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Telocytes are localized to testis of the bank vole (*Myodes glareolus*) and are affected by lighting conditions and G-coupled membrane estrogen receptor (GPER) signaling

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ABSTRACT

We aim to explore the presence of a novel cell type, telocytes (TCs), in the bank vole testis interstitium following G-coupled membrane estrogen receptor (GPER) signaling withdrawal. In addition, the involvement of interstitial cells in lipid homeostasis was investigated.

Bank voles (actively reproducing or regressed) were administered with GPER antagonist (G-15; 50 μg/kg bw) injections. To examine TC distribution, ultrastructure, function, and their connotation in the interstitial tissue lipid balance, electron microscopic observations were implemented. Immunohistochemistry and Western blot for the TC marker, CD34, and lipid balance molecules: leptin, adiponectin, and perilipin were performed. Photoperiod-regulated testis steroidogenic function was estimated *via* serum melatonin level and intratesticular cholesterol concentrations in immunoenzymatic assays.

We demonstrate the presence of TCs in bank vole testis interstitium. Distinctive TC morphology: small cell bodies with very long, slender prolongations, constituting a three-dimensional network around the interstitial cells was seen. Ultrastructurally, scarce mitochondria, a few cisternae of the endoplasmic reticulum, and lipid droplets indicated possible TC implications in lipid homeostasis. Changes in CD34 expression in TCs were seen in relation to GPER disturbances. In GPER-blocked testis, single TCs were present in the LD interstitium when in SD ones they were occasionally absent. Moreover, in TCs of SD voles, a lack of lipid droplets was revealed, likely reflecting attenuated TC function during regression. However, melatonin levels decreased in GPER-blocked LD and SD. Concomitantly, leptin, adiponectin, and perilipin expressions together with cholesterol content varied after blockage.

Based on our results we suggest TCs are an important component of the bank vole testis interstitium as they are implicated in ultramorphology maintenance, protein interactions, and lipid homeostasis.

1. Introduction

A seasonal breeder, the bank vole (*Myodes glareolus,* formerly known as *Clethrionomys glareolus*, Schreber, 1780) is a small rodent found in Europe and central Asia (Bellamy et al., 2000; Macdonald, 2001; Jonsson et al., 2000; Oksanen et al., 2001). Bank voles prefer a wide variety of habitats such as forests (coniferous and taiga), scrub forests, hedges, banks, and swamps (Yoccoz et al., 2001; Prevot-Julliard et al., 1999; Koskela et al., 1997, 1998; Ostfeld, 1985). Their activity is either diurnal or nocturnal, although they are primarily crepuscular (Macdonald, 2001). Voles are both omnivorous and herbivorous, obtaining food in the winter by burrowing underground (Ostfeld, 1985;

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Macdonald, 2001). The vole social system is characterized by a dominance hierarchy with females being dominant over the males, particularly during the breeding season (Horne and Ylonen, 1998). The hierarchy within males is also well established (Kruczek and Styrna, 2009). Females appear to prefer dominant males that show increased levels of androgens and consequently better quality of spermatozoa. The mating system of voles is found to be polygamous (Macdonald, 2001) and promiscuous (Horne and Ylonen, 1998). The offspring is born blind and helpless in an underground nest lined with grass with the female as the sole provider of parental care (Macdonald, 2001). Upon reaching maturity at approximately nine weeks of age, males disperse. Females stay in their natal area.

Bank voles breed from late April to September (Oksanen et al., 2001). Thus, the length of the daylight *via* the interaction with the hypothalamus-pituitary gonadal axis, including melatonin, is the main regulator of reproductive activity. Inhibition of reproductive function during the short-day phase (October-March) corresponds to regressive phases of the reproductive system. In the laboratory, seasonal changes of the photoperiod throughout the year can be mimicked by specific light adjustments, thus, bank voles show similar reproductive characteristics to wild populations. In this laboratory, bank voles have been bred over a 20-year span (Bilinska et al., 1996). Our previous studies demonstrated that bank vole testes exposed to a short photoperiod produce lower levels of sex hormones (androgens and estrogens) and they are highly sensitive to exogenous hormone treatment (estradiol and xenoestrogen) and modulation of estrogen signaling molecules (Gancarczyk et al., 2004; Hejmej et al., 2011, 2013; Kotula-Balak et al., 2014, 2015; Pawlicki et al., 2017). This, in turn, resulted in disturbances in tissue cytoarchitecture, spermatogenesis, including blood-testis barrier function, spermatozoa fertility, and steroidogenic Leydig cell function (sex steroid secretion).

Telocytes (TCs), a novel interstitial (stromal) cell, are responsible for supporting tissue homeostasis (Cretoiu and Popescu, 2014). Due to their extremely long prolongations, they form a multi-faceted network with cells of the interstitial tissue, thus creating a unique local microenvironment. Limited data exists on the presence of TCs in the testis (Rodríguez et al., 2008; Yang et al., 2015; Hasirci et al., 2017). Recently, we reported TC localization in mouse testis (Pawlicki et al., 2018). In human TCs, as in Leydig cells, lipid droplets were described together with calcium ion channels (Cretoiu et al., 2015). Of note, in Leydig cells, calcium is stored in the mitochondria and increased calcium concentration stimulates testosterone production from cholesterol found in lipid droplets (Haider, 2007). However, it is unknown whether TCs, *via* lipid droplets or/and calcium signaling, are implicated in Leydig cell steroidogenic function. Based on these data, the role of TCs in lipid homeostasis and metabolism requires further evaluation.

In the interstitial space of the testis loose connective tissue with blood and lymph vessels, macrophages, fibroblasts, mast cells, lymphocytes, and Leydig cells is present (Christensen, 1975). Comparative studies of various mammals revealed differences in number and distribution of Leydig cells that produce sex steroids (Fawcett, 1973; Clark, 1976; Mendis-Handagama and Ariyaratne, 2001). Steroidogenesis in Leydig cell is a multi-level process and requires the coordinated expression of related genes, proteins, signaling molecules in response to lutropin stimulation. Autocrine regulation by estrogens is involved as well (Sriraman et al., 2005). Cell-cell interactions in the interstitial tissue, including TCs, cannot be excluded either.

Estrogen signaling, regarding testis function, is more complicated than previously thought. Besides canonical estrogen receptors (ERs), the G-coupled membrane estrogen receptor is an important element of estrogen signaling in testicular cells (Isensee et al., 2009). Using qPCR, Fietz et al. (2014; 2016) showed GPER expression in Leydig and Sertoli cells of human testis. The expression level was higher in Leydig cells as compared to Sertoli cells. Our previous studies demonstrated the presence of GPER in Leydig cells of bank voles; however, no changes were observed regarding normal or physiologically decreased intratesticular estrogen concentration (Zarzycka et al., 2016). The specific role of GPER is still not fully understood.

Testicular TCs might be a new player in control of interstitial tissue lipid balance. The cross-talk between lipid balance and the metabolism-controlling molecules leptin, adiponectin, and perilipin has not been studied in regulation of testicular interstitial cells. The hormone leptin is pivotal for central reproductive axis at the hypothalamic level, and direct effects of this hormone on the testis, such as androgen synthesis modulation, have been demonstrated (Tena-Sampre et al., 2001). Recently, control of lipid accumulation and metabolism by adiponectin in lipid-rich tissues was linked to reproductive function in humans (Comim et al., 2013; Stern et al., 2016). On the other hand, perilipin is a regulator of lipid droplet formation in adipocytes and steroidogenic cells (Takahashi et al., 2013). In the latter, storage of lipid droplets determines initiation, maintenance, and efficiency of sex steroid synthesis. In lipid droplets, cholesteryl esters synthesized *de novo* are made from polar membrane lipids or derived from serum lipids.

The present study aims to investigate TC presence in bank vole testis interstitium during active and regressive phases of reproduction. In our earlier study, we showed no effect of estrogen milieu changes on expression of G-coupled estrogen receptor (GPER) in the bank vole interstitial Leydig cells. Herein, special attention is paid on the presence and role of TCs in lipid milieu regulation by examination of the leptin-adiponectin-perilipin partnership under the GPER estrogen signaling.

2. Materials and methods

2.1. Animals and housing conditions

Bank vole males isolated at the age of 3–4 weeks from mother and their sisters and bred in separate cages (five males per cage) under different light cycles (18 h light: 6 h darkness; long day; LD) and (6 h light: 18 h darkness; short day; SD) were used. In the Animal Facility of the Department of Endocrinology, LD and SD bank voles are bred in separate rooms without windows and well-isolated walls from external environment with automatic control of artificial light at 250 lx at cages level. Breeding pairs, separated pups, and older males and females are reared through the whole year at the stable temperature of 18 °C and a relative humidity of 55 ± 5%. Voles are housed in polyethylene non-transparent cages (42 cm × 26 cm × 15.5 cm) furnished with sawdust and wood shavings for bedding according to the Directive of the European Union Parliament and the Council (2010/63/EU of 22 September 2010 regarding to the protection of animals used for scientific purposes). A standard pelleted diet (LSM diet, Agropol, Motycz, Poland) supplemented with seeds of wheat or oat, red beets, apples, and water is provided *ad libitum*.

Twenty mature bank voles born in April were allotted into four experimental groups (each group including 5 animals); control (Cont.) (LD and SD voles, respectively) and treated (LD and SD voles, respectively) with a selective GPER receptor antagonist [(3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone; G-15] (Tocris Bioscience, Bristol, UK). G-15 was dissolved in DMSO and the stock solutions were kept at −20 °C. Animals from the experimental groups were injected subcutaneously with freshly prepared solutions of G-15 (50 μg/kg bw) in phosphate buffered saline (six doses each dose injected every other day). Voles from control groups received vehicle only. Dose, frequency and time of G-15 administration was based on our previous study (Kotula-Balak et al., 2018b).

Voles were sacrificed by cervical dislocation at the age of 2 months after 1 h of daylight phase was started (light is switched on at 6:30 AM). Testicular tissue was isolated and fixed for analysis in electron microscope and immunohistochemical staining. Fresh testicular tissues were used for determination of protein expression and cholesterol concentration. Blood was obtain from the liver vein and used for the melatonin (as a possible TCs regulator) level determination.

2.2. Cell topography – scanning electron microscope (SEM)

Testes of LD and SD voles (control and G-15-treated) were fixed in a mixture of 2.5% glutaraldehyde with 2.5% formaldehyde in a 0.05 M cacodylate buffer (Sigma; pH 7.2) for several days, washed three times in a 0.1 M sodium cacodylate buffer and later dehydrated and subjected to critical-point drying. They were then sputter-coated with gold and examined at an accelerating voltage of 20 kV or 10 KV using a Hitachi S-4700 scanning electron microscope (Hitachi, Tokyo, Japan).

2.3. Cell ultrastructure – transmission electron microscope (TEM)

The fixation procedure described below was based on the protocols proposed by Russell and Burguet (1977). The modification developed in our labs had important advantages: it improved the quality of fixation and enhanced the contrast of plasma membrane and the organelles (for details see Bilinska et al., 2018). Briefly, fragments of LD and SD vole testis (control and G-15-treated) were immersed in ice-cold pre-fixative containing 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3. The tissues were then rinsed and post-fixed in a mixture of 2% osmium tetroxide and 0.8% potassium ferrocyanide in the same buffer for 30 min at 4 °C. After dehydration in the graded series of ethanol and acetone the material was infiltrated in a freshly prepared mixture of acetone and Epon 812 (Serva, Heidelberg, Germany) and embedded in Epon 812. Semi-thin sections (0.7 μm thick) were stained with 1% methylene blue and examined under a Leica DMR (Wetzlar, Germany) microscope. Ultrathin sections (80 nm thick) were contrasted with uranyl acetate and lead citrate and analyzed with a JEOL 2100 HT (Japan) TEM.

2.4. Western blot analysis

Lysates of testes (control and G-15-treated) of LD and SD voles were obtained by sample homogenization and sonication with a cold Tris/EDTA buffer (50 mM Tris, 1 mM EDTA, pH 7.5), supplemented with a broad-spectrum protease inhibitors (Sigma-Aldrich). The protein concentration was estimated by the Bio-Rad DC Protein Assay Kit with BSA as a standard (Bio-Rad Labs, GmbH, München, Germany). Equal amounts of protein were resolved by SDS-PAGE under reducing conditions, transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany) and analyzed by Western blotting with antibodies listed in Table 1. The presence of the primary antibody was revealed with horseradish peroxidase-conjugated secondary antibodies diluted 1:3000 (Vector Lab., Burlingame, CA, USA) and visualized with an enhanced chemiluminescence detection system as previously described (Pawlicki et al., 2017). All immunoblots were stripped with stripping buffer containing 62.5-mM Tris-HCL, 100-mM 2-mercaptoethanol, and 2% SDS (wt:v; pH 6.7) at 50 °C for 30 min, and incubated in antibody against β-actin (loading control). Three independent experiments were performed, each in triplicate with tissues prepared from different animals. To obtain quantitative results the bands (representing each data point) were densitometrically scanned using the public domain ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) (Smolen, 1990). The data obtained for each protein were normalized against its corresponding actin and expressed as relative optical density (ROD). Results of 10 separate measurements of individual bands for each protein were expressed as mean ± SD.

Table 1
Primary antibodies used for immunohistochemistry and Western blotting.

Antibody	Host species	Vendor	Dilution
CD34	Rabbit	Abcam cat. no. ab81289	1:200 (IHC) 1:1000 (WB)
Leptin	Rabbit	Abcam cat. no. ab16227	1:100 (IHC) 1:500 (WB)
Adiponectin	Mouse	Abcam cat. no. [19F1] (ab22554)	1:200 (IHC) 1:1000 (WB)
Perilipin (PLIN1)	Rabbit	Cell Signaling Technology cat. no. 9349	1:50 (IHC) 1:200 (WB)
β-actin	Mouse	Sigma–Aldrich cat. no. A2228	1:3000 (WB)

2.5. Immunohistochemistry

Testicular sections of control and G-15-treated voles (LD and SD) voles were immersed in 10 mM citrate buffer (pH 6.0) and heated in a microwave oven (2 × 5 min, 700 W). Thereafter, sections were immersed sequentially in H_2O_2 (3%; v/v) for 10 min and normal goat serum (5%; v/v) for 30 min which were used as blocking solutions. After overnight incubation at 4 °C with primary antibodies listed in Table 1. Next respective biotinylated antibodies (anti-rabbit, anti-goat and anti-mouse IgGs; 1: 400; Vector, Burlingame CA, USA) and avidin-biotinylated horseradish peroxidase complex (ABC/HRP; 1:100; Dako, Glostrup, Denmark) were applied in succession. Bound antibody was visualized with 3,3′-diaminobenzidine (DAB) (0.05%; v/v; Sigma-Aldrich) as a chromogenic substrate. Control sections included omission of primary antibody and substitution by irrelevant IgG. Thereafter, sections were washed, and were slightly counterstained with Mayer's hematoxylin and mounted using DPX mounting media (Sigma–Aldrich) (Bilinska et al., 2018).

2.6. Melatonin and cholesterol concentrations measurements

Concentration of melatonin was measured in (100 μl) of serum of control and G-15-treated bank voles with the use of melatonin ELISA Kit (cat no ab213978; Abcam) according to manufacturer's protocol. The biological sensitivity of an assay was 162 pg/mL. For determination of optical density of the color reaction spectrophotometer (Labtech LT-4000MS; Labtech International Ltd., Uckfield, UK) with Manta PC analysis software set to 450 nm was used. Data were expressed as mean ± SD.

The Amplex® Red Cholesterol Assay Kit (Molecular Probes Inc., Eugene, OR, USA) was used for cholesterol content (μM) analysis in LD and SD vole testis (control and G-15-treated) lysates obtained by homogenization and sonication in a cold Tris/EDTA buffer. For measurement 100 μl cell lysates was used according to manufacturer's protocol. Data were expressed as mean ± SD. The fluorescence (λ = 580 nm) was measured with the use of a fluorescence multi-well plate reader SPARK Tecan, Switzerland.

2.7. Statistical analysis

Each variable was tested by using the Shapiro-Wilk W-test for normality. Homogeneity of variance was assessed with Levene's test. Since the distribution of the variables was normal and the values were homogeneous in variance, all statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc comparison test to determine which values differed significantly from controls. The analysis was made using Statistica software (StatSoft, Tulsa, OK, USA). Data were presented as mean ± SD. Data were considered statistically significant at $p < 0.05$. All the experimental measurements were performed in triplicate.

3. Results

3.1. Telocytes are present in bank vole testis – analysis by SEM and TEM

Scanning electron microscopy analysis of LD and SD vole testes (Fig. 1A) revealed the TCs present in the interstitium between seminiferous tubules (Fig. 1B). However, not all interstitial spaces had TCs, especially in SD vole testes (Fig. 1C). In LD and SD vole testes, TCs possessing small, round bodies and very long, thin telopodes were

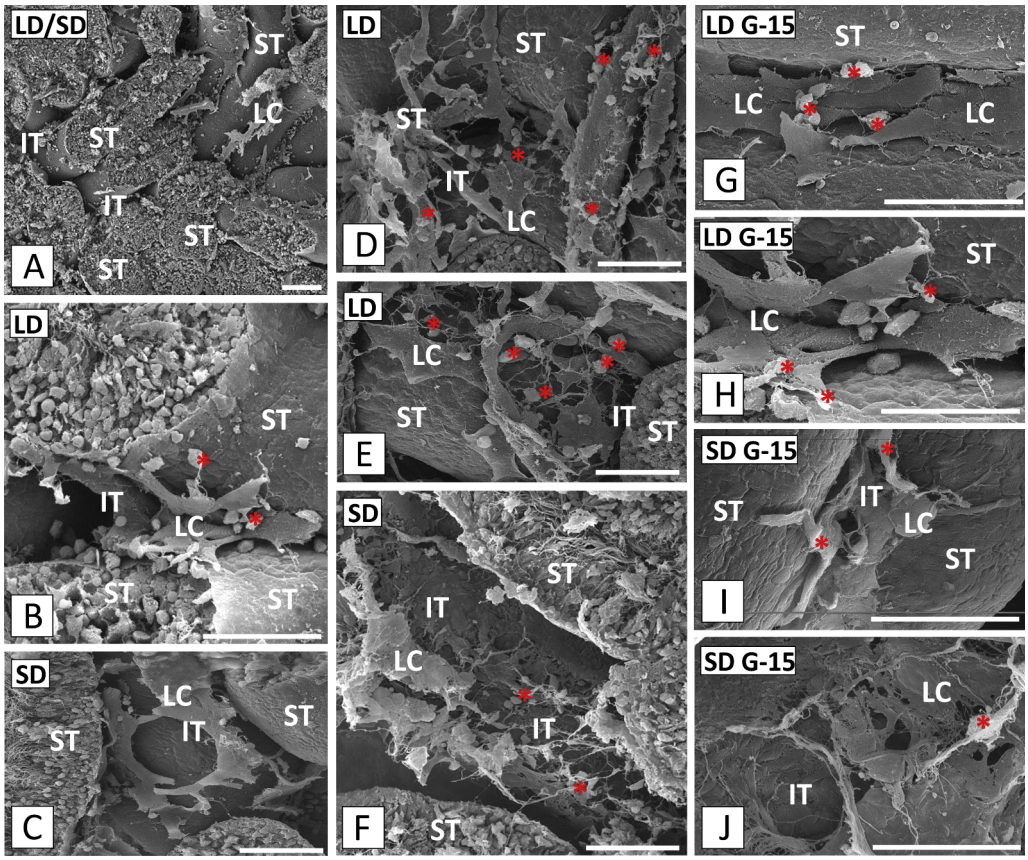

Fig. 1. Presence of telocytes in the bank vole testis. Effect of GPER blockage. SEM analysis. Representative microphotographs of sections of control (A-F) and GPER-blocked (G-J) LD and SD bank vole testes coated with gold. Bars represent 1 μm. Analysis was performed on three testicular fragments from at least three animals of each experimental group. TCs are marked with red asterisks. IT-interstitial tissue; ST-seminiferous tubules; LC-Leydig cells.

detected in close vicinity to Leydig cells (characterized by a large body and short pseudopods that were present in groups where individual cells adhered to each other) (Fig. 1D–F). Telopodes (TC protrusions) created a spatial network surrounding the interstitial cells. In LD and SD voles treated with G-15, no disturbances in TC distribution, number, or structure were observed (Fig. 1G–J).

Analyses of ultrathin, serial testis sections of both SD and LD animals revealed the number and overall structure of interstitial tissue was similar regardless of the light regime (Fig. 2A–F). Moreover, we did not observe any characteristic differences in TC ultrastructure between control and G-15-treated animals. In LD testes (both control and G-15-treated; not shown), the TCs were present in both peritubular and interstitial testis compartments. Located in both compartments TCs were markedly elongated with a relatively small cell body (Fig. 2A–F). Nearly the entire cell body was filled with an elongated nucleus surrounded by a small rim of cytoplasm. In the cytoplasm, elements of rough endoplasmic reticulum and mitochondria were present (Fig. 2B, F). The most characteristic feature of the TCs were extremely long, thin cell telopodes that formed podom-like dilated structures (Fig. 2A–F). The telopode cytoplasm contained mitochondria, elements of endoplasmic reticulum, and lipid droplets. Of note, since telopodes are extremely thin, their organelles were linearly arranged one by one (Fig. 2E, F). In TCs surrounding the seminiferous tubules, the telopodes ran parallel to peritubular cells (Fig. 2D, F). These ultrastructural features of TCs were also present in the G-15 treated LD and SD testes (not shown) with only one crucial exception. We did not detect lipid droplets in either the cell body or the telopodes in cells obtained from animals kept in the SD light regimen (Fig. 2F).

3.2. Expression and localization of CD34 in telocytes and of leptin, adiponectin and perilipin in bank vole testis

Western blot analysis revealed the presence of CD34, leptin, adiponectin, and perilipin in testicular homogenates of LD and SD bank voles (Fig. 3A, B). While the expression of CD34 was unchanged between control LD and SD males, it decreased following G-15 treatment in both groups ($p < 0.05$, $p < 0.01$). Increased leptin expression in SD voles was observed ($p < 0.05$), and a pronounced decrease was observed in LD and SD animals when GPER was blocked ($p < 0.001$). A similar pattern was observed for adiponectin expression in control animals ($p < 0.05$), and its expression was increased in SD males and decreased in GPER-blocked animals ($p < 0.01$, $p < 0.001$). No differences in perilipin expression were found in either control LD and SD voles or G-15-treated animals.

In both LD and SD animals, CD34 positive staining was revealed in a few cells surrounding the interstitial compartment (Fig. 4A–D). CD34 was localized to the thin, elongated cell body (nucleus and cytoplasm) as well as to long telopodes. Telocytes were also located near peritubular-myoid cells (Fig. 4A–C) and around vessels (Fig. 4A, B). No differences in positively stained interstitial cell number, immunoreactive signal intensity, and distribution between LD and SD testes were found. In addition, no differences in the above-mentioned parameters were seen in G-15 treated LD and SD testes (Fig. 4E).

Independent of vole group, localization of leptin, adiponectin, and perilipin was limited exclusively to interstitial cells (Fig. 5A–C and A'-B'; G-15 treated LD and SD testes not shown). As a control, no positive staining was found when primary antibodies were omitted (Fig. 5A'-C' inserts).

3.3. Serum melatonin concentration and testicular cholesterol concentration of the bank vole

Melatonin concentration was decreased in SD voles ($p < 0.001$). After G-15 treatment, it decreased in both LD and SD voles ($p < 0.05$; $p < 0.001$) (Fig. 6A). When compared to the LD regimen, SD males always maintained a lower melatonin level.

In LD and SD testes, no differences in cholesterol concentration were found (Fig. 6B). Increased cholesterol concentrations were revealed after G-15 treatment in both LD ($p < 0.05$) and SD animals.

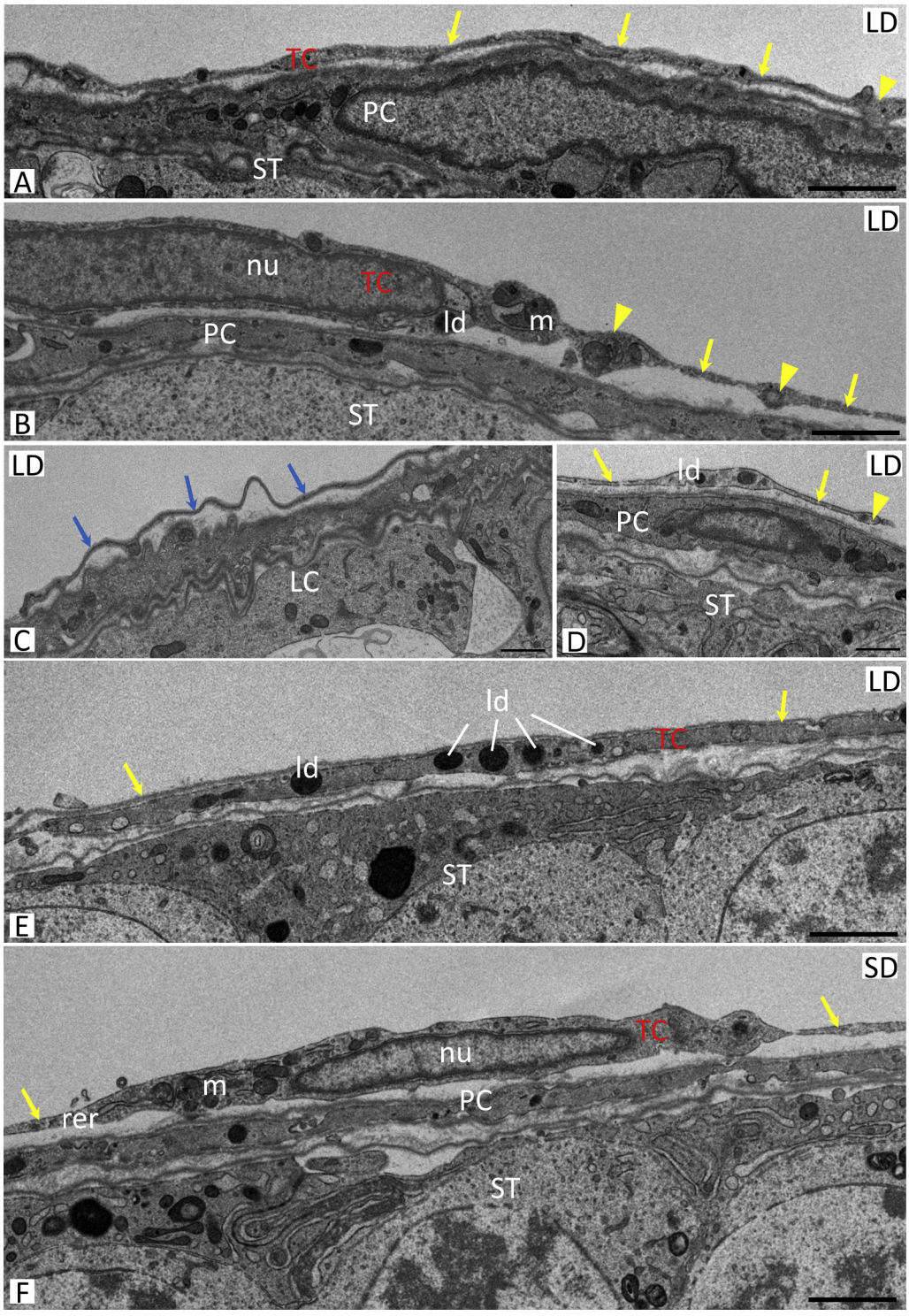

Fig. 2. Presence of telocytes in the bank vole testis. Effect of GPER blockage. TEM analysis. Representative microphotographs of ultrathin sections of interstitial tissue of control (A-F) and GPER-blocked bank vole testes (not shown). Bars represent 1 μm. Each testicular sample in epoxy resin block was cut for at least three ultrathin sections that were analyzed. Analysis was performed on samples from at least three animals of each experimental group. ST-seminiferous tubules; TC-telocyte; LC-Leydig cells; ST-seminiferous tubules; m – mitochondria; rer – rough endoplasmic reticulum; ld-lipid droplets; PC – peritubular cell. Long protrusions (telopodes) of the TCs [yellow and blue (further parts of telopodes) arrows]. Dilated fragments of the telopodes that form podomer-like structures (arrowheads).

4. Discussion

Until this study, the presence of telocytes (TCs) in the male reproductive system was only reported in a limited number of studies. In the human prostate, TCs were linked to tissue organization during postnatal development (Sanches et al., 2016). Yang et al., (2015) demonstrated TCs in testis of the Chinese softshell turtle (Pelodiscus sinensis). Further studies in humans confirmed TCs in testes of patients with prostate cancer as well as those with non-obstructive azoospermia (Rodríguez et al., 2008; Hasirci et al., 2017). The latter study showed a correlation between TC number and distribution in relation to a spermatogenesis defect. Recently, we demonstrated TCs in mouse testis following blockage of estrogen signaling *via* the G protein-coupled membrane estrogen receptor (GPER) (Pawlicki et al., 2018). While histological alterations of mouse interstitial tissue were revealed, no perturbation in spermatogenesis was detected. The number of TCs corresponded to alterations of the interstitial tissue. Here, for the first time, we revealed the presence of TCs in bank vole testes derived from reproductively active and regressed animals.

In these photoperiodic rodents, reproductive system function is endogenously-controlled by melatonin which interacts with the hypothalamic-pituitary-gonadal axis. Data by Roatesi et al. (2015) showed

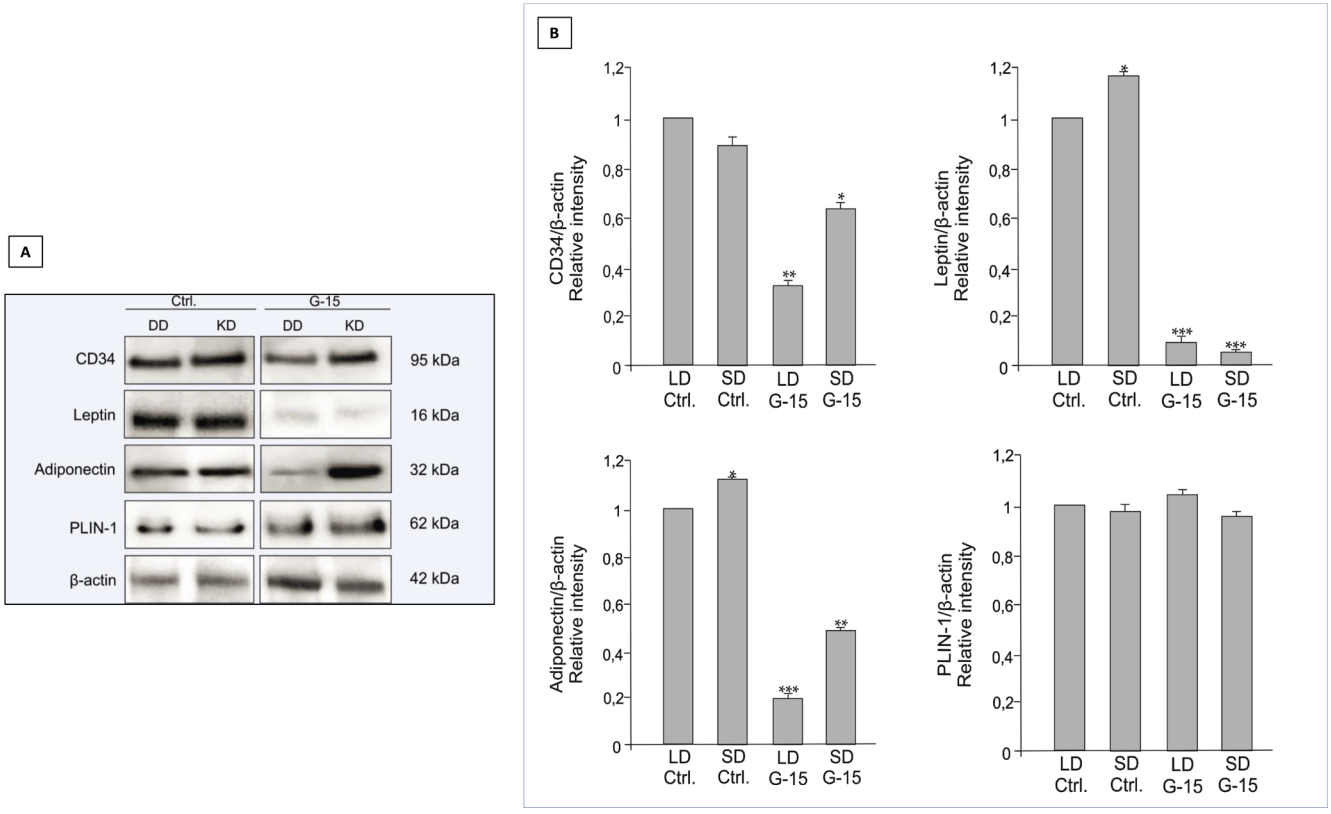

Fig. 3. The expression of telocyte specific protein markers (CD34) and leptin, adiponectin and perilipin in the bank vole testis. Representative blots of qualitative expression (A) and relative expression (arbitrary units) (B) of proteins CD34, leptin, adiponectin, perilipin in control and GPER-blocked LD and SD bank vole testes. Protein densitometry results are present below the corresponding blots. The relative amount of respective proteins normalized to β-actin. ROD (relative optical density) from three separate analyses is expressed as means. From each animal at least three samples were measured. Asterisks show significant differences control and GPER-blocked testes. Data is expressed as means. Values are denoted as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

sex steroid hormone regulation of TCs through the estrogen and progesterone receptors that are present in these cells. Recently, melatonin was also confirmed as a TC regulator (Abd-Elhafeez et al., 2017). In seminal vesicles of the Soay ram, melatonin caused a significant increase in the number of TCs and their telopode length. In the present study, a link between increased melatonin levels corresponded to irregular distribution of TCs in SD voles. Examination of TC ultrastructure of melatonin-treated rams showed an increased number and diameter of secretory vesicles. This was observed in conjunction with exaggerated secretory activity in the form of a massive release of secretory vesicles from telopodes, as well as shedding of secretory structures (exosomes, ectosomes, and multivesicular bodies) from telopodes. Herein, we found differences in TC ultrastructural components in SD bank voles as well as those where GPER was inhibited. It is possible that, in the bank vole, TC and their ultrastructural architecture remain under the control of melatonin signaling as well as other factors involved in GPER-signaling. In our previous work, we found no effect of estrogen on GPER activity in both LD and SD voles (Zarzycka et al.,

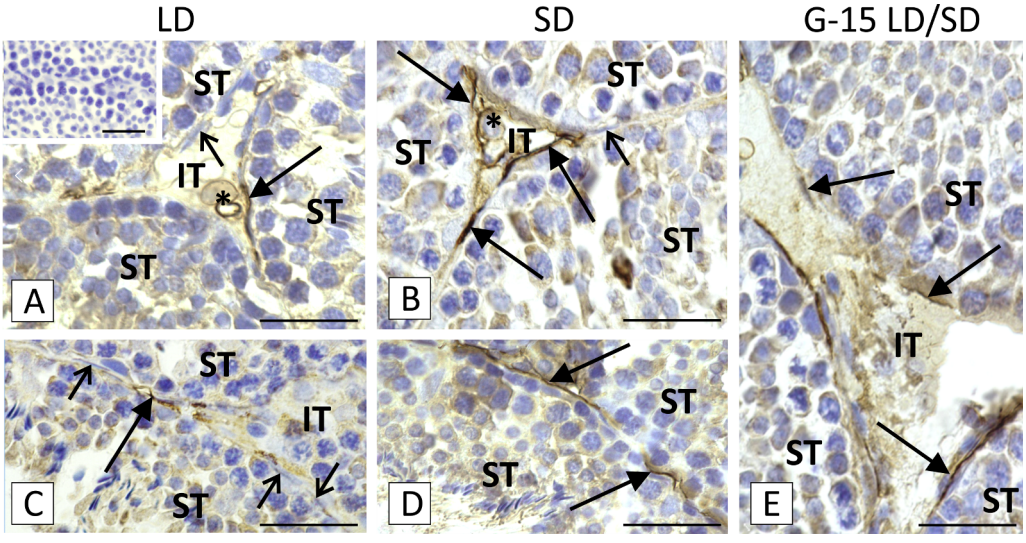

Fig. 4. Presence of telocytes in the bank vole testis. Effect of GPER blockage – immunhistochemical analysis. Representative microphotographs of CD34 immunohistochemical localization in control (A-D) and GPER-blocked (E, F) LD and SD bank vole testes. Immunostaining with DAB and counterstaining with hematoxylin. Scale bars represent 15 µm. Immunoreaction was performed on testicular serial sections from at least three animals of each experimental group. Insert at A-negative control. IT-interstitial tissue; LC-Leydig cells; PC-peritubular cells (open arrows); ST-seminiferous tubules; TC-telocyte (arrows); blood vessels (asterisks).

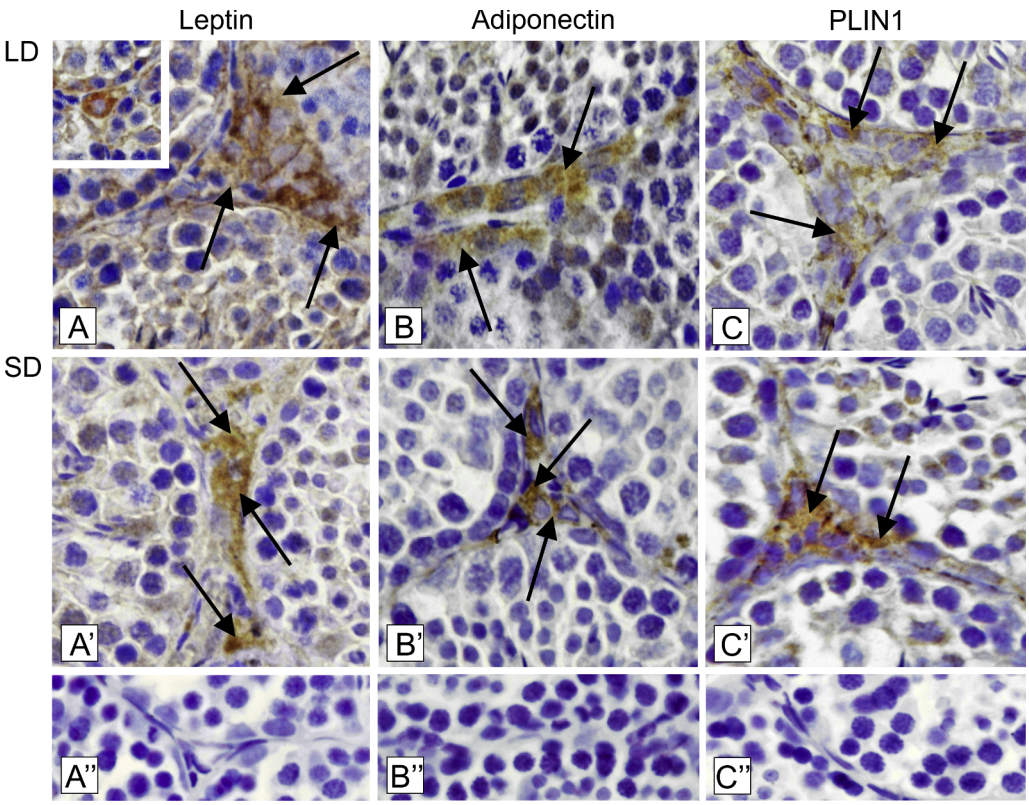

Fig. 5. Immunoexpression and localization of leptin, adiponectin and perilipin in the bank vole testis. Effect of GPER blockage – immunohistochemical analysis. Representative microphotographs of leptin (A, A' and insert with single Leydig cell at A), adiponectin (B, B') and perilipin (C, C') immunohistochemical localization in control (A-C) and GPER-blocked (A'-C') LD and SD bank vole testes. Immunostaining with DAB and counterstaining with hematoxylin. Scale bars represent 15 µm. Immunoreaction was performed on testicular serial sections from at least three animals of each experimental group. Insert at A"-C"-negative controls. Arrows depict positive staining.

2016). In this study, melatonin concentration in voles was demonstrated for the first time as well as its regulation by GPER in voles under both light regimens. This result highlights the importance of estrogen signaling for vole central endocrine testis regulation. Therefore, we suggest a direct link between estrogen level and secretory activity of the interstitial cells as indirect evidence has been previously presented (Bilinska et al., 1996; Gancarczyk et al., 2004).

In bank voles, the endoplasmic reticulum and TCs cooperate to control secretory functions. Till now three groups of extracellular vesicles shedding by TCs were identified: exosomes (from endosomes), ectosomes (from plasma membrane), and multivesicular cargo (multiple, tightly packed endomembrane-derived vesicles) (Janas et al., 2013). These vesicles mainly contain proteins, lipids, miRNAs, mRNAs, and mtDNA. This highlights a crucial function of TCs, as their intercellular signaling relates to the function and/or modification of the post-transcriptional activity of recipient cells. While the testis interstitial tissue is composed of various cell types e.g. vessel pericytes, macrophages, fibroblasts, mast cells, lymphocytes, and Leydig cells, all of which interact, TCs should be considered critical as well for their role in the cellular cross-talk. In cardiac TCs, the cytoplasm is filled with mitochondria, lipid droplets, a small Golgi apparatus, and elements of smooth and rough endoplasmic reticulum (Li et al., 2014). Bank vole TCs have poor organelle content, including only scarce mitochondria. It is possible that vole TCs are implicated in steroidogenic function and/or other activity like e.g. contractile action of interstitial tissue. Oppositely, in mouse testis, TCs not respond to GPER blockage (calcium ions concentration-signaling molecules important for steroidogenesis stimulation and tissue fluency was not changed) (Pawlicki et al., 2018) as do Leydig cells. Therefore, we propose a nutritive role of TCs in vesicles and lipid droplets. According to Kostin (2016), in the heart, TC secretory vesicles are crucial for stem cells nursing, thus they maintain the stem cell niche microenvironment, structure, and remodeling capabilities.

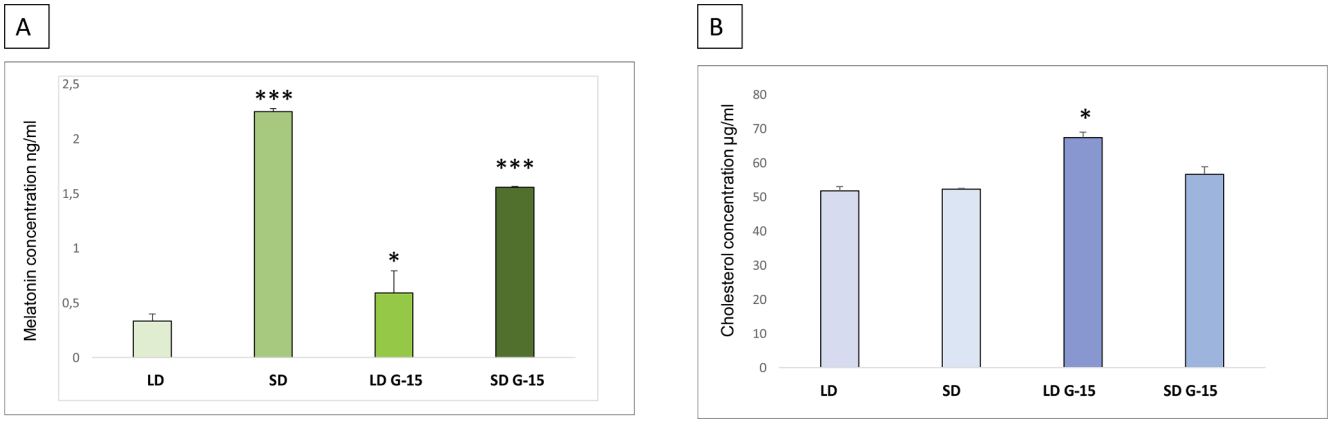

Fig. 6. Serum melatonin concentration and intratesticular cholesterol concentration in the bank vole. Effect of GPER blockage. Melatonin (A) and cholesterol (B) concentration in control and GPER-blocked LD and SD bank vole testes. Data is expressed as means ± SD. From each animal at least three samples were measured. Asterisks show significant differences between control and GPER-blocked testes. Values are denoted as $^{*}p < 0.05$ and $^{***}p < 0.001$.

Telocytes are easily distinguishable from fibroblasts and other fibroblast-like cells by their unique morphological features (Bei et al., 2015). According to our previous study, these testicular cells specifically express transmembrane protein CD34 (Pawlicki et al., 2018). In the vole interstitium, we confirmed CD34 expression in a small number of interstitial cells located close to Leydig cells, around blood vessels, and around seminiferous tubules that were morphologically distinct from other interstitial cells. Concomitantly, fibroblasts, pericytes and peritubular-myoid cells were negative for CD34 expression. Therefore, these cells were easy to distinguish from TCs. Of note, other TC markers e.g. c-kit, platelet-derived growth factor receptor α and β, and vimentin, are expressed in TCs and other interstitial cells e.g. Leydig cells, pericytes, fibroblasts, and macrophages (Feng et al., 1999; Fu et al., 2015; Zhou et al., 2015; Xiao et al., 2016). In voles, Western blot analysis revealed CD34 protein expression was regulated by GPER (Pawlicki et al., 2018). Thus, it is possible that, besides canonical sex steroid receptors (Roatesi et al., 2015), TCs found in vole testis contain the GPER receptor and respond to estrogens and/or the GPER present in other interstitial cells exert estrogen signaling on TCs. Currently, studies of novel testicular TCs are based only on observations as studies in homogenous TC cell populations are challenging. Telocyte isolation techniques have yet to be perfected due to the complicated multi-cell structure of the testis interstitium, including the neighboring multi-type seminiferous tubule compartment.

For the first time, we report leptin, adiponectin, and perilipin immunoexpression and localization in bank vole interstitial cells. Leptin was recently identified as hormone that directly influences male spermatogenic function (Soyupek et al., 2005). In infertile patients, leptin was located in spermatocytes, while its receptor was found in Leydig cells (Ishikawa et al., 2007). In obese patients, increased leptin expression correlated with dysfunctional spermatozoa production (Tena-Sempre et al., 1999; Ramos and Zamoner, 2014). Interestingly, Caprio et al. (1999) demonstrated expression of leptin and its receptor mRNA in rat Leydig cells. On the other hand, in mice, leptin mRNA and protein expression were observed in interstitial cells (Herrid et al., 2008). Herein, independent of animal reproductive status, leptin expression in Leydig cells (and probably also in TCs) was comparable while it decreased following GPER blockage. Such result indicates that the photoperiod and GPER regulated estrogen signaling may influence the action of leptin. In turn, leptin may potentially regulate the estrogen signals in the active and quiescent reproductive status of vole interstitium, with both direct and indirect involvement of TCs. In Siberian hamsters, insulin is implicated in leptin concentration regulation, as increased leptin is found in regressed animals (Garcia et al., 2010).

Our results showing adiponectin immunoexpression and distribution in bank voles mirror the results reported by Caminos et al. (2008), who revealed the presence of adiponectin in rat interstitial cells. When androgen synthesis is inhibited, adiponectin levels increase (Nishizawa et al., 2002). This finding is also reflected in our results here as reduced androgen concentration is found in SD males (Gancarczyk et al., 2004). Adiponectin is shown to promote spermatogenesis and sperm maturation (Martin, 2014). Moreover, similar to leptin, adiponectin is implicated in obesity and reproductive perturbations (Roumaud and Martin, 2015).

Lipid homeostasis is crucial for functional endocrine reproductive organs such as mammary glands, placenta, uterus, ovary, and testis, the last of which TCs were positively verified (Suciu et al., 2010; Chi et al., 2015; Petre et al., 2016). In regressed voles, estrogen level is decreased (Gancarczyk et al., 2004; Zarzycka et al., 2016) and cholesterol level is increased but only in reproducing GPER-blocked voles as related to decreased leptin and adiponectin expression. This finding reflects controlled lipid homeostasis by an adiponectin-leptin interaction with GPER. It is likely that voles have leptin and adiponectin that act in a complementary manner in the interstitium during breeding and non-breading seasons.

In adipocytes and steroidogenic cells, perilipin interacts with cAMP and hormone sensitive lipase for lipid droplet hydrolysis (Servetnick et al., 1995). Testicular lipid droplets are active cellular organelles that aid in the regulation of multiple gonadal functions (Wang et al., 2015). Our data reveals perilipin immunoexpression and localization shows similar amounts of lipid droplets (possibly of various size and content) in the interstitial cells of LD and SD males in relation to a diverse range of intratesticular cholesterol after GPER blockage. Differences in steroidogenic efficiency of the interstitium resulted from modulations of lipid homeostasis, but they were not under perilipin control. Steroidogenic cells, including TCs, in regressed vole testis are a 'reservoir' for restoration of intensive lipid metabolism, and adiponectin and leptin are involved in lipid metabolism in conjunction with newly produced *de novo* cellular lipids. These processes appear to be regulated by GPER-signaling. It was shown that perilipin interacts with leptin, and leptin overexpression leads to decreased perilipin concentration and lipolysis (Ke et al., 2003). Based on the above-mentioned results, the presence and possible role of leptin, adiponectin, and perilipin, which is not regulated by GPER signaling, in interstitial cells and TC function cannot be excluded. Similarly, TC cross-talk with other interstitial cell types maintains lipid balance and steroidogenic efficiency of the interstitial tissue by way of a leptin-adiponectin-perilipin partnership, and this is the basis for future studies.

Other interstitial tissue cells are implicated in steroidogenesis. For example, macrophages secrete various growth factors that modulate steroidogenesis in rat polycystic and mouse Leydig cells (Kmicikiewicz et al., 1997; Figueroa et al., 2012). Peritubular-myoid cells, like Leydig cells, express the androgen receptor and allow for effective androgen signaling (Welsh et al., 2012). Interestingly, GPER signaling in Leydig cells and peritubular cells regulates lipid production and metabolism, as well as steroidogenesis (Vaucher et al., 2014; Kotula-Balak et al., 2018). Estrogens exert a negative effect on steroidogenesis (Akingbemi, 2005). For example, the absence of ERβ results in an overgrowth of interstitial tissue but does not change testosterone production. In the light of above also implication of TCs and their involvement with other interstitial cells in leptin-adiponectin interaction in lipid homeostasis under estrogen signaling control should not be removed from further consideration.

Based on our results, combined with published reports, we surmise bank vole TCs are regulated *via* photoperiod. Additionally, melatonin and estrogen signaling, under the influence of reproductive conditions, control lipid homeostasis in various interstitial cells and possibly TCs.

In summary, we provide novel evidence TC localization in bank vole testis interstitium regardless of their reproductive status. In detail, TC distribution is not directly related to GPER signaling but rather to melatonin signaling. On the contrary, CD34 protein expression is GPER-dependent. In addition, as expression of leptin, adiponectin, and perilipin was found exclusively in the interstitial tissue, the potential role of these hormones is suggested to influence lipid metabolism of the interstitial tissue. Our results are the first to disclose the potential role and function of TCs, *via* molecular interactions within the vole interstitium, in lipid homeostasis regulation as related to the estrogen microenvironment.

Author contributions

Authors' contribution to the work described in the paper: A.M. P.P., E.M., B.J.P., W.T., E.G-W., M. K-B performed research. A.R., B.J. P., W.T., B.B., M. K-B analyzed the data.

M.K.-B. designed the research study and wrote the paper. All authors have read and approved the final version of the manuscript.

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Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

This study was carried out in accordance with the recommendations of the "Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes." The use of the animals was approved by the National Commission of Bioethics at the Jagiellonian University in Krakow, Poland (No. 104/2015).

Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.10.021.

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