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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), 3β-hydroxysteroid dehydrogenase (3β-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, 3β-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 3β-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.

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

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[1] *List of abbreviations:* 3β-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; hCG, human chorionic gonadotropin; 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.

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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 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 ERα can inhibit MMP-9 activity in brain tissue (Zheng et al., 2015), while ERβ 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 (3β-HSD), and aromatase (Cyp19a1), along with estrogen receptors ERα (Esr1) and ERβ (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 ($n = 6$): post-transfer 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 below the mean were removed from the study because they were considered to be non-responsive to SD. One PT designated individual that showed no sign of the brown-to-white fur color change characteristic of SD responsiveness was 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