Effects of steroid ablation and progestin replacement on the transcriptome of the primate corpus luteum during simulated early pregnancy

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ABSTRACT: Previous microarray analyses indicated that a portion of the transcriptome in the macaque corpus luteum (CL) of the menstrual cycle was regulated indirectly by luteinizing hormone via the local actions of steroid hormones, notably progesterone (P). The current study was designed to investigate this concept in the CL of early pregnancy by analyzing chorionic gonadotrophin (CG)-regulated genes that are dependent versus independent of local steroid action. Exogenous human chorionic gonadotropin treatment simulating early pregnancy (SEP) began on Day 9 of the luteal phase in female rhesus monkeys with and without concurrent administration of the 3α-hydroxysteroid dehydrogenase inhibitor trilostane (TRL) with or without the synthetic progestin R5020. Compared with SEP treatment alone, TRL altered 50 mRNA transcripts on Day 10, rising to 95 on Day 15 (P < 0.05, ≥ 2-fold change in gene expression). Steroid-sensitive genes were validated; notably effects of steroid ablation and P replacement varied by day. Expression of some genes previously identified as P-regulated in the macaque CL during the menstrual cycle were not significantly altered by steroid ablation and P replacement during CG exposure in SEP. These data indicate that the majority of CG-regulated luteal transcripts are differentially expressed independently of local steroid actions. However, the steroid-regulated genes in the macaque CL may be essential during early pregnancy, based on previous reports that TRL treatment initiates premature structural regression of the CL during SEP. These data reinforce the concept that the structure, function and regulation of the rescued CL in early pregnancy differs from the CL of the menstrual cycle in primates.

Key words: corpus luteum / microarray / chorionic gonadotrophin / steroids / early pregnancy

Introduction

Ovarian steroid hormones, including progesterone (P), function as master regulators of gene expression in the reproductive tract of primates (Gaide Chevronnay et al., 2010). Additionally, according to the Rothchild hypothesis, the ovary itself is a target organ for progestins, which then regulate the structure and function of the corpus luteum (CL; Rothchild, 1981, 1996). This hypothesis is supported by several studies in nonhuman primates. Withdrawal of luteal P by treatment of rhesus macaque females with an inhibitor of 3α-hydroxysteroid dehydrogenase (HSD3B2; trilostane, TRL) results in luteal tissue remodeling similar to that seen during the onset of luteal regression (Duffy et al., 1994; Duffy and Stouffer, 1997). Luteal proteases involved in tissue remodeling, such as ADAMTS-1 (Young et al., 2004) and matrix metalloproteinase MMP-1 (Young and Stouffer, 2004), are suppressed by HSD3B2-derived steroids during the mid-luteal phase, further supporting a role for luteal steroids in maintenance of primate luteal structure. The genomic steroid receptors for P (PGRA and PGRB) are detected and have been quantified in macaque CL (Duffy et al., 1997). In addition, chronic ablation of steroids during the mid-luteal phase results in decreased expression of PGR mRNA (Duffy and Stouffer, 1995) and increased expression of mRNA encoding the nuclear estrogen (E) receptor β (ESR2) in macaque CL (Duffy et al., 2000). Expression of ESR2 is P-dependent in luteal tissue at this point of the menstrual cycle: concurrent treatment with the synthetic progestin R5020 abolished the TRL-induced increase in ESR2 mRNA.

In many primates, including old world monkeys and women, steroid production by the CL, and especially P secretion, is obligatory for successful initiation and maintenance of pregnancy until the developing placenta assumes this steroidogenic role (Atkinson et al., 1975). Luteal steroid production during early pregnancy is stimulated by the luteinizing hormone (LH)-like molecule chorionic gonadotrophin (CG) produced by the developing conceptus and extends luteal P secretion for ~2 weeks in rhesus macaques. In a model of early pregnancy, in which increasing dosages of
human chorionic gonadotropin (hCG) are administered to mimic the rise observed during primate pregnancy (simulated early pregnancy, or SEP). CG-induced steroid production (E and P) can be abolished by concurrent administration of TRL (Duffy and Stouffer, 1997). Histological evidence indicated that systemic TRL administration also induced luteal regression in the presence of CG.

Recently the genome-wide effects of steroid ablation and progestin replacement in the macaque CL of the menstrual cycle were investigated by our research group using microarray technology (Bishop et al., 2009a). Trophic luteal support was ablated between luteal Days 9 and 11 by administering the GnRH antagonist Antide, which blocks pulsatile release of LH from the anterior pituitary, and CL were collected on luteal Day 12. In some Antide-treated females, LH support was replaced by injections of rLH three times daily. For some LH-replaced females, luteal steroids were ablated by use of TRL, and progestin levels were replaced by concurrent administration of R5020. This analysis determined that LH ablation by Antide altered the expression levels of 1415 probesets (mRNAs), 22% of which were similarly altered by ablation of HSD3B2-derived steroids; progestin replacement with R5020 prevented the effects of TRL in 7% of mRNAs regulated by LH-dependent luteal steroids. Other research groups also presented microarray data demonstrating that many genes associated with steroidogenesis in non-human primate CL are regulated by LH via local control of luteal progesterone secretion (Suresh et al., 2011).

Additional microarray analyses by our research group (Bishop et al., 2011b) compared CL rescued during SEP with time-matched CL from the menstrual cycle. This analysis identified over 4500 mRNA transcripts regulated by CG between Days 10 and 15 of the luteal phase. The following experiment was designed to investigate which of these luteal transcripts are regulated by CG-induced HSD3B2-derived steroids, and to identify P-responsive genes in the presence of CG.

**Materials and Methods**

Experiments were performed at the Oregon National Primate Research Center (ONPRC), West Campus of Oregon Health & Science University (OHSU). All protocols were approved in advance by the ONPRC/OHSU Institutional Animal Care & Use Committee, in accordance with the National Institute of Health (NIH) Guidelines for Care and Use of Laboratory Animals. A cohort of adult, female rhesus macaques (Macaca mulatta, n = 14) exhibiting regular menstrual cycles were selected for this study. All females were under the direct care of the ONPRC Division of Comparative Medicine (DCM). All surgical procedures were performed on anesthetized animals as previously described (Duffy et al., 2000) by DCM Surgical Staff.

**Ablation of steroids and replacement of progestin during SEP**

Serum E and P levels were monitored daily beginning 6 days after the onset of frank menstrual bleeding. The beginning of the luteal phase (luteal Day 1; day of LH surge) was defined as the first day of low E (<100 pg/ml) following the midcycle E surge (Bishop et al., 2009b), with a coincident rise in serum P above 0.2 ng/ml. All females were first assigned to a protocol of increasing dosages of CG mimicking the rise seen in early pregnancy (SEP) which began on Day 9 of the luteal phase as reported previously by Duffy and Stouffer (1997). Recombinant chorionic gonadotropin (hCG; Novarel™, Ferring Pharmaceuticals, Inc., Parsippany, NJ, USA) was administered in increasing dosages (15, 30, 45, 90, 180, 360 IU) twice daily by i.m. injection (SEP treatment group). In subsequent menstrual cycles, females received hCG treatment combined with concurrent administration of the HSD3B2 inhibitor TRL (SEP + TRL; Vetryny® Dechra Veterinary Products, Overland Park, KS, USA) orally in vehicle twice daily (1 g/day). Additionally, some SEP + TRL-treated females were randomly assigned to receive Promegestone (SEP + TRL + R5020; PerkinElmer, Boston, MA, USA) a non-metabolizable synthetic P by subcutaneous injection once daily (2.5 mg/day) in sesame oil vehicle.

To serve as a baseline comparison, CL were collected from a subset of randomly selected females on Day 10 (no treatment) of the luteal phase (n = 8 CL). To differentiate between early and late effects of CG-stimulated steroids on gene expression, CL were collected after 1 day of CG exposure on luteal Day 10 (1 day SEP + TRL + R5020) or 6 days of CG exposure on luteal Day 15 (6 days SEP + TRL + R5020) with and without steroid ablation and replacement (n = 4–5 CL/treatment group/day SEP). Each CL was dissected from the ovary, snap-frozen in liquid nitrogen and stored at −80°C until TRIzol® extraction of total RNA and protein from individual CL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocols.

**Microarray analyses of luteal mRNAs**

Before microarray hybridization, RNA was further purified using an RNAeasy kit® (Qiagen, Valencia, CA, USA). Total RNA integrity was assessed on the RNA 6000 LabChip® using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Aliquots of RNA from individual CL were hybridized to Affymetrix® GeneChip® Rhesus Macaque Genome Arrays by the OHSU Affymetrix Microarray Core as reported by Bishop et al. (2009a). For the complete hybridization protocol, see http://www.ohsu.edu/vxd/research/research-centers/gene-profiling-shared-resource/services/affymetrix-microarray-core/upload/AMC-3-IVT-Expression-Assay-Methods.pdf. Processed image (.CEL) files from each array are deposited in the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/; GEO Accession #: GSE29363). Hybridization data from several 1 day and 6 days of SEP only-treated females of a previously reported microarray study (Bishop et al., 2011b) were included in the current GEO dataset and are indicated by a ‘re-analysis’ designation. Files were uploaded into the web-based GeneSifter® software (Geopsia, Inc., Seattle, WA, USA) for analysis. Probeset hybridization data were Log2 transformed using the robust multi-array analysis (RMA) algorithm (Noriega & Haiman, 2009). For further details on the Rhesus GeneChip® these are also part of the GEO repository. Probesets on the Rhesus GeneChip® represent individual rhesus macaque (mRNA) transcripts [see Noriega et al. (2009) for further details on the Rhesus GeneChip®].

**Real-time PCR validation of select gene products**

Several gene products (mRNAs) were chosen for real-time PCR validation as described in the results. Aliquots of luteal RNA were rendered DNA-free by treatment with TURBOTM DNase and reverse-transcribed using a RETROscript® kit (both by Applied Biosystems/Ambion, Inc., Austin, TX, USA) to generate cDNA as per the manufacturers’ protocols. Real-time PCR for various gene products (Supplementary data, Table SI: Probes and Primers) was performed with TaqMan® MGB probes (Applied Biosystems/Life Technologies, Inc., Foster City, CA, USA) as described by Bogan and Hennebold (2010), with the exception of TIMP1 (TaqMan® Gene Expression Assays, FAM-labeled probe, assay ID: Hs00171558_m1, Applied Biosystems).

**Correlation between mRNA and protein expression**

To correlate mRNA and protein levels in steroid-ablated and P-replaced CL, expression of SERPINE1 and STAR were analyzed by western blotting (Supplementary data, Table SI: Antibodies) according to the methodology reported by Bishop et al. (2009a). Proteins were transferred onto PVDF membranes using the iBlot® Dry Blotting System (Invitrogen) and bands of interest were visualized using the WesternDot™ 625 Goat Anti-Rabbit...
western blot kit (Invitrogen) according to manufacturer’s protocols. Bands were quantified by the ImageJ Software (Abramoff et al., 2004). Protein levels were quantitated for each aliquot by calculating the area under the curve of peak band intensity and dividing by that of the loading control (GAPDH) after correcting for background staining.

Statistics
Serum hormone values from control, SEP, SEP + TRL and SEP + TRL + R5020-treated females were analyzed by repeated measures two-way ANOVA by day and treatment using a general linear model (factors investigated = SEP and TRL, day repeated effect; SAS® version 9.2, SAS Institute, Inc., Cary, NC, USA). Significant differences in RMA-transformed mRNA probeset levels of SEP CL from microarray analyses were defined as a ≥2-fold change by one-way ANOVA function of GeneSifter (P < 0.05), for comparisons between all treatment groups (SEP versus SEP + TRL versus SEP + TRL + R5020) after 1 day and 6 days of SEP. Pairwise comparisons (Welch’s t-test) were performed to identify significant changes in mRNA expression between relevant treatment groups (≥2-fold, P < 0.05). Quantitative real-time PCR and immunoblot data were log₂ transformed if needed to correct for heterogeneity of variance and then analyzed by factorial ANOVA (overall experiment factors = SEP, TRL, R5020, SEP by TRL (tests significance of interaction between SEP and TRL) and SEP by R5020 (tests significance of interaction between SEP and R5020); analyses were broken down into 1 day and 6 days of SEP, factors investigated = TRL and R5020; SAS®). Significant differences between individual treatment groups from pre-planned comparisons were determined by least squares means procedure of SAS with Tukey–Kramer adjustment for multiple comparisons.

Results
Assessment of luteal function
TRL treatment during SEP protocols (SEP + TRL) decreased circulating E and P levels in macaques when compared with SEP alone (Fig. 1); addition of TRL significantly reduced serum P levels to < 1 ng/ml after 1 day (Fig. 1B), and all SEP + TRL-treated females whose CL was collected after 6 days of SEP initiated menses prior to surgery. Administration of TRL also prevented the rise in E levels typically observed during SEP (Fig. 1A). Incidentally, TRL treatment during SEP in rhesus monkeys in previous studies induced morphological changes consistent with luteal regression after 9 days of TRL exposure (Duffy and Stouffer, 1997).

The antibody used in the current P immunoassay does not cross-react with the synthetic progestin R5020 administered to selectively replace P in TRL-treated macaques undergoing SEP (SEP + T + R5020). The P levels measured in SEP + T + R5020 females by this assay were comparable with SEP + TRL-treated females (Fig. 1). Importantly, addition of R5020 prevented onset of premature menses in this cohort. Previous experiments demonstrated that this level of progestin replacement restores ovulation (Hibbert et al., 1996) and expression of progestin-regulated genes in the CL of the menstrual cycle (Young and Stouffer, 2004).

Microarray results: steroid regulation of the luteal transcriptome during SEP
Global microarray analyses were performed using GeneSifter to identify probesets whose mRNA levels changed significantly as detailed in the Methods; first comparing RMA-transformed probeset hybridization levels of mRNA from steroid-ablated and P-replaced CL collected after 1 day of SEP with those from untreated (Day 10) CL (Supplementary data, File S1; 1 day of treatments, p < 0.05). However, principle component analysis revealed that most of these changes to luteal mRNA expression were due to differences between all SEP-treated females (SEP, SEP + TRL and SEP + T + R5020) and untreated Day 10 CL, independent of steroid ablation and replacement (data not shown). Therefore, untreated Day 10 CL were not included in additional analyses to increase the sensitivity to detect changes in luteal mRNA expression during SEP. Further global analyses were then performed within day of SEP by one-way ANOVA. When steroid-ablated (SEP + TRL) and P-replaced CL (SEP + TRL + R5020) mRNA data were compared with SEP alone, 50 probesets (mRNA transcripts) significantly changed after 1 day of SEP (p < 0.05, ≥2-fold change; Fig. 2A; Supplementary data, File S2). A similar analysis of CL collected after 6 days of SEP revealed that the number of steroid-regulated probesets increased to 95 during this longer interval (Fig. 2B; Supplementary data, File S3).

Cluster analysis of all treatment groups depicts the relationship between treatments (Fig. 2C). After 1 day of SEP, levels of some TRL-sensitive mRNAs are restored by R5020 treatment, and SEP and SEP + TRL + R5020 treated CL are most related. After 6 days of SEP, P-replacement restores levels of fewer affected mRNAs comparable with SEP CL, and at this time point SEP + TRL and SEP + TRL + R5020 treatments are now more similar.
Pairwise analyses of relevant treatment groups were performed (Welch’s t-test, GeneSifter) to further characterize steroid and P-regulated mRNAs by day of SEP (SEP versus SEP + TRL, SEP + TRL versus SEP + TRL + R5020, and SEP versus SEP + TRL + R5020; Fig. 3A and B; Supplementary data, File S4). These analyses suggest that the number of steroid-regulated mRNAs increases with longer durations of CG exposure during the SEP protocol. Analyses were further mined to identify P-regulated mRNAs/genes during SEP (Table I). Two sets of progestin-regulated genes were identified. Group 1 mRNA levels were altered by TRL (steroid ablation), and addition of R5020 significantly reversed the effect of TRL. For example, probesets with increased expression from the pairwise comparison dataset of SEP versus SEP + TRL + R5020 demonstrate decreased expression in the dataset of SEP + TRL versus SEP + T + R5020. By this definition, 9 genes are P-regulated after 1 day of SEP, and by 6 days of SEP, there are 14. Group 2 mRNA levels are not affected by TRL during SEP, but show significant changes in expression after R5020 exposure (only present in SEP versus SEP + T + R5020 pairwise analyses; Table I and Supplementary data, Table SIII).

**Gene pathways regulated by HSD3B2-derived steroids during SEP**

To identify molecular processes in SEP CL affected by steroid ablation and P-replacement, significantly altered probesets from global analyses of treatment groups were mined for gene ontologies (AmiGo gene ontology terms, The Gene Ontology Consortium http://www.geneontology.org/). This analysis differs from our previous reports that mined KEGG pathways (Bishop et al., 2011b) due to the limited number of probesets in the present study that were affected by TRL + R5020 during SEP. Significantly altered gene ontologies were identified by comparing CL from treatment groups after 1 day (Table II) and 6 days (Table III) of SEP. Note that some of the most significantly impacted ontologies also have the fewest number of mRNAs present in the gene set on the Affymetrix array. After 1 day of SEP, the most affected steroid-regulated ontology (as identified by z-score and number of impacted mRNAs) is [acyl-carrier-protein] hydrolase activity. According to the Gene Ontology database (http://www.geneontology.org/), members of this ontology catalyze the reaction: [acyl-carrier protein] + H₂O → 4′-phosphopantetheine + apoprotein. After 6 days of SEP, the most significantly affected steroid-regulated ontology is transferase activity, transferring one-carbon groups, but the ontology with the largest number of impacted mRNAs is transducer activity (molecular and signal). Members of this family are molecules that can both accept an input of one form and create an output of a different form, as well as convey a signal that triggers a change in cell function or state (does not include receptor ligands; http://www.geneontology.org/). Given the complex signaling network of the CL, effects on members of this ontology are expected.
SEP protocols, but not influenced by steroid ablation/P-replacement. Three different genes (INSL3, SFRP4 and SERPINE1) were steroid-regulated by TRL treatment after 6 days of SEP, these three genes were no longer impacted by P-replacement. After 6 days of SEP there were no significant effects of steroid ablation/P-replacement on CG-induced IL1RN mRNA expression. An example of a gene that is not affected by CG during SEP, but is negatively regulated by CG-stimulated progesterone only after 6 days of SEP, is tumor necrosis factor (ligand) superfamily, member 13b (TNFSF13B; Fig. 5B).

To quantify functional effects of steroid ablation and P-replacement on gene activity, SERPINE1 (Fig. 6) and STAR protein levels from individual CL were measured by western blot analyses and compared with real-time PCR mRNA expression. There were no effects of steroid ablation (by TRL) or P-replacement (R5020) during SEP on expression of STAR protein, demonstrating a similar pattern of expression between mRNA and protein levels (not shown). However, there were effects of all treatments on expression of SERPINE1 mRNA and protein. Levels of SERPINE1 mRNA decreased between 1 and 6 days of SEP (Fig. 6A). Steroid ablation (TRL) significantly decreased SERPINE1 mRNA after 1 day of SEP, and P-replacement (R5020) restored mRNA levels to that of CG alone. After 6 days of SEP, SERPINE1 mRNA was not affected by steroid ablation; however, P-replacement elevated mRNA levels above those of SEP and SEP + TRL CL. Protein levels of the major isoform of SERPINE1 (~50 kDa) and a second major isoform observed at ~70 kDa [which could be SERPINE1 bound to vitronectin (Declerck et al., 1988; Lijnen, 2005)] were quantified (Fig. 6B). The 70 kDa band of SERPINE1 protein significantly increased following 6 days of SEP, when compared with 1 day of SEP. Levels of the 50 kDa band of SERPINE1 decreased during TRL treatment after both 1 and 6 days of SEP protocols, whereas chronic exposure to TRL during hCG treatment had no effect on the 70 kDa band on either day of SEP. P-replacement (R5020) increased (P < 0.01) both isoforms after 6 days of SEP.

**Discussion**

Microarray analysis identified a subset of probesets in primate luteal tissue that are regulated by HSD3B2-derived steroids during CG-dependent rescue of the CL in SEP. The effects of steroid ablation during CG exposure are greatest after 6 days of SEP, at the time that CG-stimulated luteal P secretion begins to decline (Atkinson et al., 1975; Bishop et al., 2011b). Previous analyses of the luteal transcriptome during SEP indicated that 1 day of SEP treatment alone increased expression of 292 probesets and decreased expression of 127 probesets compared with untreated stage-matched CL (Bishop et al., 2011b). After 6 days of treatment, SEP induced increased expression in 2078 and decreased expression of 452 mRNAs when compared with stage-matched CL. The proportion of TRL-sensitive probesets decreases from 12% (50 of 419) and then to 3% (87 of 2530) of CG-sensitive probesets between 1 and 6 days of CG treatment during SEP. This might indicate that many genes initially regulated by LH/C G-stimulated steroids around the onset of pregnancy.
Table 1 Progesterone-regulated genes during SEP identified by GeneSifter analyses.

<table>
<thead>
<tr>
<th>Group 1</th>
<th>1 day SEP</th>
<th># TRL-regulated probesets also regulated by R5020&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Progesterone-regulated genes&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1 day SEP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Down-regulated</td>
<td>9</td>
<td>APBA1 (2 probesets)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Up-regulated</td>
<td>2</td>
<td>CES1 (2 probesets)</td>
<td>COLBAI (2 probesets)</td>
</tr>
<tr>
<td>6 days SEP</td>
<td>Down-regulated</td>
<td>5</td>
<td>ARHGAP20</td>
<td>DST</td>
</tr>
<tr>
<td></td>
<td>Up-regulated</td>
<td>13</td>
<td>C13orf33</td>
<td>FABP3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SGEF</td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>1 day SEP</td>
<td># probesets regulated only by R5020&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Down-regulated</td>
<td>38</td>
<td>APOB (2 probesets)</td>
<td>COCH (2 probesets)</td>
</tr>
<tr>
<td></td>
<td>Up-regulated</td>
<td>32</td>
<td>GATM (2 probesets)</td>
<td>FCGR3A</td>
</tr>
<tr>
<td>6 days SEP</td>
<td>Down-regulated</td>
<td>23</td>
<td>GPR109A</td>
<td>LOC100133583</td>
</tr>
<tr>
<td></td>
<td>Up-regulated</td>
<td>18</td>
<td>NFE2L3</td>
<td>PLAC8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Regulation by both TRL and R5020 is defined as mRNA levels significantly altered by pairwise comparison analyses SEP versus SEP + TRL are reversed by addition of R5020 (SEP + TRL + R5020). (For example, probesets with ↑ expression SEP versus SEP + TRL demonstrate ↓ expression SEP + TRL versus SEP + TRL + R5020.)

<sup>b</sup>Genes were identified by cross-checking probeset IDs with both Nebraska Database of rhesus gene chip (http://www.unmc.edu/rhesusgenechip/#RhesusGeneChip) and Affymetrix annotation (NetAffx™ Analysis Center). Note that a gene may have multiple probesets on the Affymetrix™ GeneChip, and not all may be similarly regulated.

<sup>c</sup>Significantly altered expression is identified only by pairwise comparison SEP versus SEP + TRL + R5020.
recognition are no longer steroid-regulated by 6 days of CG exposure. However, there are a greater number of total affected probesets after 6 days of SEP when compared 1 day of SEP, and based on cluster analyses, based on cluster analyses, and function during early pregnancy. When IL1RN expression levels in the primate CL during early pregnancy than P alone. While these experiments were conducted using an allosteric inhibitor of HSD3B2, addition of R5020 to the SEP + TRL treatments may have increased activity of HSD3B2 above that of SEP alone, which might explain some of the differences in mRNA expression. Analysis of HSD3B2 mRNA by real-time PCR showed no increase in expression after addition of R5020 between SEP and SEP + T + R5020 treatments (data not shown). However, as enzymatic activity was not measured in these CL, this remains to be investigated by future studies. A large number of mRNAs were altered by R5020 treatment independent of TRL ablation during SEP (Table I and Supplementary data, Table SIII). These changes might be due to a pharmacologic action of R5020 independent of CG-treatment during SEP, or alternatively are dependent on local levels of factors other than P that are depleted by TRL exposure. In this latter scenario once the influence of the main regulatory factor is removed, the gene then becomes responsive to exogenous P.

That many genes in the TRL-treated groups were not restored to SEP levels by P replacement provides further evidence that CG may induce/influence other steroidal factors that modulate a wider range of mRNA expression in the primate CL during early pregnancy than P alone. During SEP, the rise in serum estradiol is also prevented by TRL administration (Fig.1A). Local E signaling may be just as important for luteal

| Table II | Ontologies of steroid-regulated probesets after 1 day of SEP. |
|---------------------------------------------|-----------------|-----------------|-----------------|
| **SEP versus SEP + TRL versus SEP + R5020, P < 0.05** | **# probesets** | **z-score** |
| **Ontology (category/term)** | **Total # in dataset** | **Total # in ontology on microarray** |
| [Acyl-carrier-protein] hydrolase activity | Acyl-, myristoyl-, oleoyl- and palmitoyl- | 4 | 4 | 22.95 |
| Large conductance calcium-activated potassium channel activity | 1 | 2 | 16.2 |
| Sulfonylurea receptor activity | 1 | 2 | 16.2 |
| Flavin-containing monooxygenase activity esterase activity | 1 | 3 | 13.2 |
| Methyl indole-3-acetate, methyl jasmonate and methyl salicylate | 3 | 12 | 11.41 |
| Phosphodiesterase I activity | 1 | 4 | 11.41 |
| Nucleotide diphosphatase activity | 1 | 5 | 10.19 |
| Pattern binding | Pattern and polysaccharide binding | 4 | 274 | 3.44 |
| Protein complex binding | 2 | 219 | 2.49 |
| Receptor binding | 4 | 758 | 2.21 |
| Actin binding | 2 | 276 | 2.07 |

*a*When ontology terms are inter-related under an AmiGo category and share the same z-score, terms are combined.

*b*Prioritized by z-score, if probeset = 1 then z-score > 10.
gene regulation as P. The genomic estrogen receptor ESR2 is expressed in rhesus CL, and regulated by P during the non-gravid luteal phase (Duffy et al., 2000). In addition androgen serves as a precursor for E production in primate CL during early pregnancy (Castracane et al., 1998) and may have intraluteal actions via androgen receptors present in macaque CL (Hild-Petito et al., 1991). Serum androgen levels are increased in women following 6 days of CG exposure during early pregnancy (Castracane et al., 1998). Further studies utilizing E and androgen replacement during SEP + TRL treatment could help elucidate the local role of individual steroids in controlling luteal structure/function during establishment of early pregnancy in primates.

Unexpectedly, ontology analyses (Tables II and III) did not include many pathways classically associated with steroid hormone biosynthetic processes in the macaque CL, which were previously identified as impacted by CG treatment alone during SEP (Bishop et al., 2011b), and by LH and steroid ablation/replacement during the menstrual cycle (Bishop et al., 2009a). Thus, steroid/P control of steroidogenic genes may be limited in the CL of SEP. There is evidence for regulation of primate luteal function by non-steroidal targets of CG such as prostaglandins (Bogan et al., 2008a), luteal relaxin production (Maseelall et al., 2009) and modulation of luteal cytokines (Townson and Liptak, 2003). All of these local factors were altered by CG, as noted in our previous microarray analysis (Bishop et al., 2011b), and may regulate more probesets than steroids alone. Ablation and replacement of these non-steroidal targets during SEP would expand knowledge of factors needed to maintain luteal function during early primate pregnancy.

Alternatively, absence of classical steroidogenic pathways in this dataset could indicate nongenomic regulation of luteal processes.
during SEP, which was reported previously for both estradiol and progesterone, including regulation of sterioeogenic genes such as STAR (Jam-nongjit and Hammes, 2006). Nongenomic actions can manifest in post-translational regulation, which is suggested by the pattern of expression for SERPINE1 gene products (Fig. 6A and B). The mRNA for SERPINE1 is indeed similarly affected by TRL treatment after both 1 and 6 days of SEP (Fig. 6A), but the impact on protein expression for SERPINE1 is more complex (Fig. 6B). Studies on macaque pre-ovulatory follicles also demonstrated an increase in SERPINE1 expression following an ovulatory hCG stimulus, which was mimicked by treatment with agonists of the prostaglandin E2 receptor subtypes 1 and 3 (Markosyan and Duffy, 2009). Regulation of SERPINE1 expression in the macaque ovary may depend on the stage of the luteal lifespan and presence or absence of CG. However, several ontologies are represented by only 1 probeset in the dataset (such as sulfonylurea receptor activity in Table II, and very long-chain fatty acid-CoA ligase activity in Table III), indicating that although the analysis offers statistical significance, many of these ontologies may be of less importance for local regulation of luteal function by steroid/P during SEP.

These data also suggest that regulation of the luteal transcriptome by CG in early pregnancy is remarkably different from that by LH during the menstrual cycle (Bishop et al., 2009a), reflecting the changing functional state of the CL. Previous microarray analyses indicated that TRL ablation affected ~22% of LH-regulated probesets (311 of 1415) in the macaque CL during the mid-late luteal phase, 2% of which are P-dependent (22 of 1415; Bishop et al., 2009a). This is the same time frame in the luteal phase that the conceptus begins to secrete CG to rescue the CL during the fecund cycle (Atkinson et al., 1975). The proportion of steroid (TRL)-regulated luteal probesets under the influence of CG during SEP is fewer than in the CL of the menstrual cycle. Several genes identified in this study display differential regulation by steroids during the luteal lifespan. For example, in CL during the mid-late luteal phase of the menstrual cycle, IL1RN mRNA expression is suppressed in an LH-dependent, steroid-independent manner (Bishop et al., 2009a). This is in contrast to CG-induced P-dependent suppression of IL1RN after 1 day of SEP, while regulation by steroid/P was lost after 6 days of CG exposure (Fig. 5A). Additionally, during the mid-late luteal phase of the cycle, both STAR protein and mRNA are P-regulated in macaque CL (Bishop et al., 2009a), while in the CL of SEP, steroid ablation and P-replacement have no significant effect on either STAR mRNA or protein levels (as evidenced by absence of probesets from Supplementary data, Files S2 and S3; protein data not shown). It is well known that gene regulation by classical steroid receptors is modulated by the coactivator and corepressor cohort present in target cells/tissues (Han et al., 2007). It could be that CG is modulating gene expression through multiple pathways and, either directly or indirectly, influencing a different subset of steroid hormone coactivators and/or corepressors present in luteal tissue during SEP (Casarini et al., 2012). This could explain the differential gene regulation observed in these data.

Ablation and replacement studies conducted with rhesus females during the luteal phase involved removal of pulsatile gonadotrophin (LH) support of luteal cells, while investigations during SEP utilizing CG represent a period of increasing gonadotropin support as evidenced by the temporal patterns and rise in absolute levels of P. The initial exposure to CG results in a large increase in P, usually to much higher levels than observed during a non-fecund luteal phase (Atkinson et al., 1975). An increase in serum P was also reported in cynomolgus macaques when LH was administered in exponential doses concurrently with a GnRH antagonist during the mid-late luteal phase, similar to the dosing regimen of SEP (Zelevnik, 1998). In these experiments when hCG was administered instead of LH, this resulted in higher absolute levels of P, but similar patterns were observed for LH and hCG administration; only administration of exponentially increasing dosages of either LH or hCG was able to prevent GnRH antagonist mediated luteolysis. Therefore, the differences
in gene regulation by steroids observed in this analysis could be due to differences in the pattern of gonadotrophin support. The differences in gene regulation in the CL between fecund and non-fertile cycles could also reflect actions of other local non-steroidal factors induced by CG such as differential regulation of prostaglandins and/or cytokines as discussed previously. Differential gene regulation by luteal tissue exposed to CG versus the CL of a non-fecund cycle might indicate that CG-induced steroid production is less critical for luteal function during early pregnancy.

These array data along with others from the rhesus macaque CL are available in the public repository Gene Expression Omnibus (GEO http://www.ncbi.nlm.nih.gov/geo/) representing the ovulating follicle/forming CL (Xu et al., 2010), the CL during its functional lifespan in the non-gravid luteal phase (Bogan et al., 2008b), with emphasis on spontaneous luteal regression (Bogan et al., 2009), the CL following LH and steroid ablation/replacement protocols during the mid-late luteal phase (Bishop et al., 2009a), the CL during its rescue by CG during SEP (Bishop et al., 2011b), as well as steroid ablation/P-replacement during SEP (current database). Combining these datasets with luteal GEO microarray datasets from other primates (Priyanka et al., 2009; Fan et al., 2011; Gilbert et al., 2011; Rao et al., 2012; Zalman et al., 2012).

The microarray data presented here indicate that local and systemic factors controlling the luteal transcriptome differ both by length of CG exposure during early pregnancy and potentially as a function of LH versus CG luteotropic support. Interestingly, while the physiologic result of luteal stimulation by LH and CG is similar, i.e. increased production/release of P and E [Fig. 1 and Atkinson et al. (1975)], local steroid

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**Figure 5** Real-time PCR and microarray analyses of two gene products demonstrating the diverse effects of steroid ablation and progestin replacement during SEP. See Methods for detailed explanation of statistical tests and main effects. IL1RN (A) mRNA expression is impacted by CG after 6 days of SEP (main effect of hCG P < 0.001), and demonstrates an interaction between TRL and day of SEP (interaction between SEP and TRL; P < 0.001); after 1 day of SEP, IL1RN mRNA expression is significantly altered by both TRL and R5020 as indicated on the graph. However, there is no effect of steroid ablation and replacement after 6 days of SEP. TNFSF13B (B) mRNA expression is unaffected by CG during SEP (P > 0.5), but there is an interaction between TRL and day of SEP (interaction between SEP and TRL P = 0.03). There is no effect of steroid ablation and replacement after 1 day of SEP, but after 6 days of SEP, TNFSF13B mRNA expression is significantly altered by both TRL and R5020 as indicated on the graph. For clarity, significance associated with various treatments indicated on graphs is from real-time PCR analysis. Values denoted are means ± SEM for both real-time PCR and microarray data.
regulation of luteal genomic processes appears to be distinct. While a large portion of the luteal transcriptome is affected by SEP (Bishop et al., 2011b), only a small subset is regulated by CG-induced HSD3B2-derived steroids. The small number of steroid-regulated mRNAs and gene pathways shared in common between early and late SEP, and the non-fecund cycle [previously reported (Bishop et al., 2009a)] potentially represent master regulators of local steroid production in primate CL. These have possible implications for roles in fertility disorders such as luteal phase defect.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

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**Authors’ roles**

C.V.B. contributed to experimental design, oversaw execution of experimental protocols and processing of tissues, performed array analysis, data analysis, array validation, and prepared manuscript. R.A.A. contributed to array validation, data analysis and manuscript approval. L.M.Q. contributed to array validation, data analysis and manuscript approval. J.D.H. contributed to experimental design, data analysis and manuscript preparation. R.L.S. contributed to experimental design, funding of the

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**Figure 6** Comparison of TRL and R5020 effects on mRNA (A) and protein (B) levels of SERPINE1 during SEP (means ± SEM). Levels of SERPINE1 mRNA are dependent on CG-stimulated steroid production between Day 1 and Day 6 of SEP. (A) Real-time PCR values differing significantly are denoted by letters (main effects; hCG P < 0.001; TRL P < 0.02; R5020 P < 0.01). (B) 50 kDa protein (striped bars) values differing significantly are denoted by different letters (main effect; TRL P < 0.01), and 70 kDa protein (gray bars) values differing significantly are denoted by asterisks (main effects; hCG P < 0.005, R5020 P < 0.01; see Methods for details of statistical analyses).
study, data analysis and manuscript preparation. All authors had final approval of manuscript before publication.

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