Published in final edited form as:

Mol Reprod Dev. 2017 January ; 84(1): 55–66. doi:10.1002/mrd.22761.

Progesterone Receptor and Prostaglandins Mediate Luteinizing Hormone-Induced Changes in Messenger RNAs for ADAMTS Proteases in Theca Cells of Bovine Periovarian Follicles

ERIN L. WILLIS^{*†}, PHILLIP J. BRIDGES[‡], and JOANNE E. FORTUNE

Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York

SUMMARY

Little is known about the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family of extracellular proteases in ovarian follicles of non-rodent species, particularly in theca cells. In the present study, temporal changes in the abundance of mRNA encoding four ADAMTS subtypes and hormonal regulation of mRNA encoding two subtypes were investigated in theca interna cells during the periovarian period in cattle. Gonadotropin-releasing hormone (GnRH) was injected into animals to induce a luteinizing hormone (LH)/follicle-stimulating hormone (FSH) surge, and follicles were obtained at 0 hr post-GnRH (preovulatory) or at 6, 12, 18, or 24 hr (periovarian). *ADAMTS1*, -2, -7, and -9 transcript abundance was then determined in the isolated theca interna. *ADAMTS1* and -9 mRNA levels were up-regulated at 24 hr post-GnRH, whereas *ADAMTS2* mRNA was higher at 12–24 hr post-GnRH and *ADAMTS7* mRNA increased transiently at 12 hr post-GnRH compared to other time points. Subsequent in vitro experiments using preovulatory theca interna (0 hr post-GnRH) showed that application of LH in vitro can mimic the effects of the gonadotropin surge on mRNAs encoding ADAMTS1 and -9 and that progesterone/progesterone receptor and/or prostaglandins may regulate the levels of mRNA encoding ADAMTS1 and -9 in theca interna, downstream of the LH surge. Time- and subtype-specific changes in *ADAMTS* mRNA abundance in vivo, and their regulation in vitro by hormones, indicate that ADAMTS family members produced by theca cells may play important roles in follicle rupture and the accompanying tissue remodeling in cattle.

INTRODUCTION

Extracellular matrix remodeling occurs continually in mammalian ovaries, facilitating follicular growth, ovulation, and the formation and regression of the corpus luteum (McIntush and Smith, 1998; Smith et al., 1999; Curry and Smith, 2006). Remodeling is particularly dramatic during the periovarian period, the time between the gonadotropin surge and ovulation, because the surge initiates a complex cascade of intrafollicular changes

^{*}Corresponding author: Department of Physiological Sciences Center for Veterinary Health Sciences Oklahoma State University Stillwater, OK 74078. erin.willis@okstate.edu.

[†]Current Address: Department of Physiological Sciences, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK 74078.

[‡]Current Address: Department of Animal and Food Sciences, University of Kentucky, Lexington, KY 40546.

that lead to oocyte maturation and cumulus expansion; follicle rupture and ovulation; and the follicular-luteal transition in steroid production.

Studies with a variety of mammalian species implicate proteases and their inhibitors in the remodeling of the periovulatory follicle, including expansion of the cumulus cell matrix via degradation of cumulus matrix components, breakdown of the basement membrane separating granulosa cells from theca layers, and thinning of the follicular wall at the apex prior to expulsion of the oocyte-cumulus cell complex (McIntush and Smith, 1998; Smith et al., 1999; Curry and Smith, 2006). For example, plasminogen activator, several matrix metalloproteinases (MMPs), and their inhibitors contribute to remodeling of the periovulatory follicle in cattle; specifically, *MMP1*, *-13*, and *-14* and both tissue- and urokinase-plasminogen activator increase in a time-dependent manner after the gonadotropin surge (Bakke et al., 2002, 2004; Dow et al., 2002). Studies with rodents indicate that members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) protease family are also involved in ovulation and remodeling of the periovulatory follicle (Espey et al., 2000; Robker et al., 2000; Shindo et al., 2000; Russell et al., 2003; Richards et al., 2005; Shozu et al., 2005; Brown et al., 2010). However, knowledge of the roles of ADAMTS proteases during ovulation in non-rodent mammalian species is minimal.

Despite the many advantages of rodent models for reproductive studies, there are numerous differences in periovulatory events between rodents and larger mammals, thus emphasizing the importance of studying domestic animals and non-human primates. Cattle provide an excellent model for such studies because of their economic importance and the similarity of bovine to human ovarian follicular development (Campbell et al., 2003). Further, experimental models developed to control the timing of luteolysis and of the gonadotropin surge allow a clearer understanding of sequential changes that occur during this period (Berndtson et al., 1995).

Recent research on ovulation has focused on granulosa cells and oocytes, but theca cells are also vital to the ovulatory process (Young and McNeilly, 2010). We previously showed that the abundance of mRNA encoding multiple ADAMTS subtypes was up- or down-regulated in the theca interna of bovine periovulatory follicles 24 hr after an injection of gonadotropin releasing hormone (GnRH), which induces an endogenous surge of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Madan et al., 2003). These time-dependent changes suggest that theca-derived ADAMTS subtypes play specific roles during the bovine periovulatory period. The current study was designed to extend these findings by first characterizing the temporal profile of mRNA encoding ADAMTS subtypes 1, 2, 7, and 9 in the theca interna.

Progesterone and prostaglandins are essential for ovulation in multiple mammalian species. The effects of gonadotropins on ADAMTS1 in granulosa cells of rodents and in cumulus cells of porcine oocyte-cumulus complexes are mediated through progesterone signaling (Lydon et al., 1995, 1996; Espey et al., 2000; Robker et al., 2000; Shimada et al., 2004; Bishop et al., 2016). Furthermore, ovulation is impaired in mice null for the inducible form of prostaglandin G/H synthase and in multiple animal models after administration of prostaglandin inhibitors, such as indomethacin (Armstrong, 1981; Murdoch et al., 1993; Lim

et al., 1997). Prostaglandins are also known to regulate proteases (e.g., matrix metalloproteinases) and their inhibitors during the periovulatory period in cattle, rats, and rhesus monkeys (Tsafiriri, 1995; Duffy and Stouffer, 2003; Li et al., 2006, 2009). We therefore sought to develop an in vitro model that mimics the effects of the LH surge on abundance of mRNA encoding ADAMTS subtypes and to use the model to test the hypotheses that progesterone, via its receptor, and prostaglandins mediate the effects of the LH/FSH surge on specific *ADAMTS* mRNAs in theca cells during the bovine periovulatory period.

RESULTS

Abundance of mRNA Encoding ADAMTS Subtypes in the Theca Interna Throughout the Bovine Periovulatory Period

Messenger RNAs encoding the four ADAMTS subtypes examined were detectable at all time points in vivo (Fig. 1). The steady-state levels of *ADAMTS1* and *-9* mRNA were similar, and no time-dependent differences were observed until a 3- or 4.6-fold increase at 24 hr post-GnRH, respectively (Fig. 1A and D). In contrast, *ADAMTS2* abundance in the theca interna increased approximately 2-fold by 12 hr and remained elevated through 24 hr post-GnRH (Fig. 1B). *ADAMTS7* mRNA was transiently higher at 12 hr, but then returned to preovulatory levels (0 hr) at 18 hr through 24 hr post-GnRH (Fig. 1C). These in vivo changes indicate that the LH/FSH surge up-regulates the steady-state levels of all four *ADAMTS* mRNAs, with different temporal profiles.

Effects of LH on *ADAMTS1*, *-2*, and *-9* mRNA Abundance in Cultured Pieces of Theca Interna Isolated From Preovulatory Follicles

An in vitro model, designed to mimic the effects of the LH surge on patterns of mRNA abundance, was developed to study the regulation of ADAMTS protease mRNA in a more controlled manner (see Methods for details). Similar to what was observed in vivo, no changes were observed in the levels of *ADAMTS1* and *-9* mRNA at early time points (12 and 24 hr), whereas LH increased their respective levels 2- to 2.5-fold compared to control cultures at 36 and 48 hr (Fig. 2). LH had no direct effect on *ADAMTS2* mRNA in cultured theca interna (data not shown), in contrast to the effects observed in vivo (Fig. 1). *ADAMTS7* mRNA was not examined due to a lack of suitable primers.

Effects of Mifepristone on Abundance of *ADAMTS1* and *-9* mRNA in Cultured Pieces of Theca Interna

The same culture system was used to determine if the effects of LH on the abundance of *ADAMTS1* and *-9* mRNA are mediated through progesterone by testing the response following treatment with mifepristone, a progesterone receptor (PGR) antagonist. As expected, no effect of LH was observed for either *ADAMTS* mRNA after 12 hr of treatment (data not shown) or after 24 hr (Fig. 3). *ADAMTS1* mRNA abundance was elevated 3-fold by LH compared to control cultures at 36 hr; mifepristone completely inhibited the effect of LH, and the synthetic progestin medroxyprogesterone acetate overcame that inhibition (Fig. 3A). Similarly, levels of *ADAMTS9* mRNA were up-regulated by approximately 2-fold by LH at 36 hr; this effect was blocked by mifepristone, but was restored by co-treatment with

medroxyprogesterone acetate (Fig. 3B). Co-treatment with the synthetic glucocorticoid dexamethasone, however, did not impact *ADAMTS1* or *-9* mRNA abundance after treatment with LH plus mifepristone (data not shown).

Effects of Prostaglandins on Abundance of mRNA Encoding *ADAMTS* Subtypes in Cultured Pieces of Theca Interna

We next examined if in vitro treatment with prostaglandin $F_{2\alpha}$ or E_2 ($PGF_{2\alpha}$ or PGE_2) can mimic the effects of LH on *ADAMTS* mRNA abundance in theca cells. As expected (Fig. 2), LH increased levels of mRNAs encoding *ADAMTS1* and *-9* at 36 hr, but not at earlier time points, compared to control medium (Fig. 4). Additionally, both PGE_2 and $PGF_{2\alpha}$ up-regulated the level of mRNA encoding *ADAMTS1*, but the timing of their effects differed. In cultures with $PGF_{2\alpha}$, a slight, but significant, increase in levels of mRNA was observed at 12 hr (1.5-fold), with a more robust increase at 24 hr (2-fold), and no effect at 36 hr of culture. PGE_2 also up-regulated mRNA encoding *ADAMTS1*, with levels increasing 2- to 2.3-fold at 24 and 36 hr compared to control cultures. In contrast to the results for *ADAMTS1* mRNA, $PGF_{2\alpha}$ had no effect on the abundance of mRNA encoding *ADAMTS9* at any time point. PGE_2 , however, up-regulated steady-state levels of mRNA encoding *ADAMTS9* at 24 and 36 hr (1.7- to 2.5-fold) compared to control cultures, similar to its effects on mRNA encoding *ADAMTS1* and mimicking the effect of LH at 36 hr. Cultures of theca interna treated with a lower dose (0.1 μM) of PGE_2 and/or $PGF_{2\alpha}$ ($n = 2-3$ follicles) revealed no significant effects compared to control cultures (data not shown).

DISCUSSION

In vivo and in vitro models for studying bovine periovulatory follicles were used to generate novel information about the ability of theca interna cells to express mRNAs encoding *ADAMTS* subtypes. Specific insights into the regulation of *ADAMTS* transcript abundance by gonadotropins, progesterone/PGR, and prostaglandins were gained. The gonadotropin surge coordinately up-regulated steady-state levels of *ADAMTS1* and *-9* transcripts in theca interna cells in vivo, with mRNA abundance increasing dramatically just a few hours before ovulation. In contrast, *ADAMTS7* mRNA increased transiently, and a sustained increase in *ADAMTS2* mRNA occurred. Progesterone/PGR and PGE_2 mimicked the positive effects of LH on *ADAMTS1* and *-9* mRNA abundance, whereas $PGF_{2\alpha}$ increased levels of *ADAMTS1* but not *ADAMTS9* mRNA. Together, the different temporal profiles for the *ADAMTS* subtypes suggest that each plays a time-specific role in the thecal layer and surrounding tissue during the periovulatory period. The data provide insight into the regulation of mRNAs encoding *ADAMTS* subtypes downstream of the LH/FSH surge in theca cells, indicating that progesterone/PGR and prostaglandins, two major factors essential for ovulation, are both involved in mediating the effects of LH on the levels of mRNA encoding *ADAMTS* subtypes in theca cells. Furthermore, because prostaglandins are synthesized by the granulosa cells of bovine periovulatory follicles, changes in thecal transcripts encoding *ADAMTS* subtypes in vivo may involve an interaction between the two follicular endocrine cell types.

The dramatic up-regulation of mRNA encoding both ADAMTS1 and -9 in theca cells just prior to ovulation (24 hr post-GnRH) suggests that these subtypes may have a similar function in thecal/interstitial tissue. *ADAMTS1* mRNA in the theca interna was also up-regulated just prior to ovulation in the horse, another large mono-ovulatory species (Boerboom et al., 2003). In contrast, *Adamts1* mRNA was detected in granulosa but not in theca cells of rodents (Espey et al., 2000; Robker et al., 2000). We previously reported that *ADAMTS1* and -9 mRNAs are higher in bovine luteal cells obtained from early bovine corpora lutea compared to those from late-stage corpora lutea (Madan et al., 2003), implying that these two theca-derived ADAMTS subtypes play important roles late in the long periovulatory period of larger mammalian species. Thus, it is possible that *ADAMTS1* and -9 produced by theca cells may degrade thecal/stromal extracellular matrix components during ovulation and subsequent luteinization. ADAMTS1 and -9 share an identical enzymatic domain that targets and degrades proteoglycans such as versican (Kuno et al., 2000; Rodriguez-Manzaneque et al., 2002; Somerville et al., 2003), which is a component of the basement membrane of bovine follicles (McArthur et al., 2000). Granulosa-derived ADAMTS1 cleaves versican during the mouse periovulatory period (Russell et al., 2003; Brown et al., 2010) and *Adamts1*-null female mice exhibit incomplete luteinization and undifferentiated theca layers during ovulation (Shozu et al., 2005; Brown et al., 2010).

A luteinizing dose of LH in vitro mimicked the changes in abundance of *ADAMTS1* and -9 mRNAs observed in vivo, but more slowly than the in vivo responses. This delay is consistent with previous reports on the in vitro effects of gonadotropins on other end points (Jo et al., 2002; Bridges et al., 2006). Interestingly, we also noted a slight effect of culture on levels of mRNA encoding ADAMTS proteases, particularly at 12 hr and more consistently for *ADAMTS9* than for *ADAMTS1*. We suspect that the spontaneous elevation of mRNA encoding ADAMTS proteases was due to the isolation and preparation of the theca interna. The elevation is consistent with transient increases in matrix metalloproteinase transcript abundance previously reported over time in vitro, but, in contrast to our results, those authors found that LH treatment did not generally affect the spontaneous increases in matrix metalloproteinase mRNA in theca cells (Ohnishi et al., 2001; Jo and Curry, 2004).

Our in vitro model also revealed that *ADAMTS1* and -9 mRNA abundance was decreased by the PGR inhibitor mifepristone in the presence of LH, an effect that was reversed by a progestin but not by a glucocorticoid. The concentrations of progesterone in the follicular fluid and levels of *PGR* mRNA in the follicle wall (granulosa plus theca cells) increase, relative to 0 hr levels, both early in the periovulatory period (3.5–6 hr) and later (18–24 hr), after returning to 0 hr levels in the mid-periovulatory period (12–18 hr) (Komar, 1998; Jo et al., 2002). A luteinizing dose of LH is required for the induction of *PGR* mRNA, which occurs by 10 hr, in cultured theca interna (Jo et al., 2002), but *ADAMTS1* and -9 mRNAs increase only during the latter half of the periovulatory period. Therefore, the early progesterone peak may play a different role in the ovulatory process than the second progesterone peak. Alternatively, progesterone could have an indirect effect on *ADAMTS* mRNA levels through an unknown mediator or an early inhibitor may play a role, ensuring an appropriately timed accumulation of ADAMTS1 and -9 just prior to ovulation.

Progesterone/PGR can also modulate the gonadotropin-induced periovulatory increases in PGE₂ and PGF_{2α} (Bridges et al., 2006). Follicular production of prostaglandins occurs mainly toward the end of the bovine periovulatory period—follicular fluid PGE₂ peaks at 26 hr post-hCG (Sirois, 1994). Bovine granulosa, but not theca, cells produce PGF_{2α} and PGE in response to the LH/FSH surge (Bridges et al., 2006), whereas mRNAs encoding PGF_{2α} and PGE₂ receptors increase in both theca interna and granulosa cells in a complex time- and cell type-dependent pattern that suggests roles for these prostaglandins in both follicular cell types (Bridges and Fortune, 2007). The observed increases in vitro in *ADAMTS1* and *-9* mRNA in the presence of PGE₂ and in *ADAMTS1* mRNA with PGF_{2α} provide evidence that prostaglandins can regulate the synthesis of these proteases in theca cells during the periovulatory period, and the timing of these changes in vitro is consistent with the rise in follicular prostaglandins and *ADAMTS1* and *-9* mRNA late in the periovulatory period in vivo (Sirois, 1994; Komar, 1998). Conversely, treatment of rats with indomethacin, a general prostaglandin inhibitor, did not interfere with normal *Adams1* mRNA production during ovulation (Espey et al., 2000), emphasizing species differences in how mRNAs encoding ADAMTS proteases are controlled.

Based on the data discussed above, we propose a model in which the increases in thecal mRNA encoding ADAMTS1 and *-9* in response to the LH/FSH surge are mediated through increases in progesterone/PGR in the theca and independently, through increased secretion of prostaglandins by granulosa cells. Progesterone/PGR-dependent increases in granulosa cell prostaglandin synthesis may help maintain higher abundance of mRNA encoding ADAMTS subtypes in theca cells late in the periovulatory period. Consequently, prostaglandins could provide a redundant, additive, or synergistic effect on extracellular matrix remodeling in the later periovulatory period, perhaps ensuring sufficient ADAMTS1 and *-9* activity during the follicular-luteal transition.

Our in vivo data also suggest that ADAMTS2 and *-7* are active during the periovulatory period in cattle. An early and sustained increase was observed for *ADAMTS2* mRNA in theca cells (12 through 24 hr post-GnRH), but this profile was not replicated by a luteinizing dose of LH in the in vitro culture system. Therefore, isolated theca interna may not be adequate to reproduce in vivo effects of LH on *ADAMTS2* mRNA, or this transcript may not be as robustly regulated by the gonadotropin surge as the other ADAMTS subtypes. Alternately, the effects of LH on *ADAMTS2* in theca cells may be mediated indirectly by effects of the gonadotropin surge on granulosa cells or on stromal cells around the follicle, elements that are absent in the thecal cultures. On the other hand, the transient early increase in abundance of *ADAMTS7* mRNA in theca cells (12 hr post-GnRH) suggests that its effects may be exerted early. Unfortunately, suitable primers for real-time PCR were not obtained for *ADAMTS7*, so this subtype could not be examined using our in vitro system.

In summary, the gonadotropin surge increased steady-state levels of mRNA encoding ADAMTS1, *-2*, *-7*, and *-9* in theca interna in a time- and subtype-dependent manner during the bovine periovulatory period. A similar increase in mRNA encoding ADAMTS1 and *-9* was evoked in bovine theca interna cells in vitro in response to a luteinizing dose of LH. This study provides the first evidence that mRNAs encoding ADAMTS1 and *-9* are positively regulated in theca interna by progesterone/PGR and by prostaglandins,

particularly PGE₂. Whether or not these two intermediate signaling components regulate ADAMTS protease synthesis in the theca interna of other species remains to be determined. Future studies to determine levels of protein and enzymatic activity of ADAMTS subtypes during the periovulatory period, and to localize the activities within the follicle, will provide additional insight into the roles of these proteases during ovulation and the follicular-luteal transition.

MATERIALS AND METHODS

Animals

All animal procedures were approved by Cornell University's Animal Care and Use Committee. Holstein heifers were housed at the reproductive research farm and were assigned to an experimental group on Day 0 of the estrous cycle (Day of estrus). An experimental model validated previously by our laboratory (Komar et al., 2001) was used to induce luteal regression, differentiation of the dominant follicle of the first follicular wave of the cycle into the preovulatory follicle, and the gonadotropin surge.

For experiments on preovulatory follicles, luteal regression was induced by an intramuscular injection of PGF_{2α} (25 mg Lutalyse) (Pharmacia and Upjohn Co., Kalamazoo, MI) on the evening of Day 6 or morning of Day 7 of the estrous cycle, and the ovary bearing the preovulatory follicle was removed by colpotomy 36 hr after the injection. Periovulatory follicles were obtained from heifers injected with PGF_{2α}, as above, followed 36 hr later by intramuscular injection of a GnRH analogue (100 µg Cystorelin) (Sanofi Animal Health Inc., Overland Park, KS) to induce an endogenous LH/FSH surge. Ovaries were removed at 6, 12, 18, or 24 hr post-GnRH (periovulatory follicles). In this model, the endogenous surge of LH peaks about 2 hr after administration of GnRH while ovulation occurs ~29 hr post-GnRH (Komar et al., 2001).

Ovarian follicles were monitored by transrectal ultrasonography to observe development of the ovulatory follicle, and blood samples were taken daily, starting on Day 4 of the estrous cycle, to verify the decrease in plasma progesterone concentrations associated with luteal regression. Blood samples were collected every hour for 4 hr post-GnRH to confirm induction of the LH surge. Ovaries were transported to the laboratory (~10 min) in Eagle minimum essential medium (MEM) buffered with 25 mM Hepes (Gibco, Invitrogen, Grand Island, NY).

Isolation of Theca Interna

Pre- or periovulatory follicles were dissected from the ovary, and then theca interna and granulosa cells were separated as previously described (Fortune and Hansel, 1979). Briefly, the theca interna layer was peeled from the surrounding stroma, and then granulosa cells were separated from theca interna by scraping the theca with a fine glass needle. For experiments *in vivo*, theca interna obtained from pre- (0 hr post-GnRH) or periovulatory follicles (6, 12, 18, or 24 hr post-GnRH) (n = three follicles per time point) was cut into small pieces. The pieces were snap frozen in liquid nitrogen for later extraction of total RNA.

Culture of Theca Interna

Theca interna isolated from preovulatory follicles (i.e., 36 hr post-PGF_{2α}) was cut into small pieces, and the pieces were randomly distributed to 24-well Costar culture plates (Costar, Cambridge, MA), with three pieces per well, as described previously (Fortune and Hansel, 1979). Pieces of theca interna were cultured in duplicate in 0.5 mL of control medium (modified MEM) (Chandrasekher and Fortune, 1990) or with a luteinizing dose of LH (100 ng/mL NIH LH-S26); LH + 1 μM mifepristone (Sigma–Aldrich Corp., St Louis, MO); LH + MIFE + 10 μM medroxyprogesterone acetate (Sigma–Aldrich Corp.); LH + MIFE + 10 μM dexamethasone (Sigma–Aldrich Corp.); 0.1 or 1 μM PGE₂ (Cayman Chemical, Ann Arbor, MI); or 0.1 or 1 μM PGF_{2α} (Cayman Chemical). Concentrations of LH, mifepristone, medroxyprogesterone acetate, and dexamethasone were based on previous reports (Berndtson et al., 1995; Lioutas et al., 1997; Bridges et al., 2006); concentrations of PGE₂ and PGF_{2α} were chosen to bracket concentrations measured in bovine follicular fluid during the periovulatory period (Sirois, 1994). Pieces of theca interna were cultured for 0, 12, 24, 36, or 48 hr at 37°C with 95% air: 5% CO₂ in a humidified modular incubation chamber (Billups-Rothenburg, Del Mar, CA).

In vitro experiments were replicated with four to six preovulatory follicles, and treatments were applied in duplicate within each experiment (follicle). Pieces of theca interna obtained at 0 hr from each follicle or collected from cultures at the end of the culture period were stored in RNeasy lysis buffer (Qiagen, Crawfordsville, IN) for subsequent extraction of total RNA. Culture media were collected and replaced every 24 hr for experiments lasting longer than 24 hr. Media were also collected when thecal cultures were terminated (12, 24, 36, or 48 hr). Samples of media were stored frozen for measurement of progesterone, to confirm normal responses to LH treatment.

Semi-Quantitative Reverse Transcription PCR

The abundance of *ADAMTS1*, -2, -7, and -9 mRNA in theca interna during the bovine periovulatory period was assessed by semi-quantitative reverse-transcription PCR. Total RNA was isolated from theca interna obtained from preovulatory follicles at 0 hr and periovulatory follicles at 6, 12, 18, and 24 hr post-GnRH (n = three follicles per time point) using TRIzol (Life Technologies Inc., Grand Island, NY), according to the manufacturer's protocol; bovine corpus luteum tissue was used as a positive control. Total RNA was treated with amplification-grade DNase I (Invitrogen Corp., Carlsbad, CA) and reverse transcribed using an established protocol (Bridges et al., 2006; Bridges and Fortune, 2007).

Semi-quantitative PCR was used to quantify *ADAMTS1*, -2, -7, and -9 mRNA and the 18S subunit of rRNA. Primers for *18S rRNA*, *ADAMTS2*, and *ADAMTS7* were designed based on bovine (*RNA18S5*) or human sequences (Madan et al., 2003) and primers for *ADAMTS1* and *ADAMTS9* were designed based on human sequences (see Table 1). The number of amplification cycles was within the linear range of amplification for each primer pair. Amplified products were separated by electrophoresis in 2% agarose/tris-borate-EDTA gels and quantified based on the intensity of ethidium bromide fluorescence under ultraviolet light (Kodak Image Station 440CF, Eastman Kodak Co., Rochester, NY). The abundance of *ADAMTS1*, -2, -7, and -9 mRNA was normalized to *RNA18S5* abundance in the same

sample and standardized relative to the positive -control RNA that was included in each PCR run, as described previously (Bridges and Fortune, 2007).

PCR products were gel purified with a QIAquick Gel Extraction Kit, in accordance with the manufacturer's protocol (Qiagen Inc., Valencia, CA), and sequenced at Cornell University's Biotechnology Resource Center. Internal primers were used for sequencing with BIG Dye Terminator Chemistry and AmpliTaq-FS DNA polymerase (Applied Biosystems Automated 3730 × 1 DNA Analyzer, Cornell University, Ithaca, NY). The identity of each sequence was confirmed by BLAST query (<http://www.ncbi.nlm.nih.gov/BLAST>).

Real-Time PCR

Pieces of theca interna obtained from preovulatory follicles (36 hr post-PGF2_α; n = four to six follicles) were cultured with a high dose of LH (100 ng/mL), to mimic the LH surge (Berndtson et al., 1995), and other treatments of interest for 12, 24, 36, and 48 hr. Real-time PCR was then used to quantify *ADAMTS1*, -2, and -9 mRNA abundance in cultured pieces of theca interna. Total RNA was extracted from pieces of theca interna using the Absolutely RNA microprep kit (Stratagene, La Jolla, CA), according to the manufacturer's directions for DNase I-treated samples, with some modifications. Pieces of theca interna were homogenized with a Pro200 homogenizer (Proscientific, Oxford, CT) in 200 µL of lysis buffer with beta-mercaptoethanol. Samples were then subjected to the RNA isolation steps indicated in the manufacturer's protocol. Eluted total RNA was precipitated overnight in ethanol and 20 µg of glycogen and re-suspended in diethylpyrocarbonate-treated water. Aliquots of total RNA were reverse transcribed as described for semi-quantitative reverse-transcription PCR.

Real-time PCR with SYBR Green (Eurogentec, Seraing, Belgium) was then performed to determine the abundance of *ADAMTS1*, -2, and -9 mRNA; bovine RNA polymerase II (*POLR2A*) was amplified as a control. PCR reactions were performed using an Applied Biosystems 7300 Real-time PCR System (Applied Biosystems, Foster City, CA); melting-curve analysis verified the amplification of a single product. The threshold cycle number (C_t), defined as the cycle number at which the increase in fluorescence of the logarithmic phase crosses the threshold, was used with the 2^{-C_t} method (Livak and Schmittgen, 2001) to calculate mRNA levels for each ADAMTS subtype relative to *POLR2A* transcript abundance in the same sample. Results for the positive-control sample (bovine corpus luteum) were used to normalize values among the different PCR runs. Although mRNA from a single sample of bovine corpus luteum was used within each in vitro experiment, the same control sample could not be used for all in vitro experiments; thus, data presented within an experiment can be compared quantitatively, but not data among experiments (i.e., within Figs. 2 through 4, but not among them).

The *ADAMTS9* primer set used for analysis by real-time PCR was the same as that used for semi-quantitative PCR. Intron-spanning primers for *POLR2A* were designed based on known bovine sequences (Folger, 2007) and those for *ADAMTS1* and -2 on predicted bovine sequences. All real-time PCR primer sets were validated and optimized for use in bovine ovarian tissues. *ADAMTS7* mRNA was not examined due to a lack of suitable intron-spanning primers that passed our real-time PCR assay validation steps with

appropriate primer efficiency. Primer sequences and PCR conditions are summarized in Table 2.

The identities of PCR products were confirmed by gel electrophoresis, gel purification, sequencing, and BLAST query. All products were verified in accordance with primer design (i.e., the *POLR2A* amplification was within bovine *POLR2A*, whereas *ADAMTS9* showed sequence identity to human *ADAMTS9*; *ADAMTS1* and -2 were consistent with the sequences predicted for bovine *ADAMTS1* and -2).

Statistical Analyses

Data sets were tested for homogeneity of variance by Hartley's test (Neter and Kutner, 1985), and values were log-transformed before statistical analysis if heterogeneous variance was indicated. Data were then analyzed by ANOVA with the general linear model of SAS with random effects (animal). Relative levels of mRNA were analyzed using a two-way ANOVA with animal (follicle) and time (in vivo) as the two factors or by a three-way ANOVA with animal (follicle), treatment, and time (in vitro) as the three factors. Duncan's multiple range test was used to determine significant differences among means. Means were considered different at $P = 0.05$. All values are presented as means $\pm$ standard error of non-transformed values.

Acknowledgments

The authors thank D. Bianchi for animal care and for performing surgical procedures. We are grateful to Dr. C.D. MacCalman for critical reading of an early version of the manuscript and the National Hormone and Pituitary Program (supported by NIDDK) for providing luteinizing hormone. This research was supported by the National Institutes of Health (HD41592 to JEF) and a Lalor Foundation Fellowship (to PJB).

Grant sponsor: National Institutes of Health; Grant number: HD41592; Grant sponsor: Lalor Foundation Fellowship

Abbreviations

ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
LH	luteinizing hormone
PGE₂/F_{2α}	prostaglandin E2/F2 alpha
PGR	progesterone receptor

References

- Armstrong DT. Prostaglandins and follicular functions. *J Reprod Fertil.* 1981; 62:283–291. [PubMed: 6262509]
- Bakke LJ, Dow MP, Cassar CA, Peters MW, Pursley JR, Smith GW. Effect of the preovulatory gonadotropin surge on matrix metalloproteinase (MMP)-14, MMP-2, and tissue inhibitor of metalloproteinases-2 expression within bovine periovulatory follicular and luteal tissue. *Biol Reprod.* 2002; 66:1627–1634. [PubMed: 12021040]

- Bakke LJ, Li Q, Cassar CA, Dow MP, Pursley JR, Smith GW. Gonadotropin surge-induced differential upregulation of collagenase-1 (MMP-1) and collagenase-3 (MMP-13) mRNA and protein in bovine preovulatory follicles. *Biol Reprod.* 2004; 71:605–612. [PubMed: 15115716]
- Berndtson AK, Vincent SE, Fortune JE. Low and high concentrations of gonadotropins differentially regulate hormone production by theca interna and granulosa cells from bovine preovulatory follicles. *Biol Reprod.* 1995; 52:1334–1342. [PubMed: 7632841]
- Bishop CV, Hennebold JD, Kahl CA, Stouffer RL. Knock-down of progesterone receptor (PGR) in macaque granulosa cells disrupts ovulation and progesterone production. *Biol Reprod.* 2016; 94:109. [PubMed: 26985003]
- Boerboom D, Russell DL, Richards JS, Sirois J. Regulation of transcripts encoding ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin-like motifs-1) and progesterone receptor by human chorionic gonadotropin in equine preovulatory follicles. *J Mol Endocrinol.* 2003; 31:473–485. [PubMed: 14664708]
- Bridges PJ, Fortune JE. Regulation, action and transport of prostaglandins during the periovulatory period in cattle. *Mol Cell Endocrinol.* 2007; 263:1–9. [PubMed: 17064845]
- Bridges PJ, Komar CM, Fortune JE. Gonadotropin-induced expression of messenger ribonucleic acid for cyclooxygenase-2 and production of prostaglandins E and F2alpha in bovine preovulatory follicles are regulated by the progesterone receptor. *Endocrinology.* 2006; 147:4713–4722. [PubMed: 16825323]
- Brown HM, Dunning KR, Robker RL, Boerboom D, Pritchard M, Lane M, Russell DL. ADAMTS1 cleavage of versican mediates essential structural remodeling of the ovarian follicle and cumulus-oocyte matrix during ovulation in mice. *Biol Reprod.* 2010; 83:549–557. [PubMed: 20592310]
- Campbell BK, Souza C, Gong J, Webb R, Kendall N, Marsters P, Robinson G, Mitchell A, Telfer EE, Baird DT. Domestic ruminants as models for the elucidation of the mechanisms controlling ovarian follicle development in humans. *Reprod Suppl.* 2003; 61:429–443. [PubMed: 14635953]
- Chandrasekher YA, Fortune JE. Effects of oxytocin on steroidogenesis by bovine theca and granulosa cells. *Endocrinology.* 1990; 127:926–933. [PubMed: 2373062]
- Curry TE Jr, Smith MF. Impact of extracellular matrix remodeling on ovulation and the folliculo-luteal transition. *Semin Reprod Med.* 2006; 24:228–241. [PubMed: 16944420]
- Dow MP, Bakke LJ, Cassar CA, Peters MW, Pursley JR, Smith GW. Gonadotropin surge-induced up-regulation of the plasminogen activators (tissue plasminogen activator and uro-kinase plasminogen activator) and the urokinase plasminogen activator receptor within bovine periovulatory follicular and luteal tissue. *Biol Reprod.* 2002; 66:1413–1421. [PubMed: 11967205]
- Duffy DM, Stouffer RL. Luteinizing hormone acts directly at granulosa cells to stimulate periovulatory processes: Modulation of luteinizing hormone effects by prostaglandins. *Endocrine.* 2003; 22:249–256. [PubMed: 14709798]
- Espey LL, Yoshioka S, Russell DL, Robker RL, Fujii S, Richards JS. Ovarian expression of a disintegrin and metalloproteinase with thrombospondin motifs during ovulation in the gonadotropin-primed immature rat. *Biol Reprod.* 2000; 62:1090–1095. [PubMed: 10727282]
- Folger, JK. Heat stress in vitro affects steroid hormone production and expression of mRNA for steroidogenic enzymes, StAR and HSP90 by granulosa and theca cells from bovine preovulatory follicles [Dissertation]. Ithaca: Cornell University; 2007.
- Fortune JE, Hansel W. The effects of 17 beta-estradiol on progesterone secretion by bovine theca and granulosa cells. *Endocrinology.* 1979; 104:1834–1838. [PubMed: 446400]
- Jo M, Curry TE Jr. Regulation of matrix metalloproteinase-19 messenger RNA expression in the rat ovary. *Biol Reprod.* 2004; 71:1796–1806. [PubMed: 15286033]
- Jo M, Komar CM, Fortune JE. Gonadotropin surge induces two separate increases in messenger RNA for progesterone receptor in bovine preovulatory follicles. *Biol Reprod.* 2002; 67:1981–1988. [PubMed: 12444077]
- Komar, CM. Dissertation. Cornell University; 1998. Effects of the luteinizing hormone surge on the capacity of preovulatory follicles to secrete steroids and oxytocin and the molecular mechanisms that mediate periovulatory changes in steroidogenesis in cattle.
- Komar CM, Berndtson AK, Evans AC, Fortune JE. Decline in circulating estradiol during the periovulatory period is correlated with decreases in estradiol and androgen, and in messenger RNA

for p450 aromatase and p450 17 α -hydroxylase, in bovine preovulatory follicles. *Biol Reprod.* 2001; 64:1797–1805. [PubMed: 11369611]

Kuno K, Okada Y, Kawashima H, Nakamura H, Miyasaka M, Ohno H, Matsushima K. ADAMTS-1 cleaves a cartilage proteoglycan, aggrecan. *FEBS Lett.* 2000; 478:241–245. [PubMed: 10930576]

Li Q, Jimenez-Krassel F, Ireland JJ, Smith GW. Gene expression profiling of bovine preovulatory follicles: Gonadotropin surge and prostanoid-dependent up-regulation of genes potentially linked to the ovulatory process. *Reproduction.* 2009; 137:297–307. [PubMed: 18996975]

Li Q, Jimenez-Krassel F, Kobayashi Y, Ireland JJ, Smith GW. Effect of intrafollicular indomethacin injection on gonadotropin surge-induced expression of select extracellular matrix degrading enzymes and their inhibitors in bovine preovulatory follicles. *Reproduction.* 2006; 131:533–543. [PubMed: 16514196]

Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell.* 1997; 91:197–208. [PubMed: 9346237]

Lioutas C, Einspanier A, Kascheike B, Walther N, Ivell R. An autocrine progesterone positive feedback loop mediates oxytocin upregulation in bovine granulosa cells during luteinization. *Endocrinology.* 1997; 138:5059–5062. [PubMed: 9348240]

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]

Lydon JP, DeMayo FJ, Conneely OM, O'Malley BW. Reproductive phenotypes of the progesterone receptor null mutant mouse. *J Steroid Biochem Mol Biol.* 1996; 56:67–77. [PubMed: 8603049]

Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA Jr, Shyamala G, Conneely OM, O'Malley BW. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev.* 1995; 9:2266–2278. [PubMed: 7557380]

Madan P, Bridges PJ, Komar CM, Beristain AG, Rajamahendran R, Fortune JE, MacCalman CD. Expression of messenger RNA for ADAMTS subtypes changes in the periovulatory follicle after the gonadotropin surge and during luteal development and regression in cattle. *Biol Reprod.* 2003; 69:1506–1514. [PubMed: 12855604]

McArthur ME, Irving-Rodgers HF, Byers S, Rodgers RJ. Identification and immunolocalization of decorin, versican, perlecan, nidogen, and chondroitin sulfate proteoglycans in bovine small-antral ovarian follicles. *Biol Reprod.* 2000; 63:913–924. [PubMed: 10952939]

McIntush EW, Smith MF. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in ovarian function. *Rev Reprod.* 1998; 3:23–30. [PubMed: 9509986]

Murdoch WJ, Hansen TR, McPherson LA. A review-role of eicosanoids in vertebrate ovulation. *Prostaglandins.* 1993; 46:85–115. [PubMed: 8210447]

Neter, JWW., Kutner, MH. *Applied linear statistical models: Regression, analysis of variance, and experimental designs.* Homewood, IL: R.D. Irwin; 1985.

Ohnishi J, Ohnishi E, Jin M, Hirano W, Nakane D, Matsui H, Kimura A, Sawa H, Nakayama K, Shibuya H, Nagashima K, Takahashi T. Cloning and characterization of a rat ortholog of MMP-23 (matrix metalloproteinase-23), a unique type of membrane-anchored matrix metalloproteinase and conditioned switching of its expression during the ovarian follicular development. *Mol Endocrinol.* 2001; 15:747–764. [PubMed: 11328856]

Richards JS, Hernandez-Gonzalez I, Gonzalez-Robayna I, Teuling E, Lo Y, Boerboom D, Falender AE, Doyle KH, LeBaron RG, Thompson V, Sandy JD. Regulated expression of ADAMTS family members in follicles and cumulus oocyte complexes: Evidence for specific and redundant patterns during ovulation. *Biol Reprod.* 2005; 72:1241–1255. [PubMed: 15659705]

Robker RL, Russell DL, Espey LL, Lydon JP, O'Malley BW, Richards JS. Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. *Proc Natl Acad Sci USA.* 2000; 97:4689–4694. [PubMed: 10781075]

Rodriguez-Manzanique JC, Westling J, Thai SN, Luque A, Knauper V, Murphy G, Sandy JD, Iruela-Arispe ML. ADAMTS1 cleaves aggrecan at multiple sites and is differentially inhibited by metalloproteinase inhibitors. *Biochem Biophys Res Commun.* 2002; 293:501–508. [PubMed: 12054629]

- Russell DL, Doyle KM, Ochsner SA, Sandy JD, Richards JS. Processing and localization of ADAMTS-1 and proteolytic cleavage of versican during cumulus matrix expansion and ovulation. *J Biol Chem*. 2003; 278:42330–42339. [PubMed: 12907688]
- Shimada M, Nishibori M, Yamashita Y, Ito J, Mori T, Richards JS. Down-regulated expression of ADAMTS-1 by progesterone receptor antagonist is associated with impaired expansion of porcine cumulus-oocyte complexes. *Endocrinology*. 2004; 145:4603–4614. [PubMed: 15231699]
- Shindo T, Kurihara H, Kuno K, Yokoyama H, Wada T, Kurihara Y, Imai T, Wang Y, Ogata M, Nishimatsu H, Moriyama N, Oh-hashii Y, Morita H, Ishikawa T, Nagai R, Yazaki Y, Matsushima K. ADAMTS-1: A metalloproteinase-disintegrin essential for normal growth, fertility, and organ morphology and function. *J Clin Invest*. 2000; 105:1345–1352. [PubMed: 10811842]
- Shozu M, Minami N, Yokoyama H, Inoue M, Kurihara H, Matsushima K, Kuno K. ADAMTS-1 is involved in normal follicular development, ovulatory process and organization of the medullary vascular network in the ovary. *J Mol Endocrinol*. 2005; 35:343–355. [PubMed: 16216914]
- Sirois J. Induction of prostaglandin endoperoxide synthase-2 by human chorionic gonadotropin in bovine preovulatory follicles in vivo. *Endocrinology*. 1994; 135:841–848. [PubMed: 8070377]
- Smith MF, McIntush EW, Ricke WA, Kojima FN, Smith GW. Regulation of ovarian extracellular matrix remodelling by metalloproteinases and their tissue inhibitors: Effects on follicular development, ovulation and luteal function. *J Reprod Fertil Suppl*. 1999; 54:367–381. [PubMed: 10692869]
- Somerville RP, Longpre JM, Jungers KA, Engle JM, Ross M, Evanko S, Wight TN, Leduc R, Apte SS. Characterization of ADAMTS-9 and ADAMTS-20 as a distinct ADAMTS subfamily related to *Caenorhabditis elegans* GON-1. *J Biol Chem*. 2003; 278:9503–9513. [PubMed: 12514189]
- Tsafiriri A. Ovulation as a tissue remodelling process. Proteolysis and cumulus expansion. *Adv Exp Med Biol*. 1995; 377:121–140. [PubMed: 7484419]
- Young JM, McNeilly AS. Theca: The forgotten cell of the ovarian follicle. *Reproduction*. 2010; 140:489–504. [PubMed: 20628033]

Figure 1. Abundance of mRNA encoding ADAMTS subtypes, measured by semi-quantitative reverse-transcription PCR, in theca interna of bovine pre- and periovulatory follicles. **A:** *ADAMTS1*. **B:** *ADAMTS2*. **C:** *ADAMTS7*. **D:** *ADAMTS9*. Values were expressed relative the abundance of 18S rRNA (*RNA18S5*) in the same sample (means $\pm$ standard error) ($n =$ three follicles per time point). Within each panel, means with no common letters differ significantly ($P < 0.05$).

Figure 2.

Abundance of mRNA encoding ADAMTS subtypes, measured by real-time PCR, in theca interna isolated from bovine preovulatory follicles and cultured with LH (100 ng/mL) for 0, 12, 24, 36, or 48 hr. **A:** *ADAMTS1*. **B:** *ADAMTS9*. Values were normalized to the abundance of bovine RNA polymerase II (*POLR2A*) transcript in the same sample and standardized to a positive-control sample included in each run (means $\pm$ standard error of duplicate cultures from each of four preovulatory follicles). Means with no common letters within a time point (i.e., between treatments) differ significantly ($P < 0.05$). The same positive-control sample could not be used for all in vitro experiments, so data cannot be compared quantitatively among Figures 2–4.

Figure 3.

Abundance of mRNA encoding ADAMTS subtypes, measured by real-time PCR, in theca interna from bovine preovulatory follicles cultured with LH (100 ng/mL) $\pm$ PGR inhibitor or a synthetic progestin. **A:** *ADAMTS1*. **B:** *ADAMTS9*. Pieces of theca interna from four preovulatory follicles were cultured with control medium; with LH in the presence or absence of mifepristone (MIFE), a PGR antagonist; or with LH + MIFE + 10 μ M medroxyprogesterone acetate (MPA), a synthetic progestin. Values were normalized to the abundance of bovine RNA polymerase II (*POLR2A*) transcript in the same sample and standardized to a positive control sample included in each run (means $\pm$ standard error of duplicate cultures from each of four preovulatory follicles). Means with no common letters within a time point (i.e., between treatments) differ significantly ($P < 0.05$). The same positive-control sample could not be used for all in vitro experiments, so data cannot be compared quantitatively among Figures 2–4.

Figure 4.

Abundance of mRNA encoding ADAMTS subtypes, measured by real-time PCR, in theca interna isolated from preovulatory follicles cultured with LH (100 ng/mL), 1 μM PGE₂, or 1 μM PGF_{2α}. **A:** *ADAMTS1*. **B:** *ADAMTS9*. Values were normalized to the abundance of bovine RNA polymerase II (*POLR2A*) transcript in the same sample and standardized to a positive-control sample included in each run (means ± standard error of duplicate cultures from each of six preovulatory follicles). Means with no common letters within a time point (i.e., between treatments) differ significantly ($P < 0.05$). The same positive-control sample could not be used for all in vitro experiments, so data cannot be compared quantitatively among Figures 2–4.

TABLE 1

Primers Used in Semi-Quantitative PCR

Gene	Sequence	Product size	PCR conditions
<i>ADAMTS1</i>	5'-TGAATGGCGACTTCACTCTG 5'-CTACCCCCATAATCCCACCT	230	Denature: 95°C 30 sec Anneal: 50°C 30 sec Extend: 72°C 30 sec 40 cycles
<i>ADAMTS2^a</i>	5'-CCTATGACTGGCTGCTGGAT 5'-CTCCCAAAGTGCTGGGATAA	310	Denature: 90°C 30 sec Anneal: 55°C 30 sec Extend: 68°C 60 sec 40 cycles
<i>ADAMTS7^a</i>	5'-CCATGTGGTGTTACAAGGCGTC 5'-GGTCCTCCTCCTCATCTTCC	389	Denature: 94°C 30 sec Anneal: 55°C 30 sec Extend: 68°C 60 sec 40 cycles
<i>ADAMTS9</i>	5'-CCAGAGGGTCGTTTTAGCAT 5'-ATGGCTTCCTCTTCAAAGCA	227	Denature: 94°C 30 sec Anneal: 55°C 30 sec Extend: 68°C 60 sec 40 cycles
<i>RNA18S5^a</i>	5'-GCTCGCTCCTCTCCTACTTG 5'-GATCGGCCCGAGGTTATCTA	200	Denature: 94°C 30 sec Anneal: 55°C 30 sec Extend: 72°C 60 sec 17 cycles

^aFrom Madan et al. (2003).

TABLE 2

Primers Used in Real-Time PCR

Gene	Sequence	Product size	PCR conditions
<i>ADAMTS1</i>	5'-CGATTCTATTCACCAGACAG 5'-TAAACACGTGACCTAATTCG	164	Denature: 95°C, 15 sec Anneal: 58°C, 30 sec Extend: 80°C, 30 sec
<i>ADAMTS2</i>	5'-CTCATGAACATTGTCAACGA 5'-GTTCCCAATCTCAATGAGAC	122	Denature: 95°C, 15 sec Anneal: 57°C, 30 sec Extend: 75°C, 60 sec
<i>ADAMTS9</i>	5'-CCAGAGGGTCGTTTTAGCAT 5'-ATGGCTTCCTCTTCAAAGCA	227	Denature: 95°C, 30 sec Anneal: 53°C, 30 sec Extend: 80°C, 30 sec
<i>POLR2A^a</i>	5'-CTTCCAACAAGGCTTTCGAG 5'-GCTCAGCACATCTTTCACCA	101	Denature: 95°C, 15 sec Anneal: 57°C, 30 sec Extend: 72°C, 30 sec

^aFrom Folger (2007).