Regular paper

PELP1 and SRC kinase as important molecules in the estrogen-mediated pathway in human testis and epididymis

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Background: Estrogens have pleiotropic mechanisms of action, and their cellular transduction pathways can modulate various proteins with differential tissue expression. Proline-, glutamic acid-, and leucine-rich protein 1 (PELP1) is one such protein whose role seems important. However, very little is known about the expression of modulators involved in the estrogen-mediated pathways in the tissues of the male reproductive tract. **Methods:** In this study, we obtained autopsy specimens of testis and epididymis from 13 men of Caucasian descent. Expression levels were analyzed for both estrogen receptors (*ESR1* and *ESR2*) and their co-regulators, including *PELP1* and kinase c-Src (*SRC*). **Results:** Protein expression was confirmed with western blot and immunocytochemistry techniques. The expression of both *SRC* and *PELP1* was significantly higher in the testis compared to the epididymis (p=0.040 and p=0.002, respectively). Furthermore, a significant, positive correlation was observed between *SRC* and *PELP1*, regardless of tissue type (p<0.0001, R=0.78). In the testis, *PELP1* expression positively correlated with *ESR1* expression (p=0.0367, R=0.6). **Conclusions:** Our study suggests a possible relationship between PELP1, SRC, and ESR1 in the human testis and epididymis. This study makes a valuable contribution to the field of estrogen-mediated pathways in the male reproductive tract and describes trends of analyzed genes' expression and presence. We think our results may open some new research directions in the study of estrogen signaling in the male reproductive system.

Keywords: estrogen receptors (ESR1 and ESR2); proline-, glutamic acid-, and leucine-rich protein 1 (PELP1); proto-oncogene tyrosine-protein kinase c-SRC (SRC); steroid hormones; human testis; human epididymis

Abbreviations: BC, basal cells; CC, ciliated cells; Cr, concentration ratio; ESR1, estrogen receptor 1; ESR2, estrogen receptor 2; GPER, GPR30, G protein-coupled estrogen receptors; HPRT1, hypoxanthine-guanine phosphoribosyltransferase; IT, interstitial tissue; LC, Leydig Cells; MNAR, modulator of non-genomic action of estrogen receptor; nCC, non-ciliated cells; PELP1, proline-, glutamic acid-, and leucine-rich protein 1; Psp, primary spermatocytes; S, spermatozoa; SC, Sertoli cells; SEC, seminiferous epithelium cells; Sd, spermatids; Sg, spermatogonia; SRC, proto-oncogene tyrosine-protein kinase c-SRC

INTRODUCTION

The predominant paradigm concerning the influence of steroid hormones in the regulation of the male reproductive tract's functions was primarily focused on androgens such as testosterone. However, in 1990 knock-out animal models began to be extensively used and demonstrated that estrogens play an important role in male reproductive health (Carreau *et al.*, 2011a; Akingbemi, 2005; Carreau *et al.*, 2011b; Chimento *et al.*, 2020; Hess & Cooke, 2018). These commonly considered female hormones are indispensable for the proper development of certain structures within the male reproductive tract, such as the prostate, epididymis, and efferent ductules. Estrogens are synthesized in the testis, and 17β-estradiol is highly expressed in the rete testis fluid. Studies have shown that the male reproductive tract has extensive expression of both estrogen receptors (ESRs), namely ESR1 and ESR2, starting from the neonatal period through puberty to adulthood. However, expression level differs with regard to tissue type and age (Cooke *et al.*, 2017; Cunha *et al.*, 2021; Hess & Cooke, 2018). Several findings also confirmed an association between estradiol and a handful of physiological events such as erectile function, spermatogenesis, Leydig cell self-regulation, testosterone production control, and auto- and paracrine effects on germ cells (Hess & Cooke, 2018; Solakidi *et al.*, 2005; Carreau *et al.*, 2011a; Cooke *et al.*, 2017; Hammes & Levin, 2019; Lazari *et al.*, 2009). Furthermore, the identification of an aromatase function has been a tremendous breakthrough in defining the role of estrogens in males (Carreau *et al.*, 2006; Otto *et al.*, 2009; Akingbemi, 2005; Rago *et al.*, 2007; Carreau *et al.*, 2010; Aquila *et al.*, 2003) and has directed the attention of many researchers around the world to the influence on the male reproductive tract of estrogen-like compounds present in the environment (referred to as "xenoestrogens"). Previously, the idea of the potential negative influence of estrogens and their role in disrupting the function of the male reproductive system has become widespread (Rahman *et al.*, 2015; Mohamed *et al.*, 2011; Sikka & Wang, 2008; Hess *et al.*, 2011; Bertolla, 2020).

The commonly identified pathways that control the functional influence of estrogens on the tissues/cells of interest (including those in the male reproductive tract) classically involve either ESR or G protein-coupled estrogen receptors (GPER, GPR30). It cannot be excluded that recently discovered novel proteins, referred to as putative ESRs (estrogen-related receptor, saxiphilin-binding protein, ER-X, and ER-x), may play a similar role in mediating estrogen signals in males (Barut *et al.*, 2020; Li *et al.*, 2015; Eyster, 2016; Micevych & Dewing,

2011; Toran-Allerand *et al.*, 2002; Cooke *et al.*, 2017; Akingbemi, 2005). It is also essential to highlight the role of certain co-regulators in mediating estrogen signals in tissues/cells of interest. One of them is the scaffold protein proline-, glutamic acid-, and leucine-rich protein 1 (PELP1), also known as MNAR (modulator of nongenomic action of estrogen receptor). PELP1 was shown to interact not only with nuclear receptors and transcription factors (such as activator protein 1, specificity protein 1, or nuclear factor kappa B) but also with several key modulators of cell cycle progression, such as proto-oncogene tyrosine-protein, epidermal growth factor receptor, phosphoinositide-3 or c-SRC kinases (Vadlamudi & Kumar, 2007; Sareddy & Vadlamudi, 2016; Vadlamudi *et al.*, 2001). This previous literature has shown PELP1 to be an important mediator of steroid hormone regulation of cell or tissue functions. Moreover, our previous studies demonstrated that the percentage of PELP1+ sperm cells is correlated with decreasing sperm quality (Skibińska *et al.*, 2018). Also, our investigation revealed weak, negative *ESR2/PELP1* correlations in patients with abnormal sperm values. Interestingly, *SRC/PELP1* was moderately and positively correlated with all parameters within the WHO reference range in the subgroup of patients (Skibińska *et al.*, 2022). The literature has identified that SRC-mediated signaling is coordinated by binding PELP1 and ESR to SRCs SH3 and SH2 domains, which is then stabilized by the ESR-PELP1 interaction through PELP1's LXXLL motifs (Xiao *et al.*, 2019; Shupe *et al.*, 2011; Barletta *et al.*, 2004). SRC has been characterized as the downstream protein kinase of the non-classical testosterone signaling pathway involved in the release of sperm at spermiation. It cannot be excluded that close interactions between these two proteins have implications for estrogen signaling in males.

Despite the broad availability of animal models for research purposes, this is not the case for human tissues. Therefore, data regarding estrogens and estrogen-mediated pathways in humans do not currently provide clear answers concerning the presence, localization, and interactions between proteins involved in estrogen-mediated signaling. It appears crucial in determining the potential clinical repercussions of estrogens in the male reproductive tract, also in the context of impaired male fertility. Where and when estrogen and/or an estrogen/androgen balance might play a role in the development and function of the male reproductive tract needs to be determined. It is expected that with recent advances in molecular and cell biology techniques, many questions about estrogen-mediated signaling in men will be answered within the next decade. The aim of this study was to analyze the potential links between ESRs, PELP1, and SRC in human testis and epididymis, as these proteins are considered important factors involved in estrogen-mediated signaling in the male reproductive system.

MATERIALS AND METHODS

Testes tissue samples

Autopsy specimens of testis and epididymis were obtained from 13 men of Caucasian descent at the Chair and Department of Forensic Medicine of PUMS. The mean age of participants was 45±15 years (range: 23–76) with a 45% coefficient of variation. Tissues qualified for the study came from men with no significant spermatogenesis disorders. The assessment of tissues stained with the standard hematoxylin and eosin method was based on the microscopic evaluation, accordingly to Cerilli *et al.* (Cerilli *et al.*, 2010). Each testis and epididymal caput sample was split into three fragments (equal in mass). For RT-qPCR analyses, the tissues were immersed in RNA protective medium (Englert-Golon *et al.*, 2021; Camacho-Sanchez *et al.*, 2013), and for protein isolation were placed in phosphate-buffered saline (PBS, Chempur; Piekary Śląskie, Poland). In both cases, the tissues were stored at –80°C until further procedures. The third portion, intended for immunohistochemistry, was fixed in 4% paraformaldehyde (Chempur; Piekary Śląskie, Poland) and processed for standard formalin-fixed paraffin-embedding.

METHODS

Nucleic acid isolation and validation

Before nucleic acid isolation, the samples were thawed to room temperature. The RNA protective medium was removed, and testis and epididymis tissue samples were patted dry with a paper towel. The tissues were immediately placed in a chilled mortar and pulverized with a pestle. Aliquots of 25 mg of tissue were immersed in 800 µL fenozol reagent to deactivate endogenous RNases (A&A Biotechnology, Gdynia, Poland). The samples were dissolved by vortexing (15 sec, 1200 rpm, room temperature) and incubated in a thermal mixer to permit complete dissociation of the nucleoproteins (5 min, 300 rpm, 56°C). Then, 200 µL of chloroform (Avantor Performance Materials Poland S.A., Gliwice, Poland) was added to each aliquot, mixed by inversion, incubated (3 min, room temperature), and centrifuged (15 min, $12\,000{\times}g$, 4°C). The aqueous upper phase containing total cellular RNA was carefully transferred to a new tube, avoiding transferring the interphase.

The aqueous phases were subjected to high molecular weight RNA extraction, without microRNA fractionation, using the double-column system for microRNA and RNA isolation according to the manufacturer's protocol (A&A Biotechnology, Gdynia, Poland) as described previously (Englert-Golon *et al.*, 2021). The quality, quantity, and purity of extracted RNA were analyzed as described previously (Andrusiewicz *et al.*, 2016) with the use of NanoPhotometer NP-80 (IMPLEN, München, Germany), and the integrity was evaluated by electrophoretic separation under denaturing conditions (Andrusiewicz *et al.*, 2016).

Reverse transcription and quantitative polymerase chain reaction

Three-step reverse-transcription reactions were performed as described previously (Andrusiewicz *et al.*, 2016). In brief, a mixture of 0.5 M universal oligo(d)T_{10} primer, 1 mM random hexamer primer (Genomed; Warsaw, Poland), 1 µg RNA, and water (Thermo Fisher Scientific; Waltham, MA, USA) were incubated for 10 mins at 65°C, and samples chilled on ice. Then, 10 U transcriptor reverse transcriptase, 5 U RNasin ribonuclease inhibitor, 1× reaction buffer (Roche; Manheim, Germany), 0.1 U/µL *E. coli* poly(A) polymerase, 100 nM adenosine triphosphate (New England BioLabs; Ipswich, MA, USA), and 100 mM deoxyribonucleotide triphosphates (Novayzm; Poznan, Poland) were added with water to a final reaction volume of 20 µL. The subsequent steps of cDNA synthesis were followed as described previously (Englert-Golon *et al.*, 2021). The cDNA was synthesized in duplicate for

each sample and subsequently served as a template for qPCR reactions. The threshold cycles mean values derived from replicated samples were used for further analysis.

To determine primer sequences and hydrolysis Universal Probe positions for *ESR1*, *PELP1*, and *SRC,* the UPL Assay Design Center was used (http://qpcr.probefinder.com, last accessed on September 28, 2017, product discontinued on December 30, 2020) (Skibińska *et al.*, 2018). Probe numbers and primers' sequences are as follows: *ESR1* F: CCTTCTTCAAGAGAAGTATTCAAGG; R: ATTCCCACTTCGTAGCATTTG; probe #69 (Roche cat. no.: 04688686001; GenBank AC: NM_001122740.2, NM_001122741.2, NM_001385571.1, NM_001291241.2, NM_001385568.1, NM_001385572.1, NM_001385570.1, NM_001385569.1, NM_000125.4, NM_001328100.2, NM_001291230.2), *PELP1* F: CAAGGAGGAGACTCACAGGAG; R: CAAGGAGGAGACTCACAGGAG; probe #24 (Roche cat. no.: 04686985001; NM_014389.3, NM_001278241.2), and *SRC* F: GCCATGTTCACTCCGGTTT; R: CAGCGTCCTCATCTGGTTTC; probe #21 (Roche cat. no.: 04686942001; NM_005417.5). Either the forward or the reverse primers were designed for the exon-exon junction of the analyzed mRNAs. The amplicon lengths were similar. With regards to *ESR2*, we applied a ready-to-use assay (PrimePCR, qHsaCEP0052206, BioRad; Hercules, CA, USA). The hypoxanthine-guanine phosphoriboxyltransferase (*HPRT1*) gene assay (UPL102079, Roche; Manheim, Germany) was used as a reference gene.

The quantitative PCR reactions were carried out according to the MIQE guidelines (Bustin *et al.*, 2009) (Supplementary Materials: MIQE checklist and data set). All qPCR reactions were made in a total volume of 20 μL. Standard cycling and acquisition steps were performed with adjusted and standardized reaction mixtures for Roche UPL probes in the LightCycler 2.0 glass-capillary thermal cycler (Roche Diagnostics International AG; Rotkreuz, Switzerland) (Englert-Golon *et al.*, 2021; Andrusiewicz *et al.*, 2016). The expression level of each gene, expressed as concentration ratios (Cr), was derived from reaction efficiencies (obtained from the relevant standard curves) compared with the appropriate mean of two-reaction threshold values and normalized to reference gene expression (Englert-Golon *et al.*, 2021; Skibińska *et al.*, 2018).

Western blot analysis

Tissue samples immersed in PBS were used for protein isolation, followed by western blot. After mechanical pulverization in liquid nitrogen, tissue samples were suspended by pipetting in RIPA Lysis Buffer (Merck Millipore, Darmstadt, Germany). Then, total sample extracts were shaken in an orbital shaker (1 600 rpm, 60 min, 4°C) and centrifuged (14 000×*g*, 20 min, 4°C). The supernatant was transferred into a fresh tube and centrifuged again (3 000×*g*, 10 min, 4°C), and then the supernatant containing purified proteins was used in further analyses.

Western blot analyses were conducted as described previously (Englert-Golon *et al.*, 2021). In short, protein concentration was measured colorimetrically with Quick-Start Bradford 1 × Dye Reagent (BioRad, Hercules, CA, USA). Protein lysates (20 μg) diluted with Laemmli buffer (BioRad, Hercules, CA, USA) were denatured at 70°C for 10 min, loaded onto a 10% SDS-polyacrylamide gel (TGX FastCast Acrylamide Kit 10%, BioRad, Hercules, CA, USA), and separated by electrophoresis. After wet-transfer, PVDF membranes (Merck Millipore, Darmstadt, Germany) were incubated on an orbital shaker at 200 rpm for 60 min at room temperature in TBS-T blocking buffer (TBS with 0.1% Tween-20 and 5% bovine serum albumin, pH 7.5; LabEmpire; Rzeszow, Poland). After blocking, the membranes were incubated with primary antibodies on an orbital shaker at 200 rpm overnight at 4°C. The antibodies used are as follows: anti-ESR1 (1:1000, LS-C88420, Lifespan Biosciences, Seattle, WA, USA), ESR2 (1:1000, ab3576, Abcam, Cambridge, UK), PELP1/MNAR (1:1000, A300-180A, Bethyl, Montgomery, TX, USA), SRC (1:1000 orb379229, Biorbyt, Cambridge, UK), pSRC (1:500, orb14869, Biorbyt, Cambridge, UK), and GAPDH (1:2500, sc-25778, Santa Cruz Biotechnology, Dallas, TX, USA). Subsequent visualization processing steps were performed as described previously (Englert-Golon *et al.*, 2021). Immunoreactive bands were compared with the mass standard (3-Colour Prestained Protein Marker; Blirt, Gdansk, Poland). For protein density analysis ImageJ Software version 1.53r was used (https://imagej.nih.gov/ij/download.html, accession date 22.04.2022). The high-resolution TIFF images obtained with G:BOX (Syngen, Cambridge, UK) from chemiluminescent-visualized western blots were converted into JEPG 255 grayscale format. A defined frame size was used to measure the optical density of analyzed proteins, the background, and the GAPDH protein as the reference. The pixel density for all data was inverted and expressed as 255 − recorded by ImageJ value. Next, the net values for analyzed proteins and controls were calculated (by subtracting the value obtained for the background). Finally, the ratios of net band values for analyzed proteins and corresponding references were obtained and expressed as optical density in arbitrary units.

Immunohistochemical protein localization

The protein localization in tissue samples was assessed using immunohistochemistry as described previously (Waligórska-Stachura *et al.*, 2017; Englert-Golon *et al.*, 2021). In brief, 3 μm thick tissue sections were immersed in antigen retrieval solution (0.1 mM citric acid and 0.1 mM sodium citrate; pH 6.0; Avantor Performance Materials Poland S.A., Gliwice, Poland) and then microwaved, and this process repeated. Endogenous peroxidase activity was blocked in a 3% hydroperoxide solution (Avantor Performance Materials Poland S.A., Gliwice, Poland). The slides were blocked in a TBS-T blocking buffer for 60 min at room temperature. Immunohistochemical reactions were performed using the primary antibodies specified in the western blot analysis description at a 1:100 dilution in TBS-T buffer and visualized and assessed as described previously (Englert-Golon *et al.*, 2021). The specificity, dilutions and immunohistochemical reactions' conditions for positive controls were also adopted from the methodology referred above. The primary antibodies were substituted with a blocking buffer in the appropriate negative controls.

Statistical analyses

Results were evaluated using Statistica version 13.5.0 software for Windows (TIBCO Software Inc., Palo Alto, CA, USA). All continuous variables were checked for outliers. Min-Max normalization was used to describe and present the final data. All genes were analyzed for not only normalized C_r values but also gene-to-gene C_r values. Results were described by the median [interquartile range] (Me [IR]) values. The Shapiro-Wilk test was applied for the normality of continuous variables distribution assessment. The non-parametric two-tailed
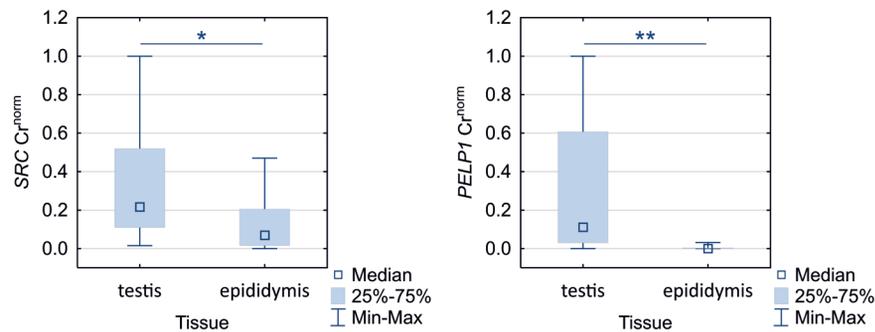
Figure 1. Normalized expression of *SRC* and *PELP1* in testis and epididymis.
$*p<0.05$, $**p<0.01$ (two-tailed Mann-Whitney U test).

Mann-Whitney U test was used for statistical analyses. The Spearman rank correlation test was applied to evaluate the strength of the correlation coefficient (R). The strength of the correlation coefficient was assessed according to Guilford's classification. Data were considered statistically significant when $p<0.05$.

RESULTS

ESRs, *PELP1* and *SRC* mRNA expression

The normalized expression level of both *SRC* and *PELP1* differ significantly between testis and epididymal tissue ($p=0.040$ and $p=0.002$, respectively), with both genes having higher expression levels in the testis (Fig. 1). We did not observe significant differences in the expression level of *ESR1* and *ESR2* in either testis or epididymal tissue in any of the analyzed samples ($p>0.05$) (not shown).

With respect to the tissue of origin, both *SRC* and *PELP1* expression was significant and very strongly positively correlated in both testis and epididymis (R=0.66, $p=0.014$, and R=0.80, $p=0.0019$, respectively). Additionally, *PELP1* expression was strongly positively corre-

lated with the expression of *ESR1* in the testis (R=0.6; $p=0.0367$) (Fig. 2). We did not observe this correlation in the case of the epididymis ($p>0.05$).

We observed a moderate positive correlation between the tissue donors' age and *ESR1* normalized expression in the testis (R=0.59; $p=0.0322$) and a moderate negative correlation of the age and *PELP1* expression in the epididymis (R=-0.61; $p=0.0354$) (Fig. 3). The expression levels of other genes were not correlated with age ($p>0.05$).

Considering the expression ratio of analyzed genes, the *ESR2/PELP1* ratio differed significantly between the testis and epididymis ($p=0.0068$). The expression ratio was lower in the testis (Fig. 4). There was no significant difference in the expression ratios of other genes between the tissue types.

Considering tissue-origin-dependent division, *ESR1/ESR2*, *ESR1/SRC*, and *ESR1/PELP1* expression ratios were moderately and positively correlated in the testis. *ESR2/SRC* was moderately positively correlated with *ESR2/PELP1*, and strongly negatively correlated with *SRC/PELP1*. We observed very strong positive correlations of *ESR1/ESR2* with *ESR1/SRC*, *ESR1/SRC* with *ESR1/PELP1*, and *ESR2/SRC* with *ESR2/PELP1* in the epididymis. Additionally, a moderately positive
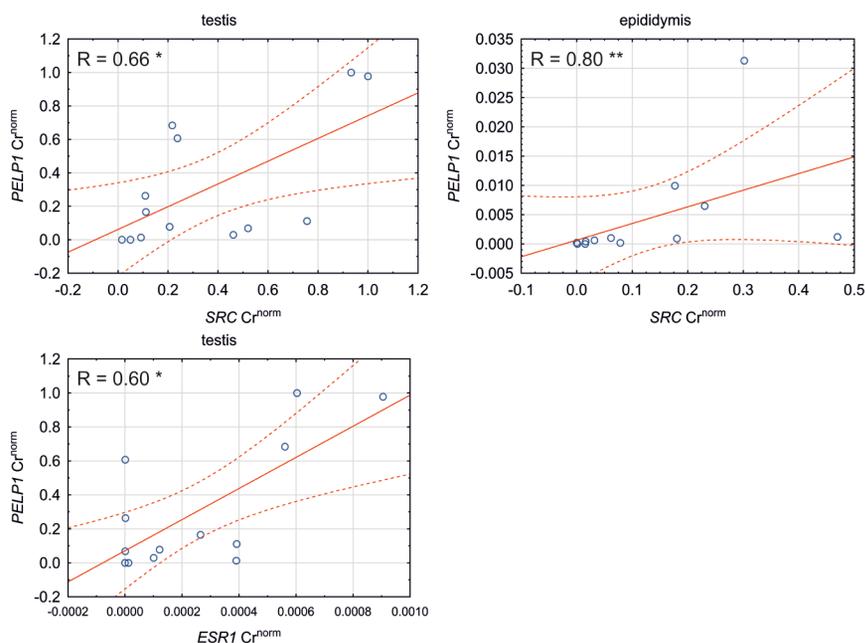
Figure 2. Dot-plot of normalized *PELP1* expression correlation with *SRC* and *ESR1* in testis and epididymis.
Spearman's rank correlation coefficient (R) is indicated as significant $*p<0.05$, $**p<0.01$.
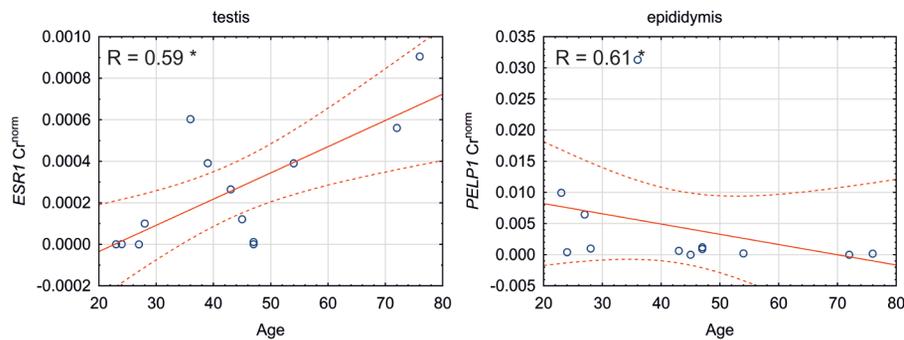
Figure 3. Dot-plot of the participants' age and correlation in testis and epididymis of *ESR1* and *PELP1* normalized expression.
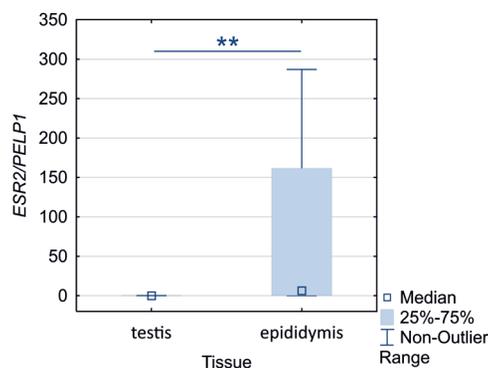Spearman's rank correlation coefficient (R) is indicated as significant *$p<0.05$.

Figure 4. Normalized ratio of the expression of *ESR2/PELP1* in testis and epididymis.
**$p<0.01$ (two-tailed Mann-Whitney *U* test).

correlation was established for the expression ratios of *ESR1/ESR2* and *ESR1/PELP1*. *ESR2/SRC* and *SRC/PELP1* in the epididymis were not correlated (Table 1).

Protein presence and localization

The presence of the analyzed proteins in the testis and epididymis was confirmed by western blotting to identify
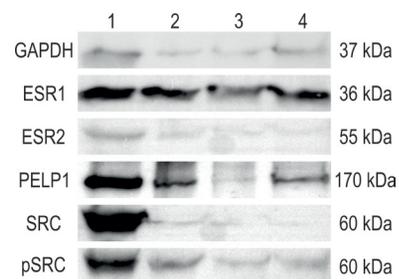

Figure 5. Western blot analysis of analyzed proteins in selected samples from different patients.
Lane 1 and 2 – testis, lane 3 and 4 – epididymis. Note that the blots displayed were cropped; see Supplementary Information for the original blots.

the presence of immunoreactive bands at the expected sizes (ESR1: 36 kDa; ESR2: 55 kDa; PELP1: 170 kDa; SRC: 60 kDa and pSRC: 61 kDa). The GAPDH reference protein bands (37 kDa) were present in all samples (Fig. 5).

The protein density of both pSRC and PELP1 differ significantly between testis and epididymal tissue ($p=0.015$ and $p<0.001$, respectively), with both proteins having a higher density in the testis (Fig. 6).

**Table 1. Spearman rank order correlations of the analyzed gene ratios in testis and epididymis.**

Pair of Variables	Testis			Epididymis		
	N	R	*p*-value	N	R	*p*-value
ESR1/ESR2 & ESR1/SRC	13	.61	.0269	9	.90	**.0009**
ESR1/ESR2 & ESR1/PELP1	12	.58	.0479	10	.82	**.0038**
ESR1/ESR2 & ESR2/SRC	13	−.54	.0546	9	−.33	.3807
ESR1/ESR2 & ESR2/PELP1	12	−.45	.1377	10	−.04	.9074
ESR1/ESR2 & SRC/PELP1	13	.25	.4154	10	−.18	.6272
ESR1/SRC & ESR1/PELP1	12	.65	.0220	10	.96	**<.0001**
ESR1/SRC & ESR2/SRC	13	.26	.3943	10	−.14	.7009
ESR1/SRC & ESR2/PELP1	12	.01	.9828	10	.08	.8287
ESR1/SRC & SRC/PELP1	13	−.37	.2159	10	−.39	.2600
ESR1/PELP1 & ESR2/SRC	12	.15	.6331	10	.07	.8548
ESR1/PELP1 & ESR2/PELP1	12	.36	.2551	11	.33	.3259
ESR1/PELP1 & SRC/PELP1	12	−.39	.2081	11	−.35	.2981
ESR2/SRC & ESR2/PELP1	12	.66	.0185	11	.95	**<.0001**
ESR2/SRC & SRC/PELP1	13	−.86	**.0001**	11	−.32	.3403
ESR2/PELP1 & SRC/PELP1	12	−.53	.0754	12	−.27	.3911

Legend: N, number of paired cases; R, Spearman's rank correlation coefficient; *p*-values <0.05 are indicated in bold.
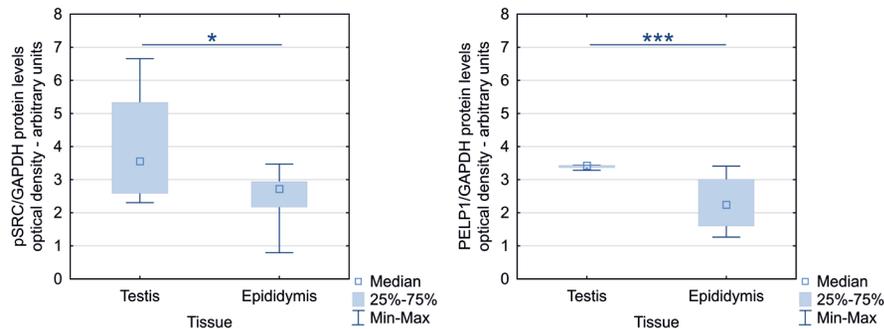
Figure 6. Protein density analysis of pSRC and PELP1 in testis and epididymis.
*p<0.05, ***p<0.001 (two-tailed Mann-Whitney *U* test).
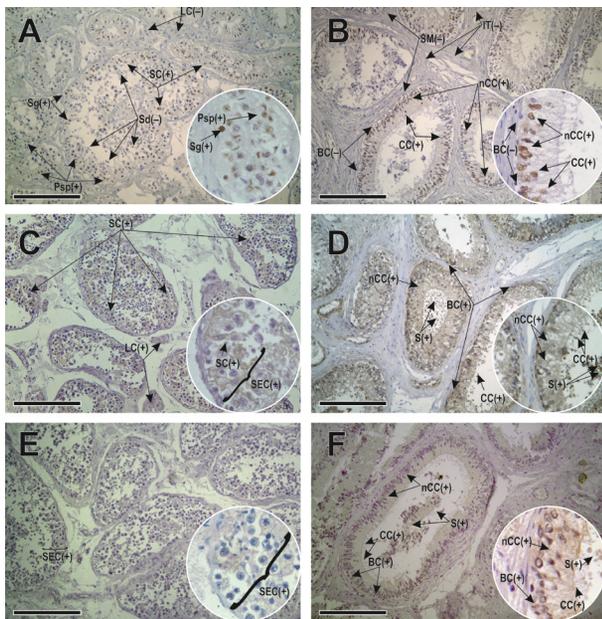
Figure 7. Immunohistochemical localization of PELP1 (A), SRC (C), pSRC (phospho-Tyr529) (E) in testis, and PELP1 (B), SRC (D), and pSRC (phospho-Tyr529) (F) in the epididymis.
Legend: BC, basal cells; CC, ciliated cells; nCC, non-ciliated cells; IT, interstitial tissue; LC, Leydig Cells; Psp, primary spermatocytes; S, spermatozoa; SC, Sertoli cells; SEC, seminiferous epithelium cells; Sd, spermatids; Sg, spermatogonia. (+), positive immunostaining; (–), negative immunostaining. Scale bar: 200 μm.

Immunohistochemical staining enabled the identification of all studied proteins in tissues of interest. The ESR1 and ESR2 expression showed similar localization as observed by other authors (Fietz *et al.*, 2014; Rago *et al.*, 2018). In the testis, ESR1 was observed in some primary spermatocytes and spermatids but not in the spermatogonia, Sertoli, or interstitial cells. The ESR2 was localized in spermatogonia, primary spermatocytes, spermatids, and Sertoli cells but not in the interstitial compartment. On the other hand, in the epididymis, ESR1 was expressed in non-ciliated cells and ESR2 in the ciliated and non-ciliated cells of the epithelium and the basal cells (Supplementary Fig. S1 at https://ojs.ptbioch.edu.pl/index.php/abp). We observed PELP1 localization in the Sertoli cells, primary spermatocytes, and single spermatogonia in the testis. We did not observe positive staining in Leydig cells or spermatids (Fig. 7A). In the magnification of Fig. 7A, positive immunostaining was indicated in primary spermatocytes and spermatogonia. On the other hand, SRC was extensively expressed in the cytoplasm of Sertoli cells and the cells of the seminiferous epithelium. SRC was also weakly expressed in the

cytoplasm of Leydig cells (Fig. 7C). A similar but weaker reaction was observed for the cells of the seminiferous epithelium for pSRC (Fig. 7E). In the epididymis, a strong PELP1 expression was demonstrated in the nuclei of the ciliated and non-ciliated cells of the epididymal ductal epithelium. Weak cytoplasmic staining was also observed in these cells. The vast majority of basal cells were PELP1 negative. There was no expression of PELP1 in smooth muscle cells of the ductal wall or interstitial tissue cells. In the magnification, the ciliated and non-ciliated cells were indicated (Fig. 7B). SRC was expressed in the basal cells' nuclei, spermatozoa, and the ciliated and non-ciliated cells of the epididymal ductal epithelium. In the magnification, the non-ciliated and ciliated cells and spermatozoa were indicated (Fig. 7D). A similar but slightly weaker reaction was observed in the case of pSRC (Fig. 7F).

DISCUSSION

There are distinct roles of ESRs throughout the entire reproductive tract in males. However, there is a lack of consensus on their detection and localization in the published literature (Hess *et al.*, 2021; Fietz *et al.*, 2014; Cooke *et al.*, 2017; Li *et al.*, 2015). There are several studies indicating that estrogen signaling may use a variety of different pathways where, for example, gene transcription may be induced by either direct binding of certain receptors to estrogen response elements (ERE) in the promotors of target genes or can involve transcription factor complexes that are able to activate transcription in the promoter regions of genes directly and without the use of EREs (Klinge, 2001). Conversely, an alternative non-genomic mechanism was also described. It uses other signaling pathways, such as growth factors, kinases, or associated co-regulators, which may activate ESRs in the absence of a ligand (Hess & Cooke, 2018). It was established that aromatase is expressed in human epithelial cells of ductuli efferentes and proximal caput of the epididymis. It suggests a putative role of estrogens produced locally in the epididymal function. Moreover, epididymal caput and cauda differed in ESR1 and ESR2 presence in humans and animals. Additionally, the possible involvement of ESR2 in estrogen modulation of the epididymal function is suggested, as ESR2 expression was confirmed in the epithelial cells of human epididymis indicates (Bilińska *et al.*, 2006; Carpino *et al.*, 2004a; Carpino *et al.*, 2004b; Kolasa *et al.*, 2003). Our study suggests that the specific ESR-mediated pathway in the male reproductive system might result from the different local interactions of estrogens with estrogen receptors and their co-regulators. Even though the tissues

are of the same type, we observed significant differences in the expression levels of the studied genes, especially PELP1 and SRC kinase in both mRNA and protein levels. This paper confirmed the presence of ESR1, ESR2, and their co-regulators, including PELP1 and SRC kinase, in human testis and epididymis. Furthermore, we analyzed potential links between them, as their interactions may have a particular impact on physiological phenomena in these tissues. However, further analyses exploring estrogen-mediated signaling supported by colocalization studies are essential to determine the nature of these interactions and the potential clinical repercussions of estrogens in the male reproductive tract.

Our study suggests that there may be a dependence between PELP1 and SRC in human testis. Our study established the significant differences in normalized expression levels of both SRC and PELP1 in the testis and epididymis, with significantly higher expression and protein density in the testis. Furthermore, our study indicated that *SRC* and *PELP1* expression levels were moderately positively correlated in the testis (R=0.66, p=0.014). Moreover, *PELP1* expression was positively correlated with the expression of *ESR1* (R=0.6; p=0.0367). It may imply the role of ESR1 in testis, as well as the involvement of PELP1 and SRC in estrogen-mediated signaling (e.g., ligand-independent signaling to induce specific effects via ESR1). As we observed PELP1 being localized in Sertoli cells and spermatocytes, it could not be excluded that PELP1 may be involved in certain stages of spermatogenesis or may somehow modulate Sertoli cells' function in this regard.

It has been confirmed that *ESR1* expression is different through certain stages of development (Hess *et al.*, 2011). In 1994, the first man lacking a functional ESR1 was reported, and subsequently, other men lacking aromatase were identified, with 13 reported cases of loss of function mutations in CYP19A1 (Cooke *et al.*, 2017). Regardless of species or investigators, *ESR1* has been shown to be highly expressed in the efferent ductule epithelium, which suggests its contribution to fluid reabsorption (Fietz *et al.*, 2014). Interestingly, the most severe histopathological changes following the disruption of ESR1 function occur in the testis and efferent ductules, likely due to its high expression, which is approximately 3.5-fold greater than in the female uterus or any other

organ independent of biological sex (Fietz *et al.*, 2014). Furthermore, ESRs involvement was suggested in the development of sperm cells (Hess, 2014). We observed age-related increasing *ESR1* and decreasing *PELP1* expression. Due to the limited number of published papers on the topic of PELP1 expression in the male reproductive tract, and because the *ESR1* and *ESR2* expression alters with age (in different tissues, not only in the male reproductive tract), it would be challenging to propose strong conclusions. Considering that we analyzed the specimens from males not only of reproductive age, further studies need to be conducted to elaborate on this topic. Moreover, a recent report confirmed the ESR1 and PELP1 presence in the human reproductive tract, including in the nuclei of ciliated and non-ciliated cells for ESR1 and principal cells of proximal epididymis for PELP1 (Rago *et al.*, 2018). As our results highlight the *SRC/PELP1* correlation in testis, it cannot be ruled out that these proteins may potentially be involved in ESR1-mediated processes surrounding rete fluid resorption in the testis, influence the composition of seminal fluids, or anyhow in the regulation of spermatogenesis. Interactions between PELP1-SRC-ESR1 seem feasible in the above-mentioned processes, especially due to the fact that the binding of PELP1 and ESR coordinates SRC-mediated signaling to SRC's SH3 and SH2 domains. It is known that the SRC family kinases (SFKs), including SRC (but also Yes, Fyn, and Lck among others) play a role in cell polarity, in the testis spermatogonial stem cell proliferation, cell adhesion or to influence the dynamics of the blood-testis barrier in the seminiferous epithelium. Even though the effects of c-SRC and c-Yes kinases may considerably overlap, and their action is thought to be limited to the cell matrix at the focal contacts, their role in the testis cannot be denied (Xiao *et al.*, 2013; Xiao *et al.*, 2019; Rago *et al.*, 2018). It is also suggested that SRC plays a crucial role in the 17β-estradiol-induced translocation of estrogen receptors from the nucleus to the cell membrane (Lucas *et al.*, 2008). Furthermore, the ability of ESRs to simultaneously bind SRC and PELP1 results in the activation of a non-genomic pathway such as cSRC/MAPK-pathway, which leads to enhanced, phosphorylation-dependent ESR1 transcriptional activity (Nieto *et al.*, 2015; Barletta *et al.*, 2004).
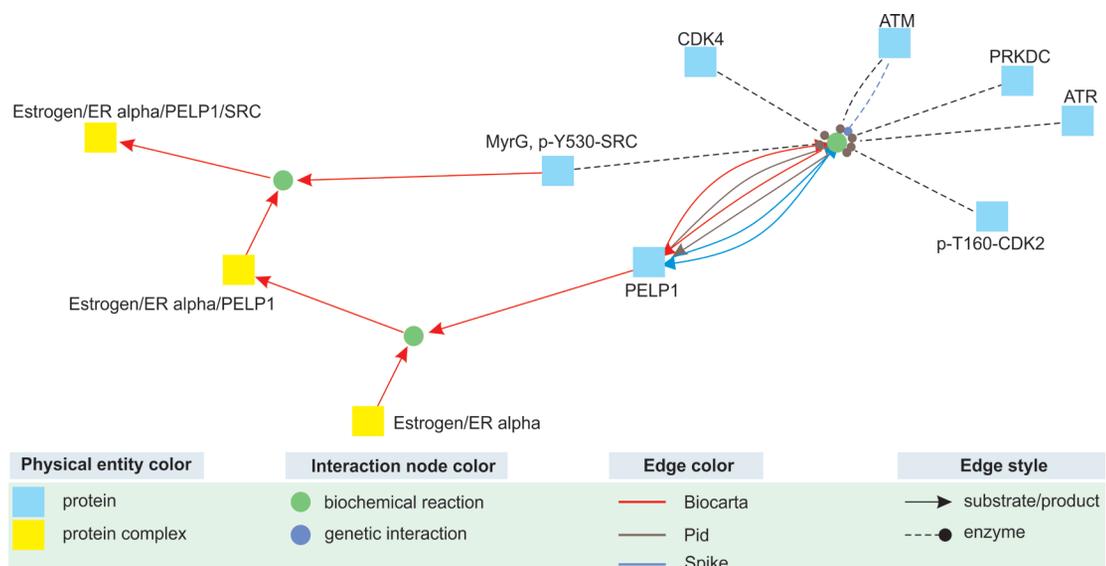

Figure 8. Map of interactions in PELP1 modulation of estrogen receptor activity (cpb.molgen.de/CPDB/cyVis).

The PELP1 biochemical interactions with other proteins are complex and involve other kinases, such as ATM, ATR, CDK2, CDK4, and PRKDC, in addition to SRC (Fig. 8). The biochemical/enzymatic reactions with these proteins directly influence PELP1 and, other protein complexes involved in estrogen signal transduction. It seems that ESR1, but not ESR2 plays a crucial role in the PELP1-mediated pathway.

Upon phosphorylation by SRC, certain cellular events may be influenced by the specific cellular localization of PELP1, SRC, and ESR1. Furthermore, it is important to mention that the extent of SRC-PELP1-ESR1 interactions in human testis may influence the availability of another estrogen receptor GPER. However, it was not analyzed in this study. These dependencies could modulate the microenvironment for spermatozoa maturation in different sites of the male reproductive tract, depending on the availability of certain receptors and their co-regulators in the tissue area of interest. It was shown that SRC phosphorylation at Tyr529 deactivates the protein function (Gonfloni et al., 2000). The Tyr529 phosphorylation status is closely related to the autophosphorylation of Tyr418, which determines the SRC activity (Xiao et al., 2017), leading to complex conformational changes (Gonfloni et al., 2000). As a consequence SH3 domain of SRC ceases to be strongly associated with the linker of the SH2 domain and the catalytic domain and becomes more accessible to other ligands (Xiao et al., 2017). Consistent with this idea would be the fact that we observed differences in the SRC and pSRC expression pattern, as this protein works bidirectionally upon phosphorylation status (Gonfloni et al., 2000). Differentiated expression patterns of SRC and pSRC in testis and epididymis, observed in our study, could be reflected in the modulation of cell events, such as cell maturation and migration, mediated by other signaling pathways (Xiao et al., 2017). Therefore, investigations of the molecular consequences and the role of these phenomena in the male reproductive tract should be further conducted.

Regarding ESR2 expression in the testis, ESR2/SRC correlated positively with ESR2/PELP1. Moreover, ESR2/SRC had a strongly negative correlation with SRC/PELP1. Until 2018, common knowledge regarding ESR2 indicated its ubiquitous expression in the male reproductive tract. However, more recent investigations have questioned antibody specificity for immunohistochemical localization of the receptor (Hess & Cooke, 2018), and therefore data concerning ESR2 distribution needs to be re-evaluated. Furthermore, it is worth pointing out that ESR2 knock-out animal models are more limited than ESR1 ones. Recent studies confirmed ESR2 expression only in ciliated cell nuclei of the efferent ductules and in a few epithelial layer cells in proximal epididymis (Rago et al., 2018).

Additionally, it cannot be excluded that PELP1, SRC, and ESR2 also take part in maintaining the function of the efferent ductule epithelium, especially as it was reported that some of the expressed genes contain both EREs. The PELP1 function may depend on other proteins' tissue-specific availability, including ESR1, ESR2, and SRC. The expression level of these proteins may be one of the limiting elements of the entire PELP1-mediated signal transduction pathway. As shown in Figure 8, some more proteins, enzymes, and factors influence this signaling network. Our results showed a differentiated expression ratio of analyzed genes, which could result from tissue-specific availability of, e.g., transcription factors. However, since androgen response elements were also identified in this location, it is clear that a hormonal milieu (estrogens vs. androgens) is likely required for maintaining the proper epithelial function. For example, the testosterone metabolite 5α-androstane-3β-17β-diol (3β-diol) was shown not to bind the androgen receptor but bind ESR2 with higher affinity than it does for ESR1. Therefore it could mediate ESR2 activity in the testis and maintain epithelial function (Hess et al., 2021). We speculate that SRC and PELP1 could be somehow involved in this phenomenon.

In the epididymis, we noted very strong positive correlation ratios of ESR1/ESR2 with ESR1/SRC and ESR1/SRC with ESR1/PELP1. These observations could suggest SRC kinase and PELP1's more intense involvement in ESR1 function in the epididymis. Immunohistochemical staining in our study indicates strong PELP1 expression in the nuclei of the principal cells of the efferent ductules epithelium. Other studies have shown that acute morphological changes, such as epithelial degeneration, may occur due to disrupting ESR1 function in the efferent ductules. As SRC and PELP1 seem to play an important role in modulating epididymal epithelial function, it cannot be excluded that impaired SRC-PELP1 interaction caused by, for example antiestrogens, may lead to a blockage of ESR activity in the efferent ductules, and consequently to the inhibition of fluid reabsorption (Hess et al., 2021). PELP1 is a scaffolding protein that enhances transcriptional activity by assembling crucial protein partners. We localized its presence in the principal cells of proximal epididymis. Thus, its function may depend on the availability of co-occurring factors in the local environment. That being said, the activity of PELP1 and other estrogen pathway-related proteins should be considered holistically (Rago et al., 2018). It is worth pointing out that previous literature focuses on the exploration of ESR1 expression patterns in the epididymis.

On the other hand, difficulties in establishing ESR2 expression are most likely caused by methodological obstacles concerning the ESR2 distribution in the male reproductive tract, and a literature review examining the expression of both ESRs in the epididymis has brought contradictory results (Cunha et al., 2021; Davis & Pearl, 2019; Aprea et al., 2021; Hess, 2014; Hess et al., 2021). Most studies in mammals report that ESR1 distribution is species-dependent, while ESR2 expression can be found the entire length of the efferent ductules (Hess et al., 2021; Hess & Cooke, 2018). Animal models have shown that ESR1 may play a prominent role in the specific cell types of the epididymis, likely during fetal development. However, the epididymal epithelium is reported to have a much lower expression of ESR1, which would likely have more significant direct estrogen and dual estrogen/androgen regulation in efferent ductules (Joseph et al., 2011). Several studies suggest that estrogens play a very important role in maintaining fluid resorption by the epithelium of the efferent ductules, mostly via ESR1 (Joseph et al., 2011; Hess et al., 2011; Hess, 2014). However, recent studies have not confirmed the presence of ESR1 in the human epididymis, stating that it is ESR2 that is more prominent in this tissue (Rago et al., 2018). It appears doubtful, considering several studies that have confirmed the presence of ESR1 in this tissue. There are also papers indicating that the loss of ESR2 in knock-out mice demonstrates no major effects on the development of testes, efferent ductules, and epididymis. Interestingly, important long-term effects were seen in the prostate and an increase in neonatal gonocytes, but not in the adult testis (Cunha et al., 2021; Davis & Pearl, 2019; Hess, 2014; Aprea et al., 2021; Hess et al., 2021).

Despite the contradictory findings regarding the expression of *ESRs* in the testis and epididymis, and considering our results, we suggest that an ESR-SRC-PELP1 relationship may be anywise important in controlling some functions of these tissues. However, our study proved the abundance of these proteins only and not their interactions. It seems that not only the level of gene expression and post-translational events influencing protein expression in a specific tissue compartment are important, but also the locally determined interaction between certain proteins and their expression ratios.

Finally, we acknowledge that our study also had some limitations. Firstly, our investigation was limited by the sample size. However, we did not find comparable data in the literature investigating the expression of modulators involved in the ESR-mediated pathways in the tissues of the male reproductive tract. This study was limited by sample size, so conclusions should be interpreted with caution. It is important to establish the proteins' presence, localization, and interplay with other proteins involved in estrogen-mediated signaling in a larger group. However, since these proteins have many different partners and functions, further analyses exploring estrogen-mediated signaling and/or estrogen signaling stimulation/inhibition analyses supported by colocalization studies are essential to determine the nature of these interactions and the potential clinical repercussions of estrogens in the male reproductive tract in the context of impaired male fertility.

Declarations

Supplementary Materials (at https://ojs.ptbioch.edu.pl/index.php/abp). Figure S1: Immunohistochemical localization of ESR1 and ESR2 in the testis and in the epididymis, Full resolution blots and MIQE checklist and data set.

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