siberian hamsters

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Abstract

Blocking matrix metalloproteinase (MMP) activity *in vivo* with inhibitor GM6001 impedes photostimulated ovarian recrudescence in photoregressed Siberian hamsters. Since direct and indirect effects of MMPs influence a myriad of ovarian functions, we investigated the effect of *in vivo* MMP inhibition during recrudescence on ovarian mRNA expression of steroidogenic acute regulatory protein (StAR), 3 β -hy-droxy steroid dehydrogenase (3 β -HSD), Cyp19a1 aromatase, epidermal growth factor receptor (EGFR), amphiregulin (Areg), estrogen receptors (Esr1 and Esr2), tissue inhibitors of MMPs (TIMP-1,-2,-3), proliferating cell nuclear antigen (PCNA), vascular endothelial growth factor A (VEGFA), its receptor VEGFR-2, and angiopoietin-2 (Ang-2). Female Siberian hamsters were randomly assigned to one of four photoperiod groups: stimulatory long (LD) or inhibitory short (SD) photoperiods, or transferred from SD to LD for 2 weeks (post-transfer, PT). Half of the PT hamsters were injected (ip) daily with GM6001 (PTG). SD exposure reduced ovarian StAR, 3 β -HSD, Cyp19a1, Esr1, Esr2, TIMPs 2–3, PCNA, VEGFR-2 and Ang-2 mRNA expression ($p < 0.05$), and 2 weeks of photostimulation restored mRNA expression of 3 β -HSD and PCNA and increased Areg and VEGFA mRNA expression in the PT group. GM6001 treatment during photostimulation (PTG) increased TIMP-1, -2 and -3 and PCNA mRNA, but inhibited Areg mRNA expression compared to PT. Neither photoperiod nor GM6001 altered EGFR expression. Results of this study suggest that *in vivo* inhibition of MMP activity by GM6001 may impede ovarian recrudescence, particularly follicular growth, in two ways: (1) *directly* by partially inhibiting the release of EGFR ligands like Areg, thereby potentially affecting EGFR activation and its downstream pathway, and (2) *indirectly* by its effect on TIMPs which themselves can affect proliferation, angiogenesis and follicular growth.

Keywords

Photoperiod; Recrudescence; MMP; TIMPs; EGFR

1. Introduction

Seasonal reproduction takes advantage of photoperiodic and other environmental cues to optimize reproductive effort. Siberian hamsters (*Phodopus sungorus*) respond to changes in photoperiod, food accessibility, and temperature to physiologically induce or cease reproductive function. In these hamsters, long photoperiods (>12 h of light per day) stimulate or maintain reproduction, whereas exposure to short photoperiods (<12 h of light per day) restricts the hypothalamic–pituitary–gonadal (HPG)¹ axis, and decreases the secretion of gonadotropin-releasing hormone (GnRH). This reduction in GnRH subsequently reduces secretion of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and the lack gonadotropin support effectively compromises both ovarian folliculogenesis and steroidogenesis (Schlatt et al., 1993). Exposure of adult, female Siberian hamsters to short photoperiods for 14 weeks reduces ovarian mass, estradiol concentrations, and both antral follicle and corpora lutea numbers (Moffatt-Blue et al., 2006), whereas transfer of photoregressed hamsters to LD for 2–8 weeks gradually restores normal reproductive activity (Salverson et al., 2008). This photostimulated ovarian recrudescence corresponds with differential expression of matrix metalloproteinases (MMPs; Salverson et al., 2008) and can be impeded with daily *in vivo* administration of pan-MMP inhibitor GM6001, as compared to non-treated or vehicle-treated (DMSO) controls (Whited et al., 2010). However, how MMPs may act to impede the return to ovarian function is not fully established.

Matrix metalloproteinases are Zn⁺ dependent endopeptidases that cleave proteins in the extracellular matrix (ECM). Degradation and synthesis of the ECM is vital for normal cyclic ovarian function; MMPs themselves are associated with processes such as follicle growth, ovulation, granulosa cell proliferation, and corpus luteum formation/degradation (Smith et al., 2002; Curry and Osteen, 2003). Indeed, blocking MMPs can prevent ovulation in primates, where preovulatory follicles injected with HCG and GM6001 fail to ovulate 72 h post injection, unlike vehicle-injected follicles (Peluffo et al., 2011).

The mechanism behind MMP activity affecting ovarian processes is multi-pronged. MMPs, via their proteolytic action, can promote the release of growth factors and cytokines critical for ovarian activity following ECM degradation, and this aspect of MMP activity can promote granulosa cell survival, proliferation, and differentiation (Curry and Osteen, 2003; Berkholtz et al., 2006). Cell proliferation, as assessed by the marker proliferating cell nuclear antigen (PCNA), fluctuates across the ovarian cycle (Kubben et al., 1994; Gaytan et al., 1996), and may respond to MMP activity. In addition to steroidogenic cell proliferation, endothelial cells increase in number in the cycling ovary, particularly during the process of new blood vessel formation, angiogenesis. Rapid angiogenesis occurs across the normal ovarian cycle, and resulting vessels provide oxygen and nutrients for follicular development

¹List of abbreviations: 3 β -HSD, 3- β hydroxysteroid dehydrogenase; Areg, amphiregulin; Ang-2, angiopoietin-2; CL, corpus luteum/corpora lutea; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; Esr1, estrogen receptor alpha; Esr2, estrogen receptor beta; ECM, extracellular matrix; FSH, follicle stimulating hormone; HPG, hypothalamic pituitary gonadal axis; i.p., intraperitoneal; LD, long photoperiod exposure 16 h light per day; LH, luteinizing hormone; MMP, matrix metalloproteinases; PCNA, proliferating cell nuclear antigen; SD, short photoperiod exposure 8 h light per day; StAR, steroidogenic acute regulatory protein; TIMP, tissue inhibitors of matrix metalloproteinases; VEGFA, vascular endothelial growth factor A; VEGFR-2, vascular endothelial growth factor receptor-2.

and corpus luteum formation (Macchiarelli et al., 2010). MMPs are also involved in angiogenesis; their ECM cleavage promotes entry of endothelial cells and may promote the release of angiogenic factors, such as vascular endothelial growth factors (VEGFs) and angiopoietins (Rundhaug, 2005).

MMP activity is also linked to ovarian steroidogenesis (Carbajal et al., 2011; Park et al., 2004; Jamnongjit et al., 2005). Inhibition of MMP2 and MMP9 by doxycycline and galardin (GM6001) inhibits LH-induced steroid production in mouse preovulatory follicles *in vitro* (Carbajal et al., 2011). Steroidogenesis in cumulus granulosa and thecal cells requires paracrine signaling from the release of MMP-mediated membrane bound EGFR ligands amphiregulin, epiregulin and betacellulin which, in turn, can stimulate the phosphorylation and activation of the EGFR receptor and induce a downstream cascade essential for ovulation and cumulus expansion (Carbajal et al., 2011; Liu et al., 2013; Jamnongjit et al., 2005). In addition to sex steroid production, estrogen itself can influence MMPs via its receptors. In mice, estrogen working through ER α , can inhibit MMP-9 activity in mouse brains (Zheng et al., 2015), while ER β activity can increase MMP-1 promoter activity in joints (Thaler et al., 2014).

Regulation of MMP activity is partially controlled by interaction with the endogenous tissue inhibitors of MMPs (TIMPs). Widely expressed, TIMPs inhibit MMP activity by forming 1:1 covalent complexes with MMPs, although the TIMPs themselves differ in their expression pattern and affinity for different MMPs (Moore et al., 2012; Boujrad et al., 1995; Lambert et al., 2004; Li and Curry, 2009). To date, four TIMPs have been identified, with TIMP-1, -2, and -4 released in the extracellular space, and TIMP-3 bound to ECM (Brew et al., 2000; review Brew and Nagase, 2010). In addition to MMP inhibition, TIMP activity can affect overall ovarian function, in part by influencing key ovarian processes. TIMPs can limit angiogenesis by reducing the response of endothelial cell to growth factors (Johnson et al., 1994; Stetler-Stevenson and Seo, 2005). Gonadal steroidogenesis is upregulated by a TIMP-1/procathepsin L complex in both Leydig cells and granulosa cells (Boujrad et al., 1995), although TIMP-1 can also inhibit progesterone production (Nothnick, 2003). It is therefore likely that inhibition of MMPs in the recrudescing ovary may alter TIMP expression and may also indirectly compromise proliferation, steroidogenesis, and angiogenesis.

MMPs influence many cellular processes that are directly or indirectly crucial to normal ovarian function, and therefore are likely critical in the recrudescence of photoregressed ovaries. As a first step to investigate the impact of MMPs on ovarian recrudescence, we investigated the effect of *in vivo* inhibition of MMP by GM6001 on mRNA expression of: (1) key steroidogenic enzymes: steroidogenic acute regulatory protein (StAR), 3-beta hydroxysteroid dehydrogenase (3 β -HSD), and aromatase (Cyp19a1), along with estrogen receptors ER α (Esr1) and ER β (Esr2) to show both steroid production and potential need for estradiol receptors, (2) epidermal growth factor receptor (EGFR) and its ligand amphiregulin, (3) TIMP-1, TIMP-2, TIMP-3, (4) angiogenic factors vascular endothelial growth factor A (VEGFA), vascular endothelial growth factor receptor-2 (VEGFR-2), and angiopoietin-2 (Ang-2), and (5) proliferation marker proliferating cell nuclear antigen

(PCNA) in ovaries of Siberian hamsters exposed to different photoperiods with and without *in vivo* inhibition of MMPs with GM6001 during photostimulated recrudescence.

2. Materials and methods

2.1. Animals

Adult female Siberian hamsters (*P. sungorus*) from our colony at California State University, Long Beach (CSULB) were used in compliance with National Research Council, and CSULB IACUC guidelines for the use of laboratory animals. Hamsters were given tap water and free access to food (a mixture of Lab Rodent Diet 5001 and Mazuri Hamster & Gerbil Diet, Purina, Brentwood, MO) and housed individually in clear polypropylene cages with temperature maintained at 20 ± 2 °C.

Hamsters were divided into four groups LD ($n = 6$): cycling ovary control, hamsters exposed to long photoperiods (16 h of light: 8 h of dark per day) for 16 weeks. SD ($n = 10$): regressed ovary control, hamsters exposed to short photoperiods (8L:16D) for 14 weeks to promote ovarian regression. PT ($n = 6$): recrudescing ovary control, hamsters first exposed to 14 weeks of SD and then transferred to LD for 2 weeks to initiate recrudescence. PTG ($n = 9$): identical conditions as the PT group, but females were given daily intraperitoneal (i.p.) injections of GM6001 (20 mg/kg body weight) for 2 weeks to inhibit MMP activity (after Whited et al., 2010). This study mirrors the design of our previous work, and because no significant differences were observed in LD and PT controls administered DMSO vehicle (Whited et al., 2010), these groups were not included in the current study. Because our current hypothesis focused on the effect of inhibiting MMPs during the recrudescence period, we also did not include an LDG group, as done previously (Whited et al., 2010). The two SD females whose uterine mass fell >2 standard deviations of the mean were removed from the study. PT designated individuals that showed no sign of the brown-to-white fur color change characteristic of SD responsiveness ($n = 1$), were also removed prior to analysis.

On the day of collection, hamsters were administered an i.p. dose of ketamine (200 mg/kg) and xylazine (20 mg/kg). Anesthetized hamsters were weighed and blood samples were taken retro-orbitally with heparinized capillary tubes. Hamsters were then euthanized via cervical dislocation and ovaries were removed, cleaned, weighed and flash frozen in liquid nitrogen.

2.2. Isolation of total RNA and real time PCR (RTPCR)

Total RNA from ovaries was isolated using Trizol LS reagent (Invitrogen Life Technologies, Carlsbad, CA) as per manufacturer protocol and as described previously (Shahed and Young, 2009). One μ g of total RNA was first treated with DNase 1 (1 unit/ μ l) and incubated for 10 min at 37 °C. The reaction was stopped by EDTA to remove any residual genomic DNA contamination, and then used for cDNA synthesis using ImProm Reverse Transcription System as per manufacturers protocol (Promega, Madison, WI). PCR primer sequences and experimentally determined conditions are listed in Table 1. Real time PCR assays were completed using an Mx3000 Stratagene thermocycler with Absolute QPCR SYBR green mix (ABgene, Surrey, UK). The real time PCR reaction mix contained 1

µl cDNA, 1 µl each of forward and reverse primers (80 nM), 6 µl SYBRgreen mix, and water to a total volume of 12 µl. RTPCR cycles consisted of 15 min hold at 95 °C (1 cycle), 40 amplification cycles at appropriate T_m (Table 1) for 30 s, and extension (30 s at 72 °C) followed by dissociation. A standard curve and non-template negative control were included in each RTPCR assay. In addition, all real time PCR products were analyzed on 1.8% agarose gels to visualize any secondary or nonspecific amplification. For the standard curve, equal amount of cDNA from all samples was pooled and diluted (5×), to obtain a 4-point curve, and the relative amount of mRNA in unknown samples was calculated based on this curve. The efficiency of the assay was typically between 80% and 100% and the correlation coefficient was >0.98. The ratios of gene of interest were normalized to reference gene HPRT1 (hypoxanthine phosphoribosyltransferase) as we have done before (Shahed and Young, 2011, 2013; Whited et al., 2010). Expression of HPRT-1 mRNA was not altered by either photoperiod or GM6001 treatment ($p = 0.995$; data not shown).

2.3. Hemotoxylin and Eosin staining and follicle counts

Formalin-fixed ovaries were dehydrated in a graded series of ethanol solutions and xylenes, and embedded in paraffin wax and 6 µm serial sections were cut from every 60 µm of tissue throughout the entire ovary and mounted onto Superfrost-plus microscope slides. Sections from all groups were stained with hemotoxylin and counterstained with eosin using a standard protocol, and preantral follicles, antral follicles, corpora lutea (CL), and hypertrophied granulosa cells (also called eosinophilic terminal atretic follicles, luteinized atretic follicles, terminology consistent with Park et al., 2014) were counted across 3–6 ovarian cross sections per animal using the criteria described previously (Moffatt-Blue et al., 2006).

2.4. Plasma estradiol concentrations

Plasma estradiol concentrations were determined by enzyme immunoassay using an Estradiol EIA kit (Caymen Chemical, MI) following manufacturers' instructions. All serum samples (50 µl) and standards curve were run in duplicate and the assay was conducted exactly as per kit instructions. Concentrations of plasma estradiol were calculated using the manufacturer's template as we have done previously (Shahed and Young, 2011).

2.5. Statistics

Data were analyzed using Prism 5 statistical software (Prism 5 statistical software 240 package; GraphPad Software, Inc., San Diego, CA, USA). One-way ANOVAs were used to analyze data that passed normality tests, and the Neuman-Keuls *post hoc* test was used for pairwise comparisons. Nonparametric analyses (Kruskal Wallis, Dunn's multiple comparison test comparing the mean rank of all data sets) were used for the corpora lutea and hypertrophied granulosa cell counts, in addition to plasma estradiol concentrations. All results are presented as mean + SEM with $p < 0.05$ considered significant.

3. Results

3.1. Photoperiod and GM6001 treatments affect plasma estradiol concentration and ovarian follicle counts

To validate new data against the previous study examining the effect of GM6001 on ovarian recrudescence, ovarian structure and plasma estradiol concentrations were assessed. Similar to our previous reports (e.g., Whited et al., 2010), exposure of Siberian hamsters to 14 weeks of short photoperiod severely compromises ovarian function as evident by a reduction in plasma estradiol levels and numbers of both antral follicles and corpora lutea compared to LD values (Table 2 and Fig. 1). In contrast, transfer of photo-regressed hamsters to LD significantly increases the numbers of antral follicles and reduces the number of hypertrophied granulosa cell patches that are characteristic in ovaries following SD exposure (Fig. 1 and Table 2). Two weeks of daily i.p. injections of MMP inhibitor GM6001 to photostimulated hamsters in the PTG group impeded full ovarian recrudescence, with a significant decrease in antral follicle numbers compared to untreated PT females ($p < 0.05$; Table 2). Plasma estradiol concentrations in both SD and PTG groups were significantly decreased from LD values ($p < 0.05$; Table 2).

3.2. Photoperiod affects the expression of ovarian StAR, 3 β -HSD, Cyp19a1, and estrogen receptor Esr1 and Esr2 mRNA

StAR, 3 β -HSD, Cyp19a1 (aromatase) and estrogen receptor Esr1 and Esr2 mRNA were expressed to some degree in the ovaries of all groups (Fig. 2). Exposure to SD decreased mRNA expression of StAR, 3 β -HSD and Cyp19a1 mRNA as compared to LD ($p < 0.05$; Fig. 2a–c). Photostimulation of regressed hamsters restored 3 β -HSD mRNA in the PT group to LD levels; however, StAR and Cyp19a1 mRNA expression did not increase within the 2 weeks of photostimulation (Fig. 2). Overall, steroidogenic enzyme gene expression was not significantly affected by GM6001 treatment, as compared to expression in the PT group (Fig. 2A–c). Expression of Esr1 and Esr2 mRNA also decreased with SD exposure and remained low in early recrudescence, with no effect of GM6001 treatment in the PTG group (Fig. 2d and e).

3.3. Expression of TIMPs-1, 2, 3 mRNA is differentially altered by photoperiod, but stimulated by GM6001

TIMP-1, -2, and -3 mRNA was expressed in ovaries from all groups (Fig. 3). Expression of TIMP-1 mRNA in SD and PT groups did not differ from LD controls; however, increased significantly with GM6001 treatment in PTG as compared to all other groups ($p < 0.05$; Fig. 3A). In contrast, expression of both TIMP-2 and TIMP-3 mRNA declined with SD exposure and remained low in PT compared to LD (Fig. 3B and C). Similar to TIMP-1, both TIMP-2 and TIMP-3 mRNA expression increased significantly in the GM6001-treated PTG group as compared to PT, although expression of TIMP-2 and -3 remained lower than the LD control group (Fig. 3B and C).

3.4. mRNA expression of epidermal growth factor receptor (EGFR) and its ligand amphiregulin

EGFR mRNA expression was not affected by either photoperiod or GM6001 treatment; mRNA levels in LD, SD, PT and PTG groups did not differ ($p > 0.05$; Fig. 4A). Areg mRNA was expressed in all groups and was not altered with SD as compared to the LD controls ($p > 0.05$; Fig. 4B). In contrast, Areg mRNA expression increased fivefold in the photostimulated PT group as compared to LD and SD (Fig. 4B). Treatment with GM6001 reversed this increase, and Areg expression was 4-fold lower in PTG as compared to PT, despite the 2 weeks of photostimulation ($p < 0.05$; Fig. 4B).

3.5. Short photoperiod and GM6001 treatment inhibit expression of PCNA mRNA

Proliferation marker PCNA was present in all groups (Fig. 5). Fourteen weeks of short photoperiod exposure reduced PCNA mRNA expression in SD, while transfer to photostimulation restored PCNA expression in the PT group ($p < 0.05$; Fig. 5). Treatment with GM6001 in the PTG group reduced PCNA mRNA as compared to both LD and PT levels, despite photostimulation ($p < 0.05$).

3.6. Short photoperiod, inhibits expression of Ang-2, VEGF-2 but not VEGFA mRNA

The angiogenic genes examined were expressed in all groups, with SD exposure inhibiting VEGFR-2, and Ang-2 mRNA in SD compared to LD ($p < 0.05$; Fig. 6B and C). Two weeks of photostimulation increased VEGFA mRNA in the PT group as compared to both SD and LD ($p < 0.05$; Fig. 6A); however photostimulation did not increase Ang-2 and VEGFR-2 above SD values. GM6001 administration did not alter mRNA expression of these angiogenic genes as compared to the PT group ($p > 0.05$; Fig. 6).

4. Discussion

Exposure to short photoperiods reduces reproductive activity and gonadal function centrally in Siberian hamsters via inhibition of adenohypophyseal gonadotropin synthesis and release. However, the intraovarian processes mediating gonadal recrudescence upon photostimulation are not fully understood. As a first step to examine the underlying mechanism(s) of ovarian recrudescence, we investigated the impact of inhibiting MMPs in recrudescing ovaries on the expression of genes associated with normal ovarian function. MMPs directly or indirectly influence several key ovarian processes: steroidogenesis, follicle growth, cyclic ECM reorganization, angiogenesis, and proliferation. We have shown previously that transfer of photoregressed Siberian hamsters to LD progressively stimulates ovarian recrudescence, and that this resumption of ovarian function can be impeded with *in vivo* administration of a pan MMP inhibitor, GM6001 (Whited et al., 2010). Results from the present study suggest that the GM6001 inhibition of ovarian recrudescence may result from reduced MMP activity interfering with an EGFR receptor ligand, TIMP expression, and cell proliferation.

Steroidogenesis is critical to reproductive function, with estrogens working both systemically and locally to promote granulosa cell proliferation that leads to follicular growth and development (Goldenberg et al., 1972), and to modulate receptor levels and

progesterone production (Richards et al., 1979). In the current study, both exposure to SD and treatment with GM6001 during photostimulation reduced plasma estradiol concentrations as compared to LD, replicating our previous results (Table 2, Whited et al., 2010). The SD reduction in plasma estradiol was concomitant with a reduction in *Esr1* and *Esr2* mRNA in SD, although expression of the receptors was not altered when MMPs were inhibited (Fig. 2d and e). In terms of estradiol production, the steroidogenic enzymes examined in this study, *StAR*, 3β -HSD, and *Cyp19a1* were all down regulated in the photoinhibited ovary, and their mRNA expression was also not altered with inhibition of MMPs (Fig. 2a–c). Transfer to stimulatory LD photoperiod restored mRNA expression of 3β -HSD within 2 weeks; however did not induce an increase in *StAR* or *Cyp19a1* (Fig. 2). *StAR* catalyzes the transfer of the precursor cholesterol from the outer to inner mitochondrial membranes at the start of the steroidogenic pathway, whereas aromatase catalyzes the final step in estradiol production. Therefore it is likely that the lack of full return observed in plasma estradiol concentrations within the first 2 weeks of photostimulation is directly linked to these lower levels of *StAR* and *Cyp19a1* mRNA in the PT group (Fig. 2 and Table 1).

In the current study, 2 weeks of GM6001 treatment during photostimulation had no direct effect on mRNA expression of *StAR*, 3β -HSD and *Cyp19a1*, suggesting that MMPs do not alter gene expression of these enzymes in this situation. However, MMPs can indirectly influence LH induced steroidogenesis by cleaving EGFR receptor ligands or EGF like growth factors amphiregulin, epiregulin, and betacellulin, from their transmembrane precursors, thus releasing them to the intraovarian environment (Carbajal et al., 2011; Conti et al., 2012). These growth factors promote phosphorylation and activation of EGFR and downstream cascade influencing folliculogenesis, proliferation and oocyte maturation (Sasseville et al., 2010). Specific inhibition of MMP-2 and -9 prevents LH induced ovarian steroidogenesis by blocking EGFR activation in mice, and treating mice with pan-MMP inhibitor GM6001 prevents EGFR receptor phosphorylation/activation and reduces plasma estradiol concentrations (Carbajal et al., 2011; Panigone et al., 2008). While neither inhibition of MMPs nor photoperiod exposure altered the expression of EGFR mRNA, treatment with GM6001 did prevent the photostimulated return of amphiregulin mRNA expression in the PTG group (Fig. 4). These observations suggest that *in vivo* administration of GM6001 may impede the full resumption of ovarian steroidogenesis associated with recrudescence by affecting EGFR signaling; an inhibition with multiple downstream effects.

TIMPs are multifunctional proteins, and affect various cellular processes that are both dependent and independent of their role as inhibitors of MMPs (review Brew and Nagase, 2010). In Siberian hamsters, SD exposure inhibits mRNA expression of TIMP-2 and -3, but not TIMP-1 (Fig. 3). Interestingly, inhibition of MMPs with GM6001 increased mRNA expression of all three TIMPs when compared to untreated controls receiving the same photostimulation (Fig. 3). While the effect of MMP inhibition by GM6001 on TIMP mRNA expression has not been widely explored, a recent study has reported that GM6001 treatment increased protein levels of TIMP-1, -2 and -3 in the kidneys of hypertensive rats when compared to normotensive controls (Pushpakumar et al., 2013). It is not known whether an increase in TIMP mRNA in GM6001 treated animals is a direct or indirect effect of

GM6001; however, high levels of TIMP-1 in the peritoneal cavity do interfere with ovarian function (Stilley and Sharpe-Timms, 2012). It may be that the inhibition of ovarian recrudescence in GM6001 treated photostimulated Siberian hamsters is linked to the consistent increases observed across TIMP mRNA.

In addition to naturally inhibiting MMPs, TIMP-1, -2 and -3 can inhibit another key ovarian process, angiogenesis (Stetler-Stevenson and Seo, 2005). Inhibition of MMP with synthetic inhibitors can block angiogenesis in tumors (Brown, 1997). Both follicular growth and corpus luteum formation both are dependent on angiogenesis for supply of oxygen, growth factors, and nutrients and therefore inhibition of angiogenesis is likely to impact recrudescence of photoregressed ovaries. In particular, TIMP-3 inhibits angiogenesis by acting as an antagonist to VEGFR-2, a receptor for angiogenic factor VEGF (Qi et al., 2003). TIMP-2 can also impede angiogenesis by preventing growth factor stimulated endothelial cell proliferation (Stetler-Stevenson and Seo, 2005). SD exposure decreased VEGF, Ang-2, and VEGFR-2 mRNA expression as compared to cycling LD ovaries (Fig. 6), similar to the reduction in VEGF protein observed in SD exposed deer mice (*Peromyscus leucopus*) (Young and Nelson, 2000). These results suggest that angiogenic processes are reduced in photoinhibited compared to functional ovaries. Two weeks of photostimulation increased VEGF but not Ang-2 or VEGFR-2 mRNA, suggesting that photostimulation induces at least some aspects of angiogenesis. Because this study examined only early recrudescence, it could be that further increases in angiogenic gene expression would be noted with additional weeks of photostimulation. Despite no direct effects of GM6001 administration observed on the above angiogenic genes, angiogenic processes could potentially be impeded by the higher TIMP mRNA expression observed in GM6001 treated PTG group compared to the untreated PT group. If the high levels of TIMPs alter endothelial proliferation directly, or inhibit VEGFA from binding to VEGFR, angiogenesis may be slowed, potentially limiting follicular growth and slowing early ovarian recrudescence. Interestingly, TIMP mRNA expression did not increase in photostimulated hamsters that were not treated with GM6001, and the resumption of ovarian function, including increased antral follicle development that requires angiogenesis, was observed in these PT females (Fig. 3 and Table 2).

The resurgence of growth factors released during ovarian recrudescence is also likely to affect cellular proliferation, another key process in the normal ovarian cycle. PCNA mRNA expression serves as a marker for cell proliferation, and the lower expression levels observed in both SD and PTG ovaries compared to LD control ovaries suggests a significant reduction in mitotic rate under these conditions (Fig. 5). Photostimulated recrudescence, a gradual process, partially restores proliferation after 2 weeks as evidenced by an increase in PCNA mRNA expression in the PT group. The lower PCNA mRNA expression in the PTG group may be a direct effect of MMP inhibition; proliferation may be reduced due to a decrease in the release of growth factors. Culturing neuronal cells with GM6001, or with another MMP-inhibitor, doxycycline, inhibits normal proliferation and differentiation (Szymczak et al., 2010). In the current study, reduced cell proliferation may directly relate to the decreased number of antral follicles observed in the PTG vs. the PT group (Table 2), suggesting that

inhibition of MMPs directly affects photostimulated ovarian recrudescence via an effect on granulosa or, potentially, stromal cell proliferation.

In conclusion, the results of this study suggest that MMPs play a significant role, directly or indirectly, in photostimulated ovarian recrudescence. Because typical ovarian function is reduced in regressed ovaries, it is likely that ovarian cells from a photoregressed ovary respond differently to the cellular signals that characterize normal ovarian cyclicity when compared to cells in a functional ovary. Additionally, because the return to full ovarian function is not instantaneous, but rather a gradual process taking up to 8 weeks (Salverson et al., 2008), it may also be that photoregressed ovaries respond differently to cellular signals in the early days or weeks of photostimulation as compared to response from these same cells later in recrudescence. Examining the effects of a more chronic MMP inhibition across multiple weeks of photostimulation will further our understanding of how MMP action contributes to resumption of ovarian function. Our results suggest that inhibition of MMP activity *in vivo* by GM6001 may impede ovarian recrudescence within the first 2 weeks of photostimulation directly by partially disabling the EGFR receptor pathway by preventing the release EGFR ligands like Areg, and indirectly by its effects on TIMPs, which subsequently may inhibit other processes such as granulosa cell proliferation and angiogenesis. The overall results from the present study highlight that recrudescence of photoregressed ovaries is a complex and multistep process that involves recruitment, growth and development of follicles, steroidogenesis, ECM reorganization, proliferation, and angiogenesis – all requiring external, endocrine, and cellular signals, including MMP activity.

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Fig. 1.

Representative cross sections of ovaries from Siberian hamsters stained with hematoxylin and eosin. (A) Long day (LD) control ovaries. (B) Short day (SD) regressed ovaries. (C) Post-transfer from SD to LD for 2 weeks (PT) recrudescing ovaries. (D) Post-transfer to LD for 2 weeks with daily i.p. GM6001 treatment (PTG). Antral follicles (AF); corpus luteum (CL); preantral follicles (PA); hypertrophied granulosa cells (H).

Fig. 2.

Ovarian mRNA expression of steroidogenesis-related genes. (A) steroid acute regulatory protein (StAR), (B) 3 beta hydroxysteroid dehydrogenase (3β -HSD), (C) aromatase Cyp19a1, (D) estrogen receptor Esr1, (E) estrogen receptor Esr2, mRNA levels during photoperiod induced regression/recrudescence and MMP Inhibitor GM6001 treatment. Photoperiod treatments include: long day (LD) control ovaries, short day (SD) regressed ovaries, post-transfer from SD to LD for 2 weeks (PT) recrudescing ovaries, post-transfer to LD for 2 weeks with daily i.p. GM6001 treatment (PTG). All graphical results are presented as mean + SEM, relative to HPRT-1, and groups with different letters are significantly different ($p < 0.05$).

Fig. 3. Ovarian mRNA expression of TIMP-1, -2, -3. (A) Tissue inhibitor of matrixmetalloproteinase-1 (TIMP-1), (B) TIMP-2, (C) TIMP-3 mRNA levels during photoperiod induced regression/recrudescence and MMP inhibitor GM6001 treatment. Details of treatment groups and data presentation are as in Fig. 2.

Fig. 4. Ovarian mRNA expression of EGFR and Areg. (A) Epidermal growth factor receptor (EGFR), (B) amphiregulin (Areg) mRNA levels during photoperiod induced regression/recrudescence and MMP inhibitor GM6001 treatment. Details of treatment groups and data presentation are as in Fig. 2.

Fig. 5.
Ovarian mRNA expression of PCNA: PCNA proliferation factor mRNA levels during photoperiod induced regression/recrudescence and MMP inhibitor GM6001 treatment. Details of treatment groups and data presentation are as in Fig. 2.

Fig. 6.

Ovarian mRNA expression of angiogenesis-related genes. (A) Vascular endothelial growth factor A (VEGFA), (B) VEGF-receptor 2 (VEGFR-2), (C) angiopoietin-2 (Ang-2) mRNA levels during photoperiod induced regression/recrudescence and MMP Inhibitor GM6001 treatment. Details of treatment groups and data presentation are as in Fig. 2.

Table 1

Primers used for real time PCR reactions.

Gene	Forward	Reverse	Tm
StAR	AAGGTATTTTCGGCTGGAGGT	TTCCCAATCTTTTGCAGGAC	60
Cyp19a1	GATCAGCGGAGAGGAGACAC	GAAAGGGCGAATTGTTTTC	60
3 β -HSD	TGCCAGCCTTCATCTACACC	TGCCTTCTCAGCCATCATT	60
Esr1	CAGGTGCCCTACTACCTGGA	CAGTCTCTCTCGGCCACTCT	60
Esr2	TGCAGAACCTCAAAAGAGTCC	AGCATCCCTCTTTGAACTCG	60
PCNA	AGCACTCGTATTTGAAGCACCA	TCACCAGAAGGCATCTTTACCA	62
EGFR	CATCAGGGGGAACACTCTGT	AGCTGCTTGGTTGGTTCTGT	60
Areg	AATGAGAACTCCGCTGCTAC	CCCCTGTGGAGAGTTCACTG	60
TIMP-1	TGTCCACAAGTCCCAGAACC	CAGGGAAACACTGTGCACAC	55
TIMP-2	GGACGTTGGAGGAAAGAAGG	GTCCGGGGAGGAGATGTAG	57
TIMP-3	GCGTGTATGAAGGCAAGATG	AATTGCAACCCAGGTGGTAG	56
HPRT-1	TGATCAGTCAACAGGGGACA	CTGGCCGATATCCAACACTT	55
Ang-2	ACTACGACGACTCAGTGCAA	TCCACCATCTCCTTCTTCAT	53
VEGFA	AAAGCCTGACATGAAGGAAG	ATAAGAAAATGGCGAATCCA	53
VEGFR-2	TGCCTACCTCACCTGTTTCC	TCTGTCTGGCTGTCATCTGG	62

Cyp19a1, Esr1 and Esr2 primers (Phalen et al., 2009); TIMP-1,-2,-3 primers (Prakbowong et al., 2009); Areg primers (Kim et al., 2011).

Table 2

Follicle counts per ovarian cross-section and plasma estradiol concentrations across photoperiod exposure groups.

	LD	SD	PT	PTG
Preantral follicles	7.08 ± 1.0	7.7 ± 0.8	8.96 ± 1.6	9.00 ± 0.7
Antral follicles	1.08 ± 0.2 ^a	0.08 ± 0.1 ^b	1.73 ± 0.6 ^a	0.33 ± 0.2 ^b
Corpora lutea	2.08 ± 0.6 ^a	0.08 ± 0.1 ^b	1.13 ± 0.5 ^{a,b}	0.67 ± 0.3 ^{a,b}
Luteinized atretic follicles	0.00 ± 0.0 ^a	12.97 ± 3.9 ^b	0.00 ± 0.0 ^a	3.00 ± 1.2 ^{a,b}
Plasma estradiol (pg/ml)	58.7 ± 7.8 ^a	4.10 ± 0.9 ^b	34.46 ± 8.5 ^{a,b}	12.26 ± 5.3 ^b

Data for follicle counts represent average (mean ± SEM) numbers of each follicle type averaged across 3–6 cross sections per animal. Data for estradiol concentrations (mean ± SEM) are averaged values from the Enzyme Linked Estradiol Assay. Means with different letters differ significantly ($p < 0.05$).