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Supplementary Materials for

Sexually Dimorphic Actions of Glucocorticoids Provide a Link to Inflammatory Diseases with Gender Differences in Prevalence

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METHODS

Quantitative RT-PCR analysis of nascent mRNA

RNA was extracted from livers by using the RNeasy Midi Kit (Qiagen) according to the manufacturer's instructions. All samples were treated with RNase-free DNase (Qiagen). Samples were aliquoted and frozen at -80°C. Reverse transcription was carried out using 2 µg of total RNA following the protocol for the Taqman Reverse Transcription Master Mix (Applied Biosystems) and 100 ng of RNA converted into cDNA of each sample was used. Quantification was achieved using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Primer sequences were designed to amplify only nascent RNA, using PCR amplicons that cross an exon/intron or untranslated region (UTR)/intron boundary. Primer sequences for qPCR validation of expression microarray data are as follows: Tat, forward primer ACAACATGAAGGTGCAGCCCAATC and reverse primer CAGGCAATACCAGCCTTCCATCAA. Ttp: forward primer ATCTCTCTGCCATCTACGAGGTGA and reserve primer TAGTCTGAGAAGGAATGGAGGCGT. Statistical analysis was performed in Prism 5.0 (GraphPad, San Diego, CA).

Histolopathology

Adult male and female intact and adult male and female adrenalectomized Sprague-Dawley rats (8-10 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA). Post operation, the adrenalectomized animals were fed ad libitum and were given 0.154 M sodium chloride to drink for 7 days. Male and female (diestrous 1) intact and

adrenalectomized rats were treated with vehicle (phosphate-buffered saline) or dexamethasone (1 mg/kg i.p.) for 6 hours. The animals were euthanized while under anesthesia by cervical dislocation and necropsied within 5 min of sacrifice. The left liver lobe was promptly removed and one cross-section was placed in 10% neutral buffered formalin for subsequent histopathology. The fixed cross sections were transferred to histology grade alcohol for 18-24 hours after necropsy. Tissues were then processed, embedded in paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin.

Western blot analysis

Procedures for preparing animal tissues and isolated hepatocytes for Western blot analysis were previously described (1). Lysates containing 10 to 50 µg of proteins were resolved on 4 to 12% NuPage Bis-Tris gels (Invitrogen), and titers for antibodies were 1:500 to 1:1,000 for anti-GR 57 antibodies, and 1:10,000 to 1:40,000 for anti-ß-actin.

[^{3}H] dexamethasone binding assay

Cytosolic glucocorticoid receptor (GR) was prepared by homogenizing tissues (1:5 w/v) in binding buffer [10 nM HEPES, 20 nM NaMoO4, 1 mM EDTA, 10 mM dithiothreitol, 10% glycerol pH 7.2 containing protease inhibitor cocktails (Roche, Indianapolis, IN) at 4°C]. The homogenization was performed for 20 seconds (24,000 rpm) in Tekmar Tissumizer SDT-1810 followed by centrifuging at 20,000 g for 15 min at 4°C. Five concentrations of [^{3}H] dexamethasone (Perkin-Elmer, Wellesley, MA) ranging from 5 to 200 nM were used for binding reactions. After incubation for 24 hours at 4°C, unbound [^{3}H] dexamethasone was absorbed and stripped using activated charcoal. The amount of [^{3}H] dexamethasone

bound to GR was counted on an LS 6500 Multi Purpose Scintillation Counter (Beckman Coulter). Nonspecific binding was defined by the addition of a 1000-fold excess of unlabeled dexamethasone. Saturable binding was normalized to protein content. The one-site binding subroutine in Prism was used to obtain the total binding (B_{max}) and apparent *Kd* (dissociation constant) values.

Hepatocytes isolation, cell culture, and dexamethasone treatment

Livers were isolated from adult male and female Sprague-Dawley rats and digested with the collagenase perfusion method developed by Berry and Friend (2). After collagenase treatment, livers were excised, minced in balanced salt solution, and centrifuged at 50 *g* for 3 min. Immediately after isolation, hepatocytes were resuspended in Williams E Medium containing penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM glutamine pH 7.4, and 10% fetal bovine serum and plated on collagen-coated 94 x 16 mm cell culture dishes and maintained for 24 hours in a 95% air–5% CO2, 37°C incubator. The medium was then changed to serum-free Williams E Medium and maintained in culture for an additional 24 hours. Hepatocytes isolated from male and rats were treated with vehicle (0.01% ethanol) or dexamethasone (100 nm) for 6 hours. Total RNA from cells were extracted by using the RNeasy Midi Kit (Qiagen) according to the manufacturer's instructions. All samples were treated with the RNase-free DNase set (Qiagen) and were kept at -80°C until labeling and hybridization.

Microarray analysis

Three replicate microarrays were completed for each group (male hepatocytes control, male hepatocytes treated, female hepatocytes control, and female hepatocytes treated) for a total of 12 microarrays. Gene expression analysis was conducted using Agilent Whole Genome Rat 4x44 multiplex format oligo arrays (014879) (Agilent Technologies).

Labeling and hybridization

Starting with an aliquot of 500 ng of total RNA from each samples, Cy3 labeled cRNA was produced using the Agilent-1 color microarray-based gene expression protocol. For each sample, 1.65 µg of Cy3 labeled cRNAs were fragmented and hybridized for 17 hours in a rotating hybridization oven. Slides were washed and then scanned with an Agilent Scanner. Data was obtained using the Agilent Feature Extraction software (v9.1), using the 1-color defaults for all parameters.

Identification of significantly expressed genes in isolated hepatocytes

The Agilent Feature Extraction Software performed error modeling, adjusting for additive and multiplicative noise. The resulting data were processed using the Rosetta Resolver® system (version 7.1) (Rosetta Biosoftware, Kirkland, WA). Replicate hybridizations were combined into intensity experiments using an error-weighted average as described in Weng *et al.* (3). Next, ratios (male DEX/vehicle, female DEX/vehicle, and vehicle female/male) were built and P values were generated representing the probability that a given gene was significantly, differentially expressed**.** A filter ($P < 0.001$) was applied to the P values obtained from the ratios above described. Thus, from the 41,012 features (probes) included on the array, 14,779 met the P value ($P < 0.001$) for at least one of the comparisons. All

microarray data are available from the Gene Expression Omnibus database with the series accession number GSE13461.

Glucocorticoid treatment of C57BL/6 mice

Male and female (diestrous 1) adrenalectomized C57BL/6 mice were treated with vehicle (phosphate-buffered saline) or dexamethasone (1 mg/kg i.p.) (Steraloids) on the same day. Six hours after dexamethasone treatment, the liver was harvested and kept in RNAlater solution at 4°C overnight. All samples were frozen and stored at -80°C.

RNA isolation

Livers were homogenized and total RNA from liver was extracted by using the RNeasy Midi Kit (Qiagen) according to the manufacturer's instructions. All samples were treated with the RNase-free DNase set (Qiagen). Samples were aliquoted and frozen at -80°C.

Microarray analysis

Three replicate microarrays were completed for each group (male control, male treated, female control, and female treated) for a total of 12 microarrays. Each microarray was hybridized using a pooled RNA sample, and each pool was created from equal amounts of total RNA from three independent RNA samples, representing individual animals (n=9 animals per group). Gene expression analysis was conducted using Agilent Whole Mouse Genome 4x44 multiplex format oligo arrays (014868) (Agilent Technologies).

Labeling and hybridization

Starting with an aliquot of 500 ng of total RNA from each pool, Cy3-labeled cRNA was produced using the Agilent-1 color microarray-based gene expression protocol. For each sample, 1.65 µg of Cy3 labeled cRNAs were fragmented and hybridized for 17 hours in a rotating hybridization oven. Slides were washed and then scanned with an Agilent Scanner. Data was obtained using the Agilent Feature Extraction software (v9.5), using the 1-color defaults for all parameters.

Identification of significantly expressed genes

The Agilent Feature Extraction Software performed error modeling and adjusting for additive and multiplicative noise. The resulting data were processed using the Rosetta Resolver® system (version 7.2) (Rosetta Biosoftware). Replicate hybridizations were combined into intensity experiments using an error-weighted average as described in Weng *et al.* (3). Next, ratios (male DEX/vehicle, female DEX/vehicle, and vehicle female/male) were built and P values were generated representing the probability that a given gene was significantly, differentially expressed**.** A filter ($P < 0.001$) was applied to the P values obtained from the ratios above described. Thus, from the 41,176 features (probes) included on the array, 11,117 met the P value ($P < 0.001$) for at least one of the comparisons. All microarray data are available from the Gene Expression Omnibus database using series accession number GSE13461.

Dose-response curves for dexamethasone

Male and female (diestrous 1) adrenalectomized rats were treated with vehicle (phosphate-buffered saline) or dexamethasone (0.0001, 0.001, 0.01, 0.1, and 1 mg/kg i.p.) (Steraloids;

Newport, RI). The liver was harvested 6 hours after dexamethasone treatment and kept in RNA later solution at 4°C overnight. Livers were homogenized and total RNA from liver was extracted by using the RNeasy Midi Kit (Qiagen) according to the manufacturer's instructions. mRNA abundance was obtained by quantitative reverse transcription PCR using selected pre-developed primer sets from Applied Biosystems (Foster City, CA) and following the manufacturer's instructions. Reverse transcription was carried out using 2 μg of total RNA following the protocol for the Taqman Reverse Transcription Master Mix (Applied Biosystems) and 100 ng of RNA converted into cDNA of each sample was used. Each primer set was analyzed in duplicate and quantification was achieved using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Statistical analysis was performed in Prism 5.0 (GraphPad, San Diego, CA).

Figure S1

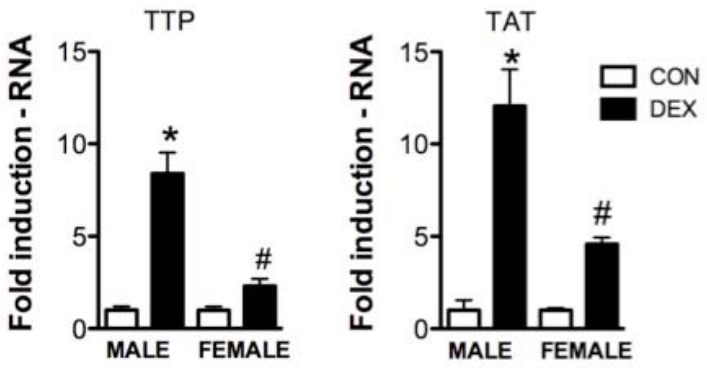

Figure S1. Nascent RNA analysis of glucocorticoid-regulated genes.

Nascent transcripts were characterized for representative glucocorticoid responsive genes in male and female rats 6 hours after dexamethasone (1mg/kg, i.p.) treatment. Total RNA was isolated from liver and quantitative RT-PCR was performed as described. Abundance of nascent RNAs was measured using primer pairs spanning intron/exon boundaries for each gene. Independent *t* tests were performed to compare values between male vehicle and male DEX (*, $P < 0.05$), and between female vehicle and female DEX ($^{\#}$, $P < 0.05$). *Ttp* = tritestrapolin and *Tat* = tyrosine aminotransferase.

Figure S2

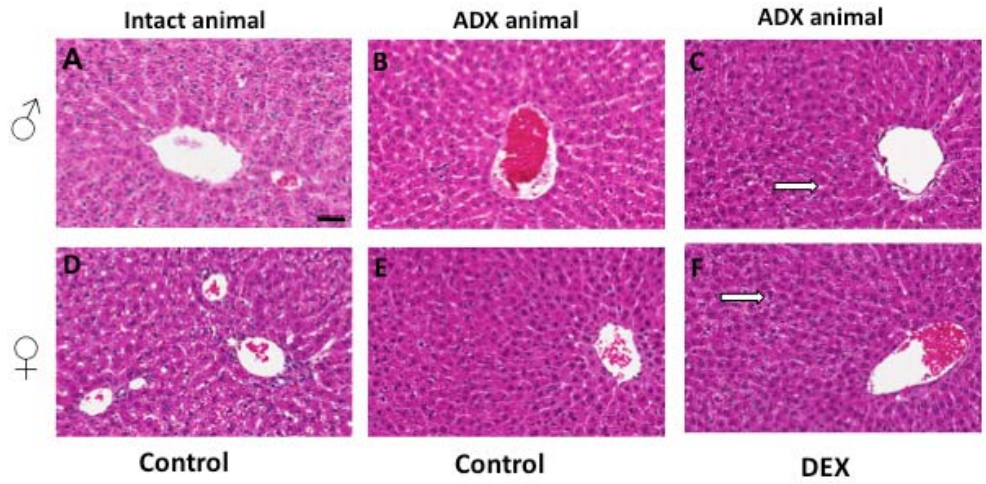

Figure S2. Comparison of male and female rat liver cross sections stained with hematoxylin and eosin. (**A** and **D**) Male and female control intact rats. (**B** and **E**) Male and female adrenalectomized rats treated with vehicle (control). (**C** and **F**) Male and female adrenalectomized rats treated with dexamethasone (DEX). White arrows point to minimal increase in periportal vacuolation of hepatocytes, which is characterized by irregular cytoplasmic clear spaces in treated male and female adrenalectomized rats. 20X magnification. The scale bar showed in Panel A represents 50μm. ADX = adrenalectomized.

Figure S3

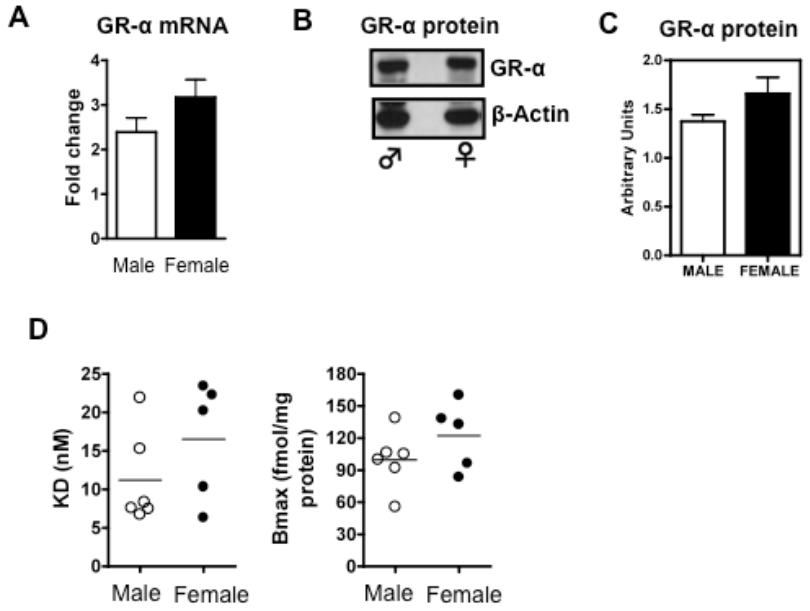

Figure S3. Comparison of glucocorticoid receptor mRNA and protein abundance and binding to [^{3}H]dexamethasone in male and female rat liver. (**A**) Total RNA was isolated from liver and quantitative RT-PCR was performed as described. (**B**) Glucocorticoid receptor (GR) protein abundance in rat livers was assessed by Western blotting protein extracts with anti-GR antibody and anti-ß-actin and is representative of four samples for each sex. (**C**) Quantification of Western blot densitometry in (B) and normalized β-actin abundance in the same lane. (**D**) Ligand binding assays were performed and B_{max} and K_d values ± standard errors of the means (SEM) were calculated. One-way ANOVA followed by Tukey post-hoc analysis was performed in (A) and (C) to compare GR mRNA abundance, B_{max}, and K_d values between males and females.

Figure S4

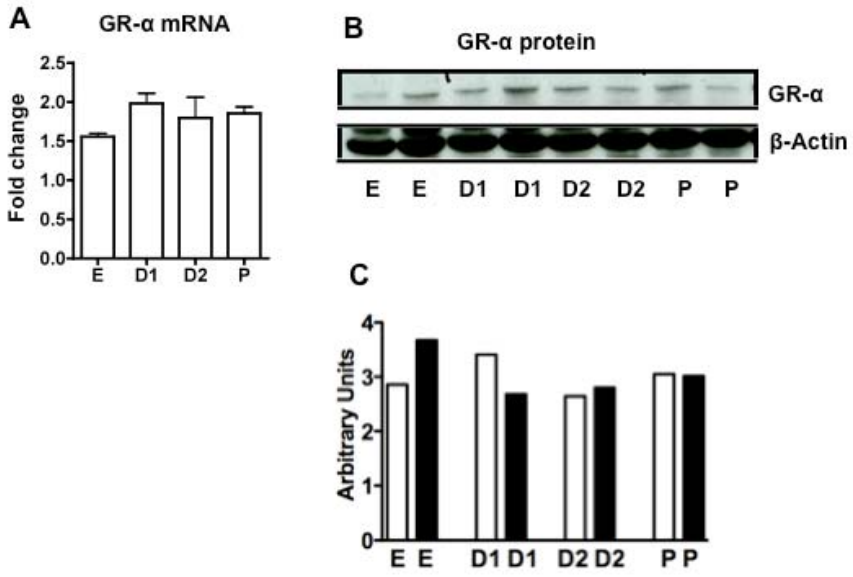

Figure S4. Comparison of glucocorticoid receptor mRNA and protein abundance in the liver of female rats in different phases of estrous cycle. (**A**) Total RNA was isolated from liver of female rats in different phases of estrous cycle and quantitative RT-PCR was performed as described. One-way ANOVA was performed to compare GR mRNA abundance from rats in different phase of estrous cycle, which was followed by Tukey post-hoc analysis. (**B**) GR protein abundance in the liver of females rats was assessed by Western blotting protein extracts with anti-GR antibody and anti-ß-actin and is representative of two samples each phase. (**C**) Quantification of Western blot densitometry in (B) normalized to β-actin abundance. E = estrous, D1 = diestrous 2, D2 = diestrous 2, and P = proestrous.

Figure S5

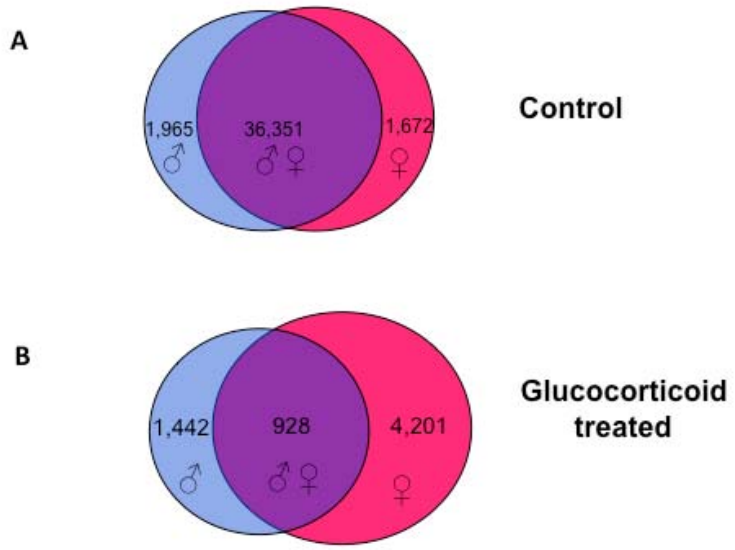

Figure S5. Sexually dimorphic regulation of gene expression by glucocorticoids in isolated hepatocytes. (**A**) Comparison of gene expression profiles in hepatocytes isolated from male and female rats. The left-hand circle indicates 1,965 probe sets that show higher abundance in adult males (female vehicle compared to male vehicle, $P < 0.001$). The right-hand circle indicates 1,672 probe sets that show higher abundance in adult females (female vehicle compared to male vehicles, $P < 0.001$). The overlapping circle indicates 36,351 probe sets that show similar abundance in males and females (female vehicle compared to male vehicle, $P > 0.001$). (**B**) Selective expression of glucocorticoid-regulated genes in male and female isolated hepatocytes. Probe sets regulated by dexamethasone (DEX) in males (male DEX compared to male vehicle) (left-hand circle) are compared to probe sets regulated by DEX in

female (female DEX compared to female vehicle) (right-hand circle). The overlapping circle represents probe sets that are commonly regulated by DEX in males and females. The microarray data has been deposited in the Gene Expression Omnibus (GEO) Database (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE24255.

Figure S6

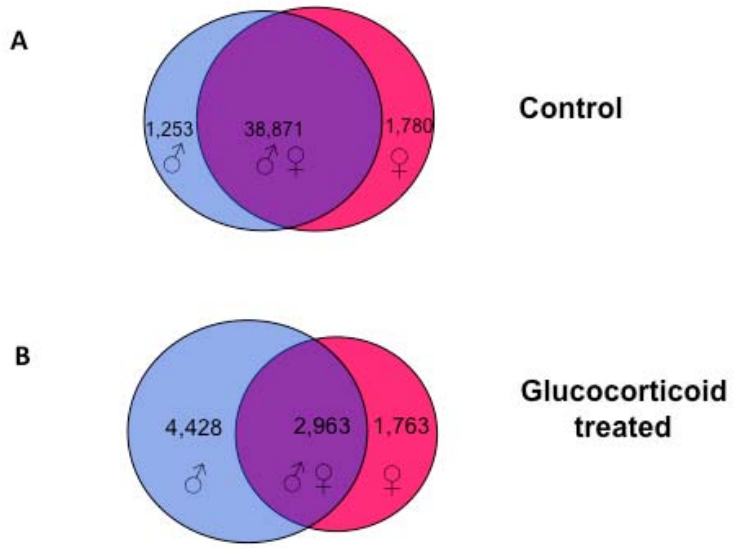

Figure S6. Sexually dimorphic regulation of gene expression by glucocorticoids in mouse liver. (**A**) Comparison of gene expression profiles in liver of male and female mice. The left-hand circle indicates 1,253 probe sets that show higher abundance in adult males (female vehicle compared to male vehicle, $P < 0.001$). The right-hand circle indicates 1,780 probe sets that show higher abundance in adult females (female vehicle compared to male vehicles, $P < 0.001$). The overlapping circle indicates 38,871 probe sets that show similar abundance in males and females (female vehicle compared to male vehicle, $P > 0.001$). (**B**) Selective expression of glucocorticoid-regulated genes in male and female mouse liver. Probe sets regulated by dexamethasone (DEX) in males (male DEX compared to male vehicle) (left-hand circle) are compared to probe sets regulated by DEX in female (female

DEX compared to female vehicle) (right-hand circle). The overlapping circle represents probe sets that are commonly regulated by DEX in males and females. The microarray data has been deposited in the Gene Expression Omnibus (GEO) Database (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE24256.

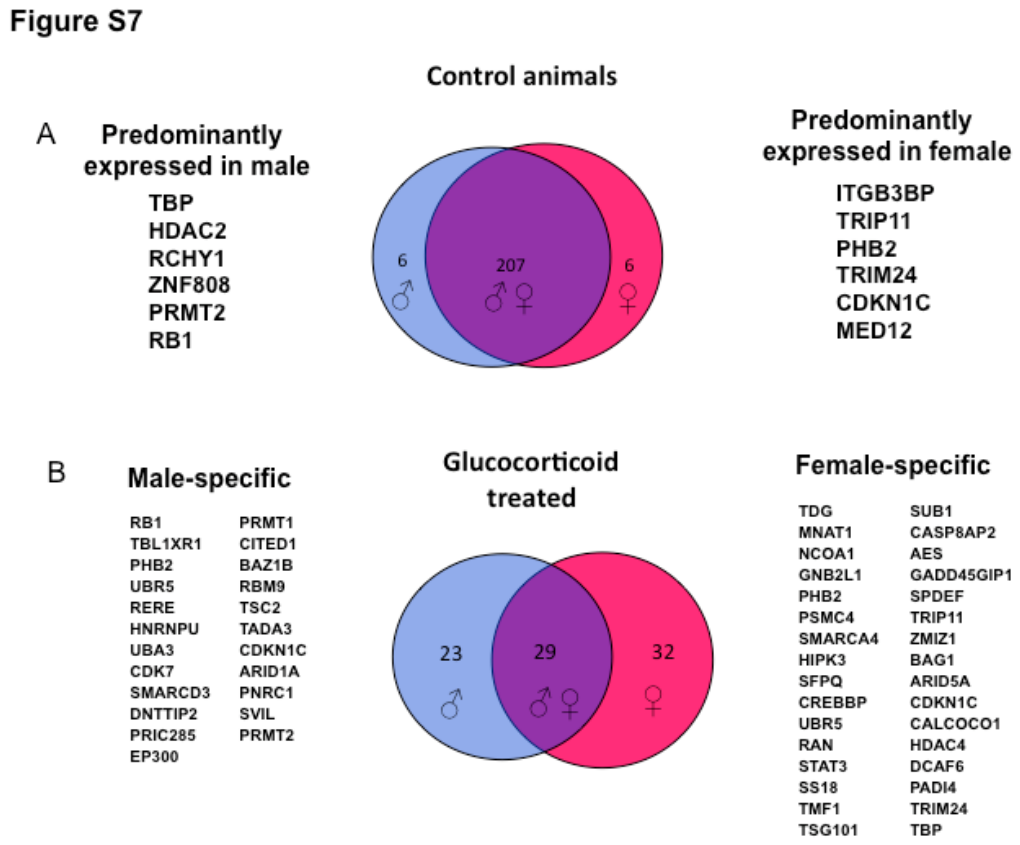

Figure S7. Analyses of gender-specific expression patterns of nuclear receptor co-regulators. (**A** and **B**) A list of co-regulators associated with nuclear receptors (248) was loaded in the Ingenuity Pathway Analysis software. Venn diagrams were built by comparing the list of co-regulators to the lists of genes predominantly expressed in males, predominantly expressed in females, and expressed in both male and female rat livers (A), and to the lists of genes regulated by glucocorticoids in both sexes, regulated only in males, and regulated only in females (B). The name and expression value of each co-regulator is given in table S4.

Figure S8

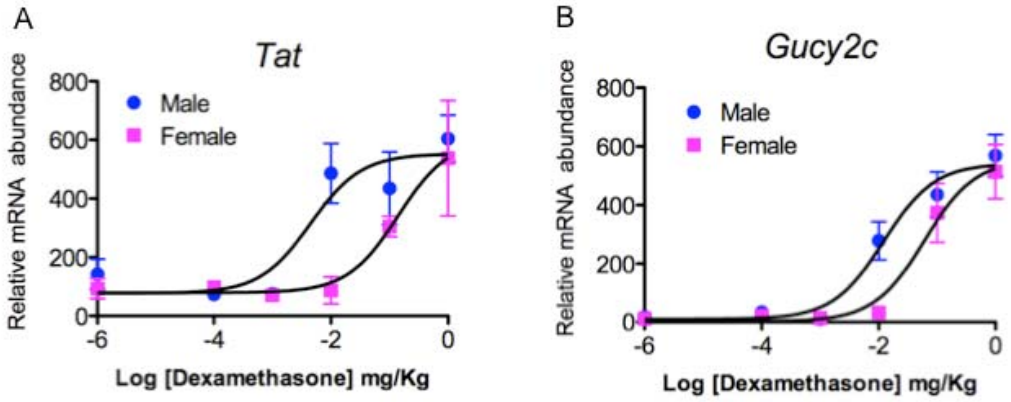

Figure S8. Analysis of dose-response curves for dexamethasone in male and female rat livers. (**A** and **B**) Animals were treated with different doses of dexamethasone (0.0001, 0.001, 0.01, 0.1, or 1 mg/kg, i.p.) for 6 hours. Quantitative RT-PCR was performed to analyze the mRNA abundance of genes commonly regulated by glucocorticoids in male and female rat livers: *tyrosine aminotransferase* (*Tat*) (A) and *Fk506 binding protein 5* (*Fkbp5*) (B). The EC50 values for each gene were calculated in Prism 5.0 and represent the average from 3 individual curves. *Tat* EC50: male = 0.006 ± 0.002, and female = 0.193 ± 0.039. *Fkbp5* EC50: male = 0.003 ± 0.001, and female = 0.046 ± 0.009.

REFERENCES

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3. L. Weng, H. Dai, Y. Zhan, Y. He, S. B. Stepaniants, D. E. Bassett, Rosetta error model for gene expression analysis. *Bioinformatics* **22**, 1111-1121 (2006).