Photoperiod-induced differential expression of angiogenesis genes in testes of adult *Peromyscus leucopus*

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Abstract

Non-pathological angiogenesis in adults is rare and is largely thought to be restricted to wound healing and female reproductive cycles. Adult male rodents, however, display seasonal angiogenesis to support seasonal changes in reproductive tissue morphology. Non-tropical rodents use photoperiod (day length) to determine the time of year. During short days, the reproductive system undergoes involution and mating behaviours stop, adaptations which presumably allow energy resources to be shifted to processes necessary for winter survival. We compared the patterns of gene expression involved in angiogenesis in testes of white-footed mice (*Peromyscus leucopus*) following 7, 14, 21 or 34 weeks of long or short day lengths. Short days decreased body mass, reproductive tract mass and seminiferous tubule diameter. Potential genes involved in seasonal angiogenesis were screened by hybridizing testicular RNA from each group to angiogenesis-specific microarrays. Genes that were \( \geq 6 \)-fold different between long- and short-day testes (i.e. hypoxia-inducible factor 1a (Hif1a), plasminogen activator inhibitor 1 (Serpine1), transforming growth factor \( \beta \) receptor 3 (Tgf\( \beta r3 \)) and tumour necrosis factor (Tnf)) were sequenced and expression differences were compared throughout gonadal regression and recrudescence using quantitative RT-PCR. Our results suggest that short days trigger expression of *Hif1a*, *Serpine1*, and *Tgf\( \beta r3 \)* to inhibit angiogenesis or promote apoptosis during testicular regression, and also trigger expression of *Tnf* to promote angiogenesis during testicular recrudescence.

Introduction

Angiogenesis is the process by which new blood vessels are formed from existing vessels. In adult mammals, angiogenesis is principally confined to the processes of wound healing, tumorigenesis and physiological changes associated with female reproductive cycles (Tomanek & Schatteman 2000, Smith 2001). Some evidence suggests, however, that adult male reproductive tissues are also capable of angiogenesis. Rodents residing in environments that undergo dramatic seasonal changes display marked morphological changes in order to adapt to the fluctuating environment (Bronson 1985). These rodents often use photoperiod (day length) for temporal information to initiate and terminate seasonally appropriate morphological, physiological and behavioural adaptations that maximize survival and reproductive success (Prendergast et al. 2001). Short day lengths trigger regression of the reproductive tract and cessation of reproductive behaviours in many rodents, an adaptation thought to shift energy reserves to survival functions (Nelson et al. 2002). Regrowth of the reproductive tract commences after prolonged exposure to short day lengths so that full reproductive development precedes the onset of the mating season (Gram et al. 1982). In the laboratory, seasonal changes can be triggered in many seasonally responsive rodents solely by manipulation of photoperiod (Smale et al. 1988, Prendergast & Nelson 2001); photoperiod information is transduced into a physiological signal via the pineal hormone, melatonin (Goldman 2001).

The mass of the reproductive tract of male white-footed mice (*Peromyscus leucopus*) decreases following 6–12 weeks of exposure to short photoperiods (Lynch et al. 1981). Testicular cells (primarily spermatids and spermatocytes) undergo apoptosis in short days, which may contribute to decreased tissue mass (Young et al. 2000, Young & Nelson 2001). Seasonal changes in testicular capillary blood flow (Joffre & Joffre 1973), permeability of the blood–testis barrier (Pelletier 1986), and volume and density of testicular microvasculature (Mayerhofer et al. 1989) have been reported in several species that breed seasonally. The molecular mechanisms that regulate vascular development and demolition in response to environmental cues remain unspecified. However, the collapse and regrowth of the testes positively correlates with vascular endothelial growth factor (VEGF) protein expression (Young & Nelson 2000). VEGF
is a growth factor that is present during blood vessel growth of tumours, wound healing and uterine vascularization (Neufeld et al. 1999).

The photoperiod-driven cycle of VEGF protein levels in the testes of white-footed mice is evidence that changes in tissue mass may be accompanied by changes in the supporting vasculature. Tissue growth may precede growth of the vascular bed and tissue demise may precede vascular regression or vice versa. For example, excessive tumour growth can be inhibited by prevention of angiogenesis suggesting that tissue growth precedes angiogenesis (Brem 1976). However, seasonal changes in testicular blood vessel volume precede measurable changes in testes morphology in Syrian hamsters (Mesocricetus auratus), suggesting that angiogenesis precedes tissue morphology (Mayerhofer et al. 1989). Also, a seasonal increase in testicular blood flow precedes the presence of spermatozoa in testes of dormice (Glis glis), but not ferrets (Mustello furo) and foxes (Vulpes vulpes) (Joffre & Joffre 1973). Regardless of the timing of angiogenesis, the process consists of four main steps: (1) degradation of the epithelial layer of the existing vessel by matrix metalloproteinases; (2) recruitment of growth factors; (3) proliferation and recruitment of new epithelial cells; (4) stabilization of the new vessel by smooth muscle cells (Klagsbrun & Moses 1999). To maintain vasculature, many blood-borne factors are involved and are expressed in a balance between promoting and inhibiting angiogenesis (Klagsbrun & Moses 1999). When the signal to expand the vasculature is initiated, this balance swings in favour of the pro-angiogenic factors. We hypothesized that expression of angiogenesis genes would differ in the testes between long- and short-photoperiod-treated white-footed mice and that these differences would correspond to the pattern of testicular regression and regrowth.

Materials and Methods

Animals

White-footed mice (P. leucopus) from our breeding colony were used in this study (116 adult males; >55 days of age). Animals were housed individually in polypropylene cages (27.8 × 7.5 × 13 cm) with a constant temperature and humidity of 21 ± 5°C and 50 ± 5% respectively; food (Harlan Teklad 8640 rodent diet, Indianapolis, IN, USA) and filtered tap water were available ad libitum. Mice were either housed in a reverse long (16h light per day; lights illuminated at 2300h Eastern Standard Time (EST)) photoperiod (n = 36), or in a short (8h light per day; lights illuminated at 0700h) photoperiod (n = 80 males). Mice exposed to short days that failed to regress their reproductive tract (approximately 35%) were included in the study as a separate group described below as ‘short-day, non-responsive’ mice. Mice were maintained in their respective photoperiods for 7, 14, 21 or 34 weeks. All studies were conducted after approval by the Ohio State University Institutional Animal Care and Use Committee and were in compliance with all US federal animal welfare requirements.

Experimental procedures

Body mass and reproductive responses to photoperiod were assessed bi-weekly under light isoflurane anaesthesia. Reproductive status was assessed by external measurement of the left testis with calipers. The product of testis length times testis width squared provides a measure of estimated testis volume (ETV) that is highly correlated with testis weight (Gorman & Zucker 1995). After 7, 14, 21 and 34 weeks of photoperiod treatment, 4–34 male mice (from each photoperiod) were killed via rapid cervical dislocation and tissues were collected.

Tissue collection

Right testis, epididymides, epididymal fat pads and seminal vesicles were collected from mice under aseptic conditions and submerged in 10 volumes (10 µl per mg tissue) of RNAlater RNA stabilization reagent (Qiagen) and stored at 4°C for 24 h. All surgical instruments were washed consecutively with 70% alcohol, RNase Away (Fisher, Pittsburgh, PA, USA) and sterile deionized water between mice. Seminal vesicle fluid was expressed prior to storage. Following 24 h of RNAlater submergence, tissues were blotted briefly, weighed and frozen at −70°C until RNA processing. Prior to weighing, connective tissue and fat were cleaned from epididymides. The left testis was collected from each mouse and fixed in 10% neutral buffered formalin for at least 2 weeks until processed for histology. The mean testes mass for long-day (LD) mouse was determined and all short-day (SD) males with testes mass equal to or greater than 1.5 standard deviations below this mean were considered reproductively responsive to short days (SDR), whereas those mice that did not differ from the LD mean were considered non-responsive to short days (SDNR).

Histology

Testes were fixed in 10% neutral buffered saline, paraffin embedded and cut into 5 µm sections on a rotary microtome. Sections were progressively stained with eosin Y and Harris haematoxylin, and coverslips were affixed with Permount. Diameters of 10 seminal vesicles chosen semi-randomly using a fixed line across the section were visualized with light microscopy at ×20 or ×30 power and measured on five randomly selected sections of each testes using SpotBasic software (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Diameters were measured only if their length and width differed by <25%. Seminiferous tubule measurements of SDNR mice at 21 weeks were excluded due to improper embedding processing.
**RNA extraction**

Total RNA was extracted from ≤30 mg of individual testes using a rotor-stator homogenizer with an RNeasy Mini Kit according to the manufacturer’s protocol (Qiagen). Extracted RNA was suspended in 30–50 μl RNase-free water and RNA concentration was determined by spectrophotometer. For gene array analysis, aliquots of equal mass (e.g. 1 μg) from individual RNA samples at the 14-week time point were pooled to create a single pooled sample representative of each photoperiod treatment group. Individual samples with concentrations <100 μg/ml were excluded from the pooled sample (n = 1, LD at week 21). For quantitative PCR (qPCR), individual RNA samples were used. All RNA samples were stored at −80°C until gene expression analysis.

**Microarrays**

As a preliminary screen for differences in angiogenesis gene expression, microarrays were hybridized with testicular RNA after 14 weeks of photoperiod treatment. Non-Radioactive Mouse Angiogenesis GEArray Q Series microarrays with the GEArray RT-labeling method (Super-Array, Frederick, MD, USA) were used according to the manufacturer’s protocol with a few modifications. Briefly, 4 μg of each pooled RNA sample were reverse transcribed to cDNA and used for probe synthesis and the reverse transcription step was extended to 4 h. Hybridization of labeled cDNA to the microarray occurred at 59°C for 24 h. On a sheet of plastic wrap, the chemiluminescent substrate was incubated on the array for 4 min while covered by a piece of Parafilm (Fisher) to equalize the substrate distribution. Immediate, side-by-side chemiluminescent imaging of the microarray from each treatment was made possible by a CCD camera imaging system and detection was stopped upon pixel saturation. Quantity One software (Applied Biosystems, Foster City, CA, USA) was used to capture images and measure relative density of gene spots after background subtraction. Individual gene spot density was quantified following normalization to the density of the Gapdh spot. We plotted the distribution of the fold differences in gene expression against the number of genes and designated a cut-off point based on the mean fold difference (mean = 5.7). Therefore, genes with expression levels that differed ≥6-fold among treatment groups were examined throughout testicular regression and recrudescence via qPCR.

**Sequencing**

In order to design species-specific primers and probes for qPCR, a portion of each gene of interest was sequenced. Semi-degenerate primers were designed based on conserved regions between Mus musculus and Rattus norvegicus gene sequences using PrimerExpress software (Applied Biosystems, Table 1). Forty cycles of PCR were conducted on 1 μl cDNA from pooled P. leucopus RNA with Taq DNA Polymerase enzyme (Invitrogen), according to the manufacturer’s protocol, in a thermocycler with melting temperatures of 59–60°C. Gene-product amplification was visualized on 2% 40 mM Tris acetate and 2 mM EDTA (TAE)-agarose gels containing ethidium bromide using a CCD camera. To verify amplification of the correct gene, PCR products of the expected molecular size were purified (Centricon-100, Millipore, Billerica, MA, USA), and sequenced at the Plant-Genomics Centre at Ohio State University. Resulting amplicon sequences were >90% homologous to the Mus gene of interest and therefore were assumed to be the correct P. leucopus gene of interest.

**Quantitative RT-PCR**

We examined and quantified potentially functional gene expression differences of hypoxia-inducible factor 1α (Hif1α), transforming growth factor β receptor 3 (Tgfbr3), plasminogen activator inhibitor 1 (Serpine1) and tumour necrosis factor (Tnf) using qPCR. Expression of these genes was quantified for individual animals of each photoperiod group at all four time points in duplicate or triplicate. Individual RNA samples (n = 4 per photoperiod group per time point) were reverse transcribed into cDNA with MMLV Reverse Transcriptase enzyme (Invitrogen) according to the manufacturer’s protocol. Primers and probes for qPCR were designed based on the attained sequencing information using PrimerExpress. Primers and probes were synthesized as follows, with probes labeled with 6-FAM

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences for sequencing</th>
<th>Tm</th>
<th>Accession created</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia-inducible factor 1α</td>
<td>Forward: AGAAACCRCCYATGACGTGC</td>
<td>60</td>
<td>AY591916</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCACCTCCTTTTGGCAAGCAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasminogen activator inhibitor 1</td>
<td>Forward: ACRTSTGGAACTGCTCTAC</td>
<td>60</td>
<td>AY591915</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAGCTGCTCTTGTCGAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transforming growth factor β receptor 3</td>
<td>Forward: GCACCAAGAAGGTCTGTGTT</td>
<td>59</td>
<td>AY591914</td>
</tr>
<tr>
<td></td>
<td>Reverse: ATGGATTGCAAACATGGACTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor necrosis factor (Tnf)</td>
<td>Forward: GGAGGCACTTCCCCAAAAAG</td>
<td>59</td>
<td>AY608911</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCCCTTGCCCTTGAAGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>Forward: CACGCGCCGTACAGTGAAC</td>
<td>60</td>
<td>AY591913</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGTTGATAGGGCGAGCCTCGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tm, melting temperature.

Table 1 Sequencing information for genes differentially expressed ≥6-fold in microarray analysis.
(fluorescent dye) and MGB (non-fluorescent quencher dye) at the 5’ and 3’ ends respectively: HIf1α forward 5’-CTGG-
GATGAAAGATTACGATTACGATG-3’, HIf1α reverse 5’-
CATGATATGGCAGCTGCAGG-3’, HIf1α probe 5’-TATGA-
CCAGAAGAC-3’, Tgfb3 forward 5’-CAGGACACGCTC-
GATGGA-3’, Tgfb3 reverse 5’-CACCAAGAGGTCT-
TGTTTATTACA-3’, Tgfb3 probe 5’-CATCACCTCTCAAC-
ATGG-3’, Serpine1 forward 5’-CTCACCCACGATCTTGGA-
TGCT-3’, Serpine1 reverse: 5’-CGAGGCAGTCTGTAT-
TGTC-3’, Serpine1 probe 5’-CTCATCCCCACATGGAA-3’;
Tnf forward 5’-CCCACCAAGGGGAGGAAGT-3’, Tnf reverse
5’-GCCATGGAGCCGGATGAT-3’, Tnf probe 5’-CCCAAC-
AACTCTC-3’. A TaqMan 18S Ribosomal RNA primer and
probe set (labeled with VIC (fluorescent dye); Applied
Biosystems) was used as the control gene for relative
quantification. Ninety-six-well PCR plates were composed
do duplicate or triplicate wells for all five genes of each
individual sample. On each plate, batches of samples were
randomized for photoperiod treatment and time point, and
sample identification was encoded to eliminate potential
experimental bias. Amplification was performed on an ABI
7000 Sequencing Detection System by using TaqMan
Universal PCR Master Mix. The universal two-step RT-PCR
cycling conditions used were: 50°C for 2 min, 95°C for
10 min, followed by 40 cycles of 95°C for 15 s and 60°C
for 1 min. Relative expression was calculated by
comparison to a relative standard curve consisting of serial
dilutions of pooled P. leucopus cDNA (1, 1:10, 1:100,
1:1000) followed by normalization to 18S rRNA gene
expression.

Statistical analysis
ANOVA repeated measures tests were used to compare all
repeated measures among time points. Two-tailed
Student’s t-tests compared treatment groups of interest.
Data with unequal variances were log transformed and
compared by ANOVA (within-week comparisons for PCR
data) or compared using the non-parametric Kruskal–
Wallis test (between-week comparisons for PCR data).
All comparisons were considered significant when $P < 0.05$.
StatView software was used for all analyses (Cary, NC,
USA).

Results

Body mass
SDR mice weighed less than LD mice after 10, 12, 14 and
17 weeks of photoperiod treatment (Fig. 1; $P < 0.05$ in all
cases). SDR mice also weighed less than SDNR mice after
2, 4, 10, 12 and 14 weeks of photoperiod treatment
(Fig. 1; $P < 0.05$ in all cases).

Estimated testicular volume
SDR mice displayed decreased ETV as compared with LD
mice after 6, 8, 10, 12, 14, 17, 19, 21 and 29 weeks of
photoperiod treatment (Fig. 2; $P < 0.05$ in all cases). SDR
mice displayed decreased ETV as compared with SDNR
mice after 0, 6, 8, 10, 12, 14, 17, 19, 21 and 23 weeks of
photoperiod treatment (Fig. 2; $P < 0.05$ in all cases).

Tissue masses
Secondary reproductive tissue absolute masses and masses
relative to body mass (absolute mass/body mass $\times 1000$)
are shown in Table 2. Testes and epididymides absolute
masses decreased in SDR mice as compared with LD
mice after 7, 14 and 21 weeks of photoperiod treatment,
whereas absolute seminal vesicles mass decreased after 7,
14, 21 and 34 weeks ($P < 0.05$ in all cases). Calculation
of masses of these tissues after adjusting for body mass
yielded similar statistically significant differences except
for epididymides mass at 7 weeks and testes mass at 14
weeks (see Table 2; $P < 0.05$). Absolute and relative epidi-
dymal fat pad mass also decreased in SDR mice as com-
pared with LD mice, but only after 14 weeks of treatment

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**Figure 1** Effect of photoperiod treatment on body mass (means $\pm$ s.e.m.). Weeks 0–7: LD (n = 38), SDR (n = 53), SDNR
(n = 28). Weeks 8–14: LD (n = 29), SDR (n = 43), SDNR
(n = 19). Weeks 15–21: LD (n = 11), SDNR (n = 9), SDNR (n = 8). Weeks
22–34: LD (n = 4), SDNR (n = 4). *, $P < 0.05$ between LD and SDR; +, $P < 0.05$ between SDR and SDNR.

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**Figure 2** Effect of photoperiod treatment on reproductive status (means $\pm$ s.e.m.). Weeks 0–7: LD (n = 38), SDR (n = 53), SDNR
(n = 28). Weeks 8–14: LD (n = 29), SDR (n = 43), SDNR (n = 19). Weeks
15–21: LD (n = 11), SDNR (n = 9), SDNR (n = 8). Weeks
22–34: LD (n = 4), SDNR (n = 4). *, $P < 0.05$ between LD and SDR; +, $P < 0.05$ between SDR and SDNR. Time points of
tissue collections are indicated by arrow-heads.
Table 2: Mean values (± S.E.M.) of absolute and relative male secondary reproductive tissue masses (mg) after 7, 14, 21 and 34 weeks of photoperiod treatment.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mass</th>
<th>7 weeks</th>
<th>14 weeks</th>
<th>21 weeks</th>
<th>34 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD (9)</td>
<td>SDR (10)</td>
<td>SDNR (9)</td>
<td>LD (18)</td>
<td>SDR (34)</td>
</tr>
<tr>
<td>Testes Absolute</td>
<td>221.0 ± 16.1</td>
<td>115.6 ± 20.9*</td>
<td>204.7 ± 24.3</td>
<td>221.0 ± 12.4</td>
<td>77.1 ± 7.0*</td>
</tr>
<tr>
<td>Testes Relative</td>
<td>10.0 ± 0.7</td>
<td>5.8 ± 1.1#</td>
<td>8.3 ± 0.9</td>
<td>9.1 ± 0.4</td>
<td>3.9 ± 0.3*</td>
</tr>
<tr>
<td>Seminal vesicles</td>
<td>117.9 ± 13.8</td>
<td>52.9 ± 13.1*</td>
<td>117.8 ± 16.7</td>
<td>142.7 ± 16.0</td>
<td>40.9 ± 15.4*</td>
</tr>
<tr>
<td>Relative</td>
<td>5.2 ± 0.5</td>
<td>2.7 ± 0.7*</td>
<td>4.7 ± 0.6</td>
<td>6.1 ± 0.6</td>
<td>2.0 ± 0.7*</td>
</tr>
<tr>
<td>Epididymides Absolute</td>
<td>48.5 ± 4.1</td>
<td>34.2 ± 2.6*</td>
<td>46.0 ± 2.6</td>
<td>52.5 ± 3.5</td>
<td>27.6 ± 3.6*</td>
</tr>
<tr>
<td>Relative</td>
<td>2.2 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>1.9 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>1.4 ± 0.1*</td>
</tr>
<tr>
<td>Epididymal fat pads</td>
<td>184.0 ± 35.3</td>
<td>201.4 ± 56.7</td>
<td>324.2 ± 74.7</td>
<td>207.1 ± 54.2</td>
<td>54.3 ± 10.1*</td>
</tr>
<tr>
<td>Relative</td>
<td>8.0 ± 1.2</td>
<td>9.5 ± 2.1</td>
<td>11.8 ± 2.3</td>
<td>7.7 ± 1.6</td>
<td>2.6 ± 0.4*</td>
</tr>
</tbody>
</table>

* P < 0.05 between short-day responders (SDR) and both long-day (LD) and short-day non-responders (SDNR).
# P < 0.05 between (SDR) and (LD).
þ P < 0.05 among all groups.
Sample sizes are indicated in parentheses.

Average seminiferous tubule diameters decreased in SD mice after 7 and 14 weeks of photoperiod treatment (Fig. 2A–D; Table 1). SDR mice had significantly reduced seminal vesicles mass compared with LD mice at all time points. Additionally, stained nuclei in LD and SDNR mice depicted active spermatogenesis in these groups (Fig. 3A and D).

Microarrays

Microarray expression differences of at least 2-fold are summarized in Table 3. Based on the distribution of the fold differences, gene expression in LD mice at week 7 as compared with LD mice at week 14 was statistically significant (Fig. 4A, B and D; P < 0.05). Microarray expression differences of at least 2-fold were reversed at 14 weeks such that expression in LD mice was significantly greater than all other groups (Fig. 4C, D; P < 0.05). SDR and LD mice had similar gene expression levels of Serpine1 and Tnf at all time points. Although it was only statistically significant between LD and SDNR mice at 7 weeks, Serpine1 expression levels of LD mice were numerically higher than those of SDNR mice at 21 weeks. Furthermore, Serpine1 expression levels of SDNR mice were similar to those of LD mice at week 34.

Microarray expression differences of at least 2-fold were observed in Tgfbr3 expression levels of LD mice at week 7 as compared with LD mice at week 14 (Fig. 4A, B and D; P < 0.05). Additionally, relative seminal vesicles and epididymides masses were decreased in SD mice after 34 weeks as compared with LD mice (P < 0.05). Both absolute and relative testes and epididymides masses of SD mice depicted active spermatogenesis in these groups (Fig. 3A and D).

Histology

Histology samples were examined for seminiferous tubule diameters as compared with SDNR mice at 7 weeks (Fig. 2A–D). SDR mice had significantly reduced seminiferous tubule diameters as compared with SDNR mice at 7 weeks (Fig. 2A–D; P < 0.05). Additionally, relative seminal vesicles and epididymides masses were decreased in LD mice (P < 0.05). Also at week 21, although SDNR mice had significantly higher expression of Hif1b in LD mice, this expression was significantly lower after 14 weeks such that expression was not statistically significant (Fig. 4A, B and D; P < 0.05). However, the Serpine1 gene expression levels of LD mice at week 14 were comparable with those of SDNR mice at week 7 (Fig. 4A–C; P < 0.05). Furthermore, the Serpine1 gene expression levels of LD mice were numerically higher than those of SDNR mice at week 21 (Fig. 4A–D; P < 0.05).

All data were log-transformed due to unequal variances. Relative gene expression of Tgfbr3 was increased in all SD mice at week 7 compared with LD mice (Fig. 4A–D; P < 0.05). Additionally, relative seminal vesicles and epididymides masses were decreased in SDR mice after 34 weeks (Fig. 2A–D; P < 0.05). Furthermore, relative seminal vesicles and epididymides masses were decreased in SDR mice after 34 weeks (Fig. 2A–D; P < 0.05). Furthermore, relative seminal vesicles and epididymides masses were decreased in SDR mice after 34 weeks (Fig. 2A–D; P < 0.05). Furthermore, relative seminal vesicles and epididymides masses were decreased in SDR mice after 34 weeks (Fig. 2A–D; P < 0.05).
34 weeks of photoperiod treatment (Fig. 4B; \( P < 0.05 \)). No differences in Hif1a, Serpine1 or Tnf expression existed among groups at 34 weeks (Fig. 4A, C and D; \( P > 0.05 \)).

There were significant effects of week on Tgfbr3, Serpine1 and Tnf expression in LD, SDR and SDNR mice (\( P < 0.05 \) in all cases). Additionally, there was a significant effect of week on Hif1a expression in LD only (\( P < 0.05 \)).

**Discussion**

The present study confirms and extends previous results that short days decrease reproductive tract mass, body mass and seminiferous tube diameter in adult male white-footed mice (Whitaker 1940, Johnston & Zucker 1980, Glass 1986). Additionally, expression of genes involved in angiogenesis differs in testes of LD and SD mice and corresponds to the photoperiod-driven pattern of regression/recrudescence of the reproductive tract. Specifically, SD mice display high expression levels of Hif1a, Tgfbr3 and Serpine1 during testicular regression and high expression levels of Tnf during testicular recrudescence. These data are consistent with the hypothesis that photoperiod alters expression of angiogenesis genes in adult males.

Angiogenesis in adult mammals has been studied in the context of tumour growth, wound healing and the cyclical changes of the female reproductive tract. Studies of angiogenesis specific to adult males are sparse and are limited to models of seasonal breeders including dormice (G. glis), ferrets (M. furo), and foxes (V. vulpes) (Joffre & Joffre 1973). Dormice and foxes increase capillary blood flow in the testes during the breeding season. Short days decrease blood vessel volume and transfer to long days increases vascular permeability in the testes of Syrian hamsters.

**Table 3** Two fold or greater angiogenesis gene expression differences in the testes after 14 weeks of photoperiod treatment.

<table>
<thead>
<tr>
<th>Gene</th>
<th>14 weeks</th>
<th>Fold difference</th>
<th>RT-PCR</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiopoietin 2</td>
<td>↑</td>
<td>2</td>
<td></td>
<td>Vessel remodeling and sprouting</td>
</tr>
<tr>
<td>Hypoxia-inducible factor 1a (Hif1a)</td>
<td>↑</td>
<td>19.4</td>
<td>*</td>
<td>Pro-angiogenic or pro-apoptotic</td>
</tr>
<tr>
<td>Endothelial nitric oxide synthase</td>
<td>↑</td>
<td>5.1</td>
<td></td>
<td>Vessel permeability</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor 1 (Serpine1)</td>
<td>↑</td>
<td>6.4</td>
<td>*</td>
<td>Matrix remodeling; pro- or anti-angiogenic</td>
</tr>
<tr>
<td>Secreted acidic cysteine-rich glycoprotein</td>
<td>↓</td>
<td>4.2</td>
<td></td>
<td>Cell detachment, matrix remodeling</td>
</tr>
<tr>
<td>TGF ( \beta ) receptor 3 (Tgfbr3)</td>
<td>↑</td>
<td>69.4</td>
<td>*</td>
<td>Inhibit formation of new vessels by TGF ( \beta )</td>
</tr>
<tr>
<td>Tenasin C</td>
<td>↑</td>
<td>5.5</td>
<td></td>
<td>Cell adhesion and detachment</td>
</tr>
<tr>
<td>Tumor necrosis factor (Tnf)</td>
<td>↑</td>
<td>20.9</td>
<td>*</td>
<td>Differentiate cells to support new vessels</td>
</tr>
</tbody>
</table>

Fold difference expression difference between LD and SDR at 14 weeks.

* Examination of gene expression by RT-PCR.
(M. auratus) (Mayerhofer et al. 1989). Similarly, testicular blood vessel volume of adult male red stags (Cervus elaphus) and camels (Camelus dromedaries) was greater when measured in the breeding season than during the non-breeding season (Hochereau-de Reviers & Lincoln 1978, Zayed et al. 1995). In mink (Mustela vison), the blood–testis barrier competency changes across seasons, although the pattern of competency does not completely match the seasonal progression of spermatogenesis (Pelletier 1986). These studies provide evidence for structural changes in the testicular vasculature of seasonally breeding adult males.

The mechanisms by which testicular vasculature varies across seasons have not been established. However, Vegf may be implicated in this process based on the positive relationship between VEGF protein expression and testes mass in SD and LD white-footed mice (Young & Nelson 2000). No differences (≥2-fold) in Vegf gene expression were found in this study. However, the time points at which gene expression were assessed are ‘snapshots’ of a 34-week process of regression and recrudescence. It is possible that the differential gene expression necessary for morphological changes in the testes was relatively transient, and therefore missed by our sampling schedule. Additionally, gene transcription and translation of gene products may not overlap in time, and therefore, VEGF protein expression may be high at particular time points whereas, Vegf transcription may have already returned to basal levels. Studies using models in which more abrupt changes in testes mass are measured are necessary to examine changes in angiogenesis gene expression over a more concentrated window of time. Our study implicates several other genes in the process of seasonal angiogenesis of the testes.

Directed by our preliminary microarray findings after 14 weeks of photoperiod treatment (the nadir of testicular regression), we investigated expression patterns of four genes involved in angiogenesis. Hif1a is an oxygen-regulated subunit of the HIF transcription factor that has been implicated in both angiogenesis and apoptosis (Piret et al. 2002). The overlap of the timing of testicular apoptosis in SD white-footed mice and the expression pattern of Hif1a suggests that expression of Hif1a in SD mice may be an indicator of apoptosis associated with testicular regression (Young et al. 2001). Tgfbr3, also known as betaglycan, in its soluble form, is a potent antagonist of TGFβ, a promoter of angiogenesis. Additionally, expression of the soluble extracellular domain of TGFβ inhibits angiogenesis

Figure 4 Effect of 7, 14, 21 and 34 weeks of photoperiod treatment on Hif1a (A), Tgfbr3 (B), Serpine1 (C) and Tnf (D) gene expression relative to 18S rRNA expression in the testes. *, P < 0.05 among all photoperiod groups of a single time point unless specified by a bar. n = 4 per photoperiod group per time point.
in vivo and in vitro (Bandyopadhyay et al. 2002). Therefore, it is likely that the initial increase in Tgfβr3 expression in SD mice represents an inhibition of testicular angiogenesis. The effect of Serpine1 on tumour growth and angiogenesis depends on the level of expression. High SERPINE1 doses inhibit tumour growth and angiogenesis in vivo, whereas, low doses increase angiogenesis (McMahon et al. 2001). Consistent with this bi-modal function of SERPINE1, SD mice display high expression levels of Serpine1 at week 7 during regression, which suggests an inhibitory role of Serpine1 on angiogenesis. Lastly, Tnf promotes proliferation and differentiation of mesenchymal cells into pericytes or smooth muscle cells that support newly formed vessels during angiogenesis (Distler et al. 2003). The late increase in Tnf in SD testes suggests that Tnf may promote new vessel stabilization during testicular recrudescence. Overall, the timing of the expression of these four genes in SDR mice suggests that Hif1α, Tgfβr3 and Serpine1 are involved in testicular regression, whereas Tnf is involved in testicular recrudescence.

The effect of time (weeks) on gene expression in LD testes was unexpected. We predicted that the vasculature in LD animals would be stable. However, many genes (including the ones examined in the present study) have multiple functions and it is possible that the changes measured in LD testes may be associated with extra-angiogenic processes. Alternatively, the trend of increasing gene expression peaking around week 21 in LD mice may be due to a fluctuation in overall body growth (including the testes). Between 14 and 21 weeks, the LD animals display a slight drop in body mass followed by a recovery that may account for slight changes in testicular gene expression at this time.

In this study, microarrays designed for Mus musculus were used for P. leucopus which may have resulted in false positive and negative findings based on possible sequence differences between the species. Therefore, the microarray analysis was solely used as a screening procedure whereas the specificity and sensitivity of qPCR enabled precise examination of specific genes of interest. Common laboratory animals that have been selectively bred to ignore seasonal cues – such as Mus musculus and Rattus norvegicus – and for which molecular tools are readily available, are not suitable for this type of comparative study. Because of the genomic homology among species, however, it is possible to work around some of the molecular roadblocks to address ecologically relevant questions in genetically heterogeneous populations.

The SDNR group represents the population of mice that fails to regress their gonads in response to short day lengths. This population is not merely a laboratory anomaly because winter breeding has been reported in field studies and is thought to confer fitness advantages during mild winters (Prendergast et al. 2001). We compared the SDNR animals to the LD and SDR phenotypes. Body mass, testes size and testes morphology of the SDNR group were more similar to LD than to SD mice. With respect to gene expression of our four candidate genes, the SDNR profile initially (Fig. 4A–D; week 7) was similar to that of the SDR group. However, by week 14, SDNR expression of all four genes switched to resemble that of the LD mice. These data suggest that there is some breakdown between the transcription of the gene and the function of the gene product. Potential differences between SDNR and SDR post-transcriptional pathways may exist. However, in SDNR mice the uncoupling between large, sperm-filled testes (characteristic of LD animals) and their initial SDR-like gene expression profile suggests that testosterone is not the only factor influencing angiogenesis gene expression in the testes. Additionally, long durations of melatonin secretion do not appear sufficient to induce SDR-like angiogenesis expression profiles in the testes because SDNR and SDR white-footed mice display the same long melatonin durations (Carlson et al. 1989) but different gene expression patterns.

The present study is among the first to describe seasonal plasticity of angiogenesis in adult testes on a molecular level. Changes in angiogenesis gene expression differ between testes of LD and SD white-footed mice and correspond to changes in testicular morphology. The gene expression profile of SDNR mice initially resembled that of SDR mice, but subsequently became similar to LD mice. Future studies are necessary to determine the intervening role of testosterone and melatonin in photoperiod-induced changes in testicular angiogenesis gene expression. It is likely that seasonal melatonin secretion exerts its effects on testicular angiogenesis via an indirect central mechanism that may implicate testosterone. Melatonin receptors have not been localized in the testes in this species and melatonin does not affect testosterone secretion from testicular cells in vitro (Knotts et al. 1988). However, binding of melatonin to receptors in the hypothalamus does modulate reproductive hormone secretion (Glass & Dolan 1988). Therefore, melatonin-induced changes in testosterone secretion may regulate seasonal angiogenesis (or possibly gonadotrophin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH) or some combination of these peptides). Because non-pathological angiogenesis in adults is rare (particularly in males) understanding the mechanisms underlying this model of seasonal angiogenesis may prove to have therapeutic value.

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