Mouse *Pxt1* expression is regulated by Mir6996 miRNA

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ABSTRACT

Mouse *Pxt1* gene is expressed exclusively in male germ cells and encodes for a small, cell death inducing protein. However, upon PXT1 interaction with BAG6, cell death is prevented. In transiently transfected cell lines the PXT1 expression triggered massive cell death, thus we ask the question whether the interaction of PXT1 and BAG6 is the only mechanism preventing normal, developing male germ cells from being killed by PXT1. The *Pxt1* gene contains a long 3′UTR thus we have hypothesized that *Pxt1* can be regulated by miRNA. We have applied *Pxt1* knockout and used *Pxt1* transgenic mice that overexpressed this gene to shed more light on *Pxt1* regulation. Using the ELISA assay we have demonstrated that PXT1 protein is expressed in adult mouse testis, though at low abundance. The application of dual-Glo luciferase assay and the 3′UTR cloned into p-MIR-Glo plasmid showed that *Pxt1* is regulated by miRNA. Combining the use of mirDB and the site-directed mutagenesis further demonstrated that *Pxt1* translation is suppressed by Mir6996-3p. Considering previous reports and our current results we propose a model for *Pxt1* regulation in the mouse male germ cells.

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1. Introduction

Programmed cell death (apoptosis) is an active, highly regulated biological process that enables the maintenance of tissue homeostasis by elimination of aged, overproduced or dysfunctional cells. Apoptotic loss of germ cells during testicular development is very common in both normal and pathological conditions [1] and it is critical to maintain the proper germ cells-Sertoli cells ratio and to prevent generation of defected sperm [2,3]. Despite increasing number of reports regarding the pro-apoptotic factors acting during spermatogenesis the mechanisms underlying this important event in the male gonad remains poorly understood.

The *peroxisomal testis specific 1* (*Pxt1*, Gene ID: 69 307) is a gene expressed in a testis-specific manner and encodes for a small protein consisting of only 51 amino acids. Our previous studies showed that the expression of *Pxt1* is developmentally regulated during spermatogenesis and restricted to male germ cells in the mouse testis [4]. The protein encoded by this gene contains a domain similar to the BH3 domain at the N-terminus of the PXT1.

The ability of this domain to induce cell death has been demonstrated in transiently transfected cells [5]. In addition, massive degeneration of the male germ cells leading to permanent infertility of adult males was observed in a transgenic mouse model overexpressing *Pxt1* [5]. On the other hand, targeted knockout of *Pxt1* resulted in the increased number of sperm with high DNA strand breaks (increased DFI), thus it is suggested that the function of *Pxt1* is to eliminate male germ cells with damaged chromatin [6].

PXT1 protein interacts with the well characterized anti-apoptotic protein BAG6 and this interaction prevents cell death when the cells are co-transfected with both *Pxt1* and *Bag6* expression constructs [5]. Therefore, one control mechanism of the PXT1 activity is the interaction between PXT1 and BAG6. We have addressed the question, whether *Pxt1* transcription and translation might be regulated by some additional mechanism(s). Mouse *Pxt1* is located at the chromosome 17 and a long 3′UTR region was described within its transcript [4]. Genes containing long 3′UTRs are common targets of miRNAs [reviewed in 7]. Growing numbers of reports emphasize the role of miRNAs in the control of spermatogenesis [reviewed in 8–10]. The involvement of miRNA in the mitotic, meiotic and postmeiotic phases of the male germ cells development was demonstrated by inhibiting the activity of target genes [11–14]. Here we demonstrate that the mouse *Pxt1* transcript

is a target for Mir6996 which results in reduced translation of PXT1 protein.

2. Material and methods

2.1. Animals

Mice were maintained at the Laboratory of Genetics and Evolution, Institute of Zoology and Biomedical Research, the Jagiellonian University in Krakow. Mice were kept under a 12 h light—dark cycle with free access to water and standard laboratory diet. Three mice lines were applied in this study. Namely, *Pxt1* knockout line (designated in this work as Pxt1-KO) was generated and characterized as described in Ref. [6]; we also used transgenic line with the overexpression of *Pxt1* gene (designated as Pxt1-TR and described in Ref. [5]) and a control mice from ICR strain (designated as WT). According to the local regulations the post-mortem tissue collection does not require the approval of the Local Ethical Committee. The generation and the use of genetically modified organisms (GMO), namely Pxt1-KO and Pxt1-TR lines, was approved by the Polish Ministry of Climate and Environment under the license number 21/2018.

2.2. Mouse genotyping, testes collection, cell culture, RNA and protein isolation

DNA samples for PCR genotyping were isolated from the mouse tail biopsies using standard phenol/chloroform isolation technique. Animals from the heterozygous breeding of Pxt1-KO mice were genotyped by PCR with the following primers: Pxt1FP (5′-GAA-GAACGGGAGGAACAGAA-3′), Pgk-nRP (5′-AGGAGCAAGGTGA-GATGACAG-3′) and Pxt1RP (5′-CAGACAGCGGTTTACAACCAT-3′). The product size was of 464 base pairs (bp) for the wild-type allele and of 697 bp for the mutant allele. Homozygous knockout males at the age of 3—4 months were applied in this work. Transgenic mice from the Pxt1-TR line were obtained from the heterozygous females mated with the wild type males and identified by PCR using the gen_hPGK2_F1 (5′-CCAGGAAGTTGGAATCTTCACC-3′) and the gen_Pxt1ex.2—3_R (5′-GTGCAAGATGCTCCTGAATCAC-3′) primers. Samples demonstrating the expected PCR product with the size of 674 bp represented transgenic animals. To verify the quality of genomic DNA the PCR was used with *Tnp2* specific primers: TP2_F1 (5′-AACCAGTGCAATCAGTGCACC-3′) and TP2_R1 (5′-ATGGACA-CAGGAACATCCTGG-3′) that amplified a 450 bp long product. Transgenic males at the age of 3—4 months were used in this work. Testes of three adult animals from each mouse line: ICR, Pxt1-KO and Pxt1-TR were collected post-mortem, frozen in liquid nitrogen and stored at −80°C until further protein analyses. For RNA analyses five males from each line were used. RNA was isolated from 30 mg of testes with NuceloSpin miRNA mini kit (Macherey-Nagel) according to the manufacturer's instructions. This procedure allows to isolate simultaneously total RNA and small RNA fractions. For isolation of RNA from MC3T3 cells, an immortalized mouse osteoblast precursor cell line [15], cells were cultured in 10 cm^2 flasks with 5 ml MEM (BioShop) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Scientific), 2 mM Glutamine and penicillin-streptomycin antibiotics mixture (100 units/ml penicillin, 0,1 mg/ml streptomycin, Thermo Scietific) at 37 °C and 5% CO_2. Cells were collected upon the confluence of approximately 90% and RNA was isolated with NucleoSpin miRNA mini kit (Macherey-Nagel) according to the manufacturer's instructions. RNA concentration and purity was measured with NanoDrop 2000 spectrophotometer (Thermo Scientific). For protein isolation, approximately 50—100 mg of tissue was placed in 500 μl of RIPA lysis buffer with Halt™ Proteases Inhibitor Cocktail 100x (Thermo Scientific) at the concentration of 10 μl/ml, followed by homogenization in Bio-Sprout-Vortex V1 homogenizer (Biosan). Each sample was then sonicated for 30 s (3 × 10 s impulses) and centrifuged at 16 000 rpm for 15 min at 4 °C. Then, protein concentration was measured with Micro BCA™ Protein Assay Kit (Thermo Scientific) following the manufacturer's instruction.

2.3. Reverse transcription, RT-PCR and RT-qPCR

To synthesize cDNA, 1 μg of total RNA was oligo-T primed and the High Capacity Reverse Transcriptase Kit (Applied Biosystems) was used according to the manufacturer's instructions. For cDNA synthesis from miRNA, MiScript reverse transcriptase kit (Qiagen) and 10 μl of isolated miRNA fraction was used. The miRNA was primed with oligo-T primer with an 5′ adapter that served as a template for further PCR analyses. The cDNA synthesis was performed according to manufacturer's instruction. To test primer specificity cDNA generated without the use of reverse transcription (designed as −RT) was used as a template in PCR amplification.

The expression of Mir6996 was analyzed using specific forward primer Mir6996FP (5′-AGGACAGAGCACAGTCAGCA-3′) and the reverse primer complementary to the oligo-T adaptor (5′-CCAGT-TACGCATGCCGAGGT-3′). Kapa Taq PCR Kit was applied for the above (Kapa Biosystems). The quality of the cDNA synthesized from miRNA was verified with the U6 specific primers (U6FP 5′-TGGCCCCTGCGCAAGGATG-3′, and oligo-T adaptor RP).

For the quantification of *Pxt1* expression the RT-qPCR analysis was used. First, a standard curve was generated using a series of diluted cDNA samples to determine amplification efficiency of *Pxt1* specific primers (Pxt1_ex2_FP 5′-CAGCTTAGACACATTGGGGACA-3′ and Pxt1_ex3_RP 5′-ACCTGGCCTCTCACGAACAC-3′) and the primers specific to several selected housekeeping genes. Next, from all tested primers specific for the housekeeping genes, the primers amplifying the *Sdha* (SdhaFP: 5′-GCTTGCGAGCTGCATTTGG-3′ and SdhaRP: 5′-CATCTCCAGTTGTCCTCTTCCA-3′) were chosen as a control. To determine the relative expression levels, the deltadelta cT method was used. RT-qPCR was performed with the SybrGreen quantitative PCR kit (Applied Biosystems) using a Step One thermocycler (Applied Biosystems).

2.4. Cloning of Pxt1 3′UTR sequence into pMIR-Glo plasmid

In order to amplify the 3′UTR of *Pxt1* gene, DNA from the WT ICR mouse was amplified in PCR with primers containing restriction sites at the 5′ ends (Pxt1_3UTRPmeIFP − 5′GGGTTTAAACAGGA AGGACAGACAGACA3′; Pxt1_3UTRNheIRP − 5′ATATGCTAGCAG GGTGAGGGATGGGAT3′). PCR reaction was performed using PCR Mix Kit (A&A Biotechnology). PCR products were cut out from 1% agarose gels and DNA was cleaned using Gel-Out Concentrator (A&A Biotechnology). Purified DNA fragments were ligated into pGEM-T Easy Vector (Promega) plasmid using the kit reagents and the manufacturer protocol and then transfected into JM109 *E.coli* cells. Bacteria were distributed on agar plates containing ampicillin, IPTG and X-Gal, and they were cultured overnight at 37 °C. The white colonies were inoculated into 3 ml of LB medium containing ampicillin. After the overnight incubation at 37 °C, the plasmids were isolated using Plasmid Mini Kit (A&A Biotechnology) and they were sequenced using the Big Dye Terminator 3.1 kit (Applied Biosystems) and the primers T7: 5′TAATACGACTCACTATAGGG3′ and SP6: 5′ATTTAGGTGACACTATAG3′. Terminators were removed using the ExTerminator kit (A&A Biotechnology) and the sequencing readouts were performed in Genomed SA, Warsaw, Poland. Sequences were confirmed by the analysis in Chromas program.

Plasmids with proper sequences were digested with enzymes

NheI and PmeI (ThermoFisher), then separated in 1% agarose gel. The 419 bp fragment of *Pxt1* 3′UTR was next cut out, isolated using the Gel-Out Concentrator (A&A Biotechnology) and ligated into the NheI, PmeI digested pMIR-Glo Dual Luciferase Vector (Promega cat. No E1330) using T4 ligase. The obtained constructs were transfected into JM109 *E.coli* cells and cultured overnight on plates with agar and ampicillin at 37 °C. Next day, colonies were inoculated to 3 ml of liquid LB medium with ampicillin and incubated overnight at 37 °C. Afterwards, the pMIR-Glo plasmids with *Pxt1* 3′UTR were isolated using Plasmid Mini Kit (A&A biotechnology) and subjected to the restriction analysis with NheI, PmeI enzymes to confirm cloning correctness. Inserts were sequenced using the Big Dye Terminator 3.1 kit and the primers SV40pro-F: 5′TATTTATG CAGAGGCCGAGG3′ and RenSeqR: 5′TCATTTCGAACCCCAGAGTC3′ to exclude any mutation as described above.

2.5. Luciferase assay

To obtain the pMIR-GLO-Pxt1-3′UTR constructs without bacterial endotoxin, 100 ml of LB medium with ampicillin was inoculated with conformed bacteria containing the constructs. After overnight incubation at 37 °C, pMIR-GLO-Pxt1-3′UTR plasmids were isolated using the Plasmid Midi AX kit (A&A Biotechnology). MC3T3 cells were seeded onto 96 well TC White plates (Greiner) at a density of 1000 cells/well in 100 μl of MEM alpha (Gibco) with 10% FBS and 1% P/S. Next day, the pMIR-GLO-Pxt1 plasmids were transfected into the cells using the Lipofectamine 3000 kit (Invitrogen) according to manufacturer's protocol and they were incubated at 37 °C and 5% CO_2 for 72h. Thereafter, 100 μl aliquots of Dual-Glo Luciferase Assay Reagent (from Dual-Glo Luciferase Assay System, Promega) were added to cells and the cells were incubated in RT for 10 min. Subsequently, the luminescence of firefly luciferase was measured in SpectraMax L Luminescence Microplate Reader (Molecular Devices). Next, 100 μl aliquots of Dual-Glo® Stop & Glo Reagent (Promega) were added and samples were incubated likewise. The luminescence of renilla luciferase was measured and analyzed according to the manufacturer's protocol. Firefly luciferase activity was dependent on the cloned 3′UTR interactions with the miRNA while renilla luciferase activity was the internal control of transfection efficiency. Results were given as a ratio of the firefly to renilla luciferase activity.

2.6. mirVana miR6996-3p inhibitor analysis

mirVana miRNA inhibitors are single-stranded oligonucleotides designed to specifically bind to and inhibit endogenous miRNAs. To test whether Pxt1 3′UTR is targeted by Mir6996-3p the assay mmu-miR-6996-3p (ThermoFisher Scientific, kat nr 4464084) was used accordingly to manufacturer's instruction. MC3T3 cells were transfected as described in paragraph 2.5 with pMIR-GLO-Pxt1-3′UTR plasmids and 30 ng of Mir6996-3p inhibitor. After incubation at 37 °C and 5% CO_2 for 72h luciferase activity was measured as described in paragraph 2.5.

2.7. Site-directed mutagenesis

Mutagenesis was performer using the Q5 Site Directed Mutagenesis (New England Biotechnology) and the primers Mir6996_mut_F: 5′CAACCCACCTGTTCCTGGTCTATTT3′ and Mir6996_mut_R: 5′TGAGAATGGAAGGGAAGGTGGC3′ according to the manufacturer's instruction. After mutagenesis, plasmids were transfected into DH5-alpha competent *E.coli* and cultured overnight at 37 °C on agar with ampicillin. Next day, colonies were inoculated to the liquid LB medium with ampicillin and incubated overnight at 37 °C. Then, plasmids were isolated using Plasmid Mini Kit (A&A

Biotechnology) and sequenced as described in paragraph 2.4. Clones with the confirmed mutation were used for the endotoxin free plasmid isolation as described in paragraph 2.5.

2.8. ELISA

The content of the mouse PXT1 protein in testis was measured by the Mouse Peroxisomal testis-specific protein 1 (PXT1) ELISA Kit (Abbkine). For this analysis, testicular protein extracts (as described in paragraph 2.2) of the WT, Pxt1-TR and Pxt1-KO mice were used. The latest served as a control for the ELISA test specificity and an additional calibrator for the test sensitivity. The assays were carried out as indicated by the manufacturer. In each assay a series dilution of PXT1 peptide (provided in ELISA kit) was used to estimate protein concentration.

2.9. Statistical analyses

All data were initially tested for normality using the Shapiro-Wilk test. Data of luciferase activity assays were not normally distributed and could not be normalized by any known transformation, therefore the nonparametric alternative for the *t*-test, the U-Mann-Whitney test or the nonparametric alternative for ANOVA, the Kruskal-Wallis test were used for these analyses. Data of the RT-qPCR and ELISA were not significantly different from normal distribution and thus they were analyzed using the *t*-test. A p-value below 0.05 was considered significant. All statistical analyses were performed using the Statistica v.13 software package (TIBICO Software Inc.).

3. Results

3.1. Detection of the PXT1 protein in mouse testis

Although the *Pxt1* transcript expression was well studied during mouse spermatogenesis [4] the demonstration of the PXT1 protein has not been reported to date. The ELISA test was used to detect PXT1 protein in testicular extracts. To monitor test specificity two controls were used, namely the protein extracts from 1) the testes of knockout mice with confirmed lack of transcription of *Pxt1* gene [6], genotyped by PCR (Fig. 1A) and 2) the transgenic mouse line overexpressing *Pxt1* (Pxt1-TR, [5]), genotyped by PCR (Fig. 1B). Ahead of protein analyses we analyzed the *Pxt1* transcript level in Pxt1-TR line, as this had not been determined before. RNA was isolated from the adult transgenic and control males, reverse transcribed and quantitative PCR analysis with primers Pxt1_ex2_FP and Pxt1_ex3_RP was performed. In the testis of transgenic mice the 2.5 fold increase of *Pxt1* expression was detected compared to the control mice (*t*-test, p < 0.05, Fig. 1C). Then, protein extracts from the testes of adult Pxt1-KO, WT and Pxt1-TR mice were isolated and the PXT1-ELISA kit was used to measure protein levels. To assure specificity of the assay, the protein extracts from Pxt1-KO mice were used for additional background correction. In the testis of WT mice, the PXT1 protein content was determined at 0.05 ± 0.03 pg per 1 mg of tissue that had been used initially for protein extraction while in the testes of the Pxt1-TR mice the PXT1 protein content was determined at 0.56 ± 0.15 pg/mg of tissue (*t*-test, p < 0.005, Fig. 1D). We thus demonstrated for the first time that PXT1 protein is produced in mouse testis but in the WT animals the protein content is 10 fold lower as compared with transgenic mice. As the transcript level was only 2.5 fold lower in the WT we have concluded that *Pxt1* translation might be suppressed.
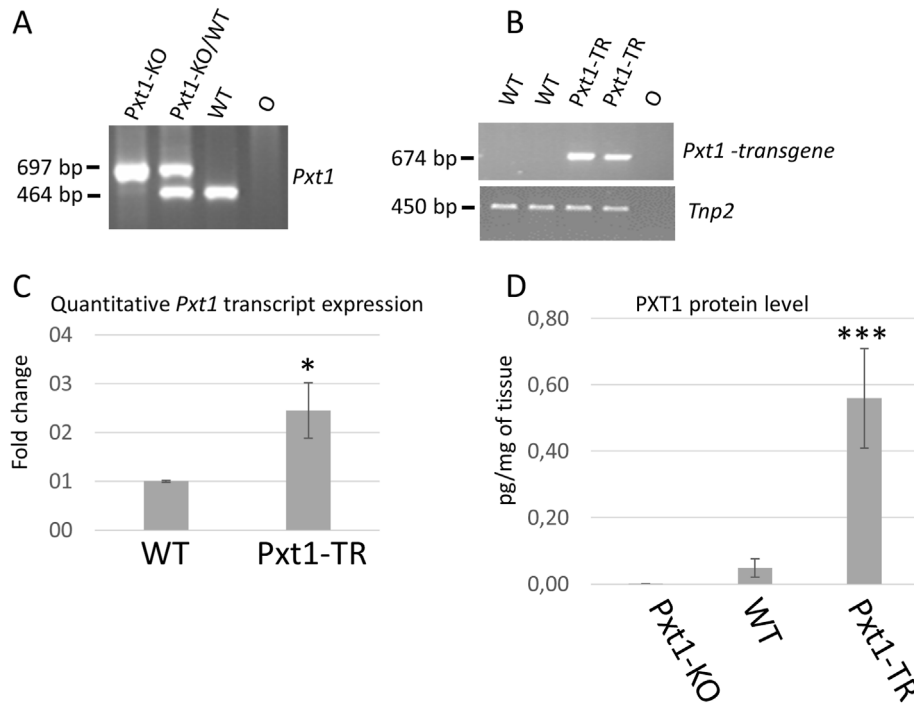
Fig. 1. The expression of *Pxt1* transcript and protein. (A) Representative result of genotyping of mice from Pxt1-KO line by PCR. The band 697 bp in size represents knockout allel and the band 464 bp in size represents wild type (WT) allel. (B) Representative result of Pxt1-TR line genotyping. The band 674 bp in size represents transgenic allel. *Tnp2* specific primers amplifying a PCR product of 450 bp were used to verify DNA quality. O − negative control (C) Quantitative RT-qPCR analysis of the *Pxt1* transcript expression in testes of WT and Pxt1-TR mice (n = 5). (D) PXT1 specific ELISA test of testicular proteins from WT, Pxt1-KO and Pxt1-TR animals (n = 3).

3.2. Pxt1 expression is regulated by miRNA

From the 1015 nucleotides building the *Pxt1* transcript, the 3′UTR is 718 nucleotides long. Genes with long 3′UTR are prone to regulation by miRNAs. To analyze whether the *Pxt1* is regulated by miRNA, the luciferase assay was applied. The 3′UTR of this gene was amplified in PCR, cloned into the pMIR-Glo dual luciferase vector (Fig. 2A), verified by restriction analyses for the presence of insert (Fig. 2B) and sequenced. Positive clones were used in the luciferase assay to analyze the 3′UTR of *Pxt1* gene. Next, the generated pMIR-

Glo-Pxt1-3′UTR construct was transfected into the cells to measure the luciferase activity. Giving the deficiency of cell lines reflecting the spermatogenesis progress we sought for appropriate somatic cell line. It has been found that in the osteoblastic mouse MC3T3 cells that had been transfected with the pMIR-Glo-Pxt1-3′UTR construct, the luciferase activity was significantly reduced as compared to the MC3T3 cells transfected with the pMIR-Glo control vector (U-Mann-Whitney test, p < 0.001, Fig. 2C). The reduced luminescence, as measured in the luciferase assay, indicated that in this cell line the 3′UTR of the *Pxt1* gene is targeted by the miRNA
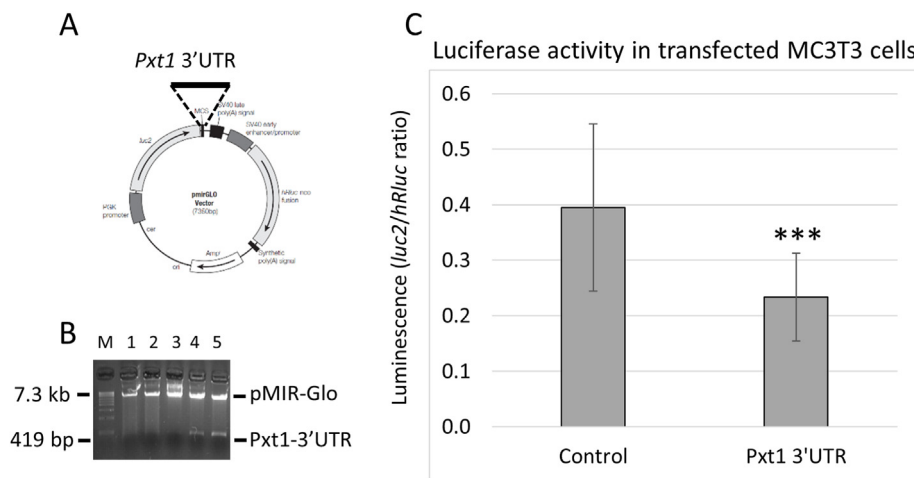

Fig. 2. Generation of p-MIR-GLO-Pxt1-3′UTR construct and luciferase assay. (A) Pxt1 3′UTR was cloned into the p-MIR-Glo dual luciferase plasmid downstream the firefly luciferase ORF. (B) Restriction analysis of the cloning correctness. Clones 4 and 5 contain the expected 419 bp insert. (C) Luciferase activity measured in transiently transfected MC3T3 cells. Significant downregulation of luciferase activity was observed in cells transfected with p-MIR-GLO-Pxt1-3′UTR construct (Pxt1 3′UTR) as compared with MC3T3 cells transfected with p-MIR-Glo plasmid without the *Pxt1* 3′UTR (control).

and the translation of firefly luciferase is reduced.

3.3. Identification of miRNA regulating the expression of Pxt1 gene

The sequence of the 3′UTR of the mouse *Pxt1* gene was analyzed by the miRDB on-line tool [16] (www.mirdb.org) and the 32 miR-NAs were predicted to target this gene with the target score above 50. The strategy used in this work allowed us to narrow down the number of candidate miRNAs. It has been assumed that a candidate miRNA must be expressed in MC3T3 cells (since in this cell line the *Pxt1* 3′UTR was targeted by miRNA) and in the testis, because *Pxt1* is exclusively expressed in testis. Consequently, RT-PCR analysis was performed and the Mir6996-3p (accession number: MIMAT0027895 in the mirbase.org database) was found to be expressed in both MC3T3 cells and testis (Fig. 3A). To analyze whether this miRNA indeed targets the 3′UTR of the *Pxt1* gene, the predicted Mir6996-3p seed sequence AGACACCA (the positions 577—585 in the *Pxt1* sequence with NCBI accession number NM_153390) was mutated into CTCACAAC sequence by the use of site-directed mutagenesis protocol and the Mir6996_mut_F and Mir6996_mut_R primers. The obtained clones were verified by sequencing (Fig. 3B) and the construct containing the confirmed mutation of the Mir6996-3p seed sequence was used for the transfection of MC3T3 cells. Native p-MIR-GLO-Pxt1-3′UTR construct and the p-MIR-Glo plasmid without *Pxt1* 3′UTR were used as controls and the luciferase activity was measured in transfected cells. As demonstrated in Fig. 3C, the mutation of the predicted Mir6996-3p binding site restored the luciferase activity to the level detected in cells transfected with p-MIR-Glo plasmid without the *Pxt1* 3′UTR. The luciferase activity in cells transfected with the native p-MIR-GLO-Pxt1-3′UTR construct was significantly lower as compared with the cells transfected with mutated construct and with the control p-MIR-Glo plasmid (Kruskal-Wallis test, $p < 0.005$). In contrast, there was no significant difference between the luciferase activity in cells transfected with the mutated construct and the control p-MIR-Glo plasmid (Kruskal-Wallis test, $p = 0.99$). To further analyze the Mir6996-3p interaction with the *Pxt1* 3′UTR, MC3T3 cells were co-transfecetd with p-MIR-Glo-Pxt1-3′UTR construct and Mir6996-3p inhibitor. The luciferase activity was significantly increased in cells co-transfected with Mir6996-3p inhibitor together with p-MIR-Glo-Pxt1-3′UTR construct as compared with cells transfected with p-MIR-Glo-Pxt1-3′UTR construct only (Fig. 3C, Kruskal-Wallis test, $p < 0.01$). It is worth to note that Mir6996-3p inhibitor restored the luciferase activity to the level observed in cells transfected with p-MIR-Glo plasmid and with construct containing the mutation of Mir6996-3p seed sequence (Fig. 3C, Kruskal-Wallis test, $p = 0.2$ and $p = 0.55$, respectively). The miRNA inhibitors are highly specific single stranded oligonucleotides that bind to target miRNAs preventing them to interact with target mRNAs. Thus, our results verified that the Mir6996-3p targets the 3′UTR of the mouse *Pxt1* transcript and that this interaction can downregulate *Pxt1* translation.
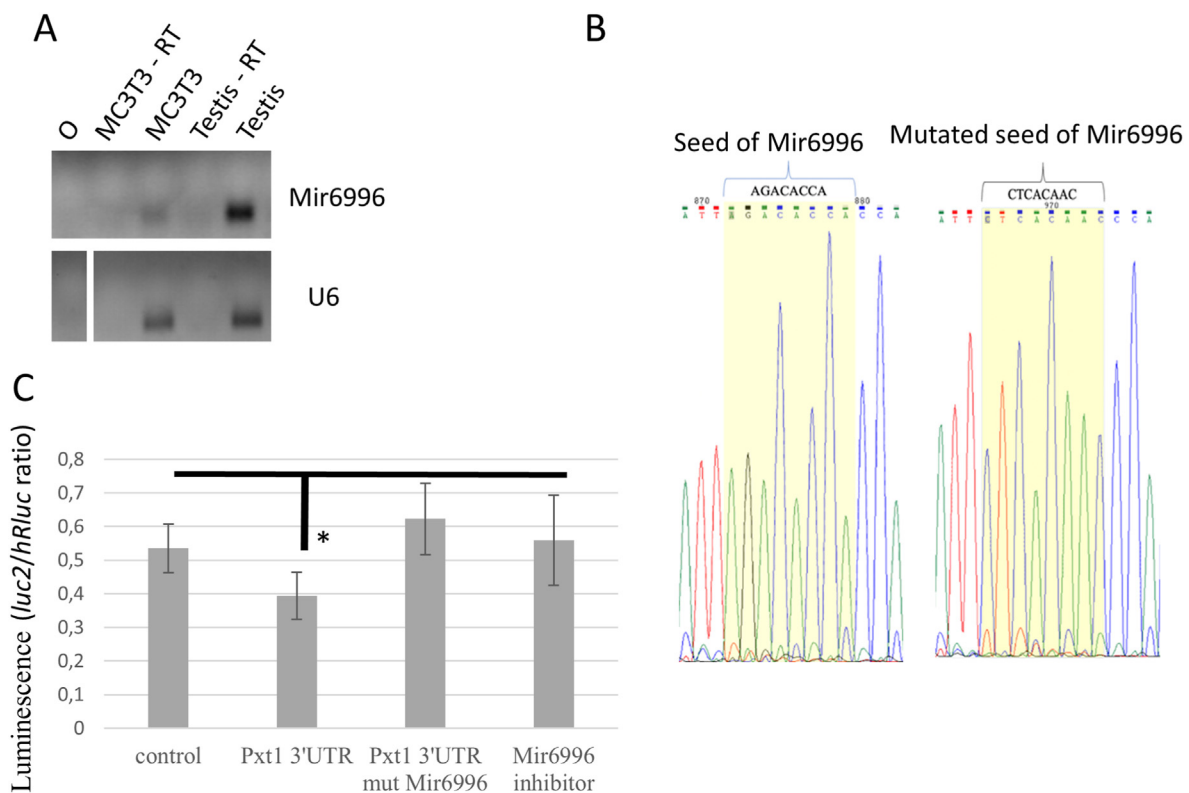
Fig. 3. The analysis of *Pxt1* regulation by Mir6996. (A). Expression analysis of the Mir6996 by the use of RT-PCR. PCR products were observed in both, RNA extracts from MC3T3 cells and from testis of adult WT mice (upper panel). To verify starters specificity control reaction with cDNA synthesized without the use of reverse transcriptase was applied (-RT). To verify cDNA quality U6 specific primers were used in PCR reaction (lower panel). O − negative control. (B) Site-directed mutagenesis was use to introduce mutations into Mir6996 seed sequence in the *Pxt1* 3′UTR cloned in p-Mir-Glo plasmid. Sequencing of the plasmid after mutagenesis confirmed introducing of the mutation. (C) Luciferase activity was measured in MC3T3 cells transfected with: p-Mir-Glo plasmid (control), p-Mir-Glo plasmid containing Pxt1 3′UTR fragment (Pxt1 3′UTR), p-Mir-Glo plasmid containing *Pxt1* 3′UTR with the mutation of Mir6996 binding sequence (Pxt1 3′UTR mut Mir6996) and co-transfected with p-Mir-Glo plasmid containing *Pxt1* 3′UTR and Mir6996-3p inhibitor (Mir6996 inhibitor). Luciferase activity in Pxt1 3′UTR samples differed significantly from control, Pxt1 3′UTR mut Mir6996 and Mir6996 inhibitor samples. No significant difference was noticed between control, Pxt1 3′UTR and Mir6996 inhibitor samples.

4. Discussion

The enhanced expression of *Pxt1* leads to cell death in both transiently transfected cells and the male germ cells of transgenic mouse line overexpressing this gene [5]. On the other hand, the activity of *Pxt1* has been suggested to be required to eliminate aberrant testicular germ cells with DNA strand breaks [6]. To shed more light on the mechanism regulating the *Pxt1* mediated cell death, we have asked whether this gene is controlled by miRNA. We demonstrated that the PXT1 is indeed expressed in mouse testis but the protein abundance is much lower as compared with the testis of transgenic mice, the latest characterized by a degeneration of germ cells and infertility [5]. We have also found that the Mir6996 suppresses *Pxt1* translation and this effect is completely abolished when the Mir6996 binding site is mutated at the *Pxt1* 3′UTR or the Mir6996 inhibitor is applied.

The expression of *Pxt1* gene is well studied at the level of its transcription. Its expression is limited to the mouse testis [4,5]. Within the testis the expression is restricted to the germ cells as in the testis of W/Wᵛ mutants, that lack any germ cells [17] no expression of *Pxt1* was detected [4]. However, as it comes to the protein encoded by *Pxt1* gene, all data so far come from the fusion protein studies. The PXT1 protein expression was detected by the use of different tags added to this protein and then analyzed either directly (thanks to e.g. GFP) [4] or using tag-specific antibodies (e.g. against E2 or c-Myc) [5]. Here we have utilized ELISA assay with the anti-PXT1 specific antibodies to demonstrate for the first time that this protein is expressed in the mouse testis. Although at the transcription level the *Pxt1* was only approximately 2.5 fold higher in the testis of the PXT1-TR line, the protein content was elevated as much as 10 fold compared to the WT. These results suggest that in the testis of the WT mice the translation of PXT1 is suppressed, whereas in the PXT1-TR, the elevated mRNA expression of *Pxt1* cannot be efficiently suppressed by the physiological level of Mir6996 and thus the translation of this gene is much more effective. Considering that the protein-per-mRNA ratio in eukaryotic cells may differ and it is a result of complex regulatory mechanism [18] and taking into account that the building of polysomes usually enhance translation efficiency [19] the 10-fold increase of PXT1 protein level accompanied by a 2.5-fold *Pxt1* mRNA increase is self-explanatory. However, we cannot exclude that other miRNAs regulate *Pxt1*.

Growing numbers of reports emphasize the role of miRNA in the control of spermatogenesis [reviewed in 8–10]. Micro RNAs together with the PIWI-associated RNAs (piRNAs), both belonging to small non-coding RNAs (sncRNAs), have been reported to regulate the number of genes by the mechanism termed RNA silencing and this process is crucial for proper spermatogenesis [20–22]. In the study of Liu et al. [23] the 559 miRNAs were identified to be distinctively expressed by human spermatogonia, pachytene spermatocytes and round spermatids, thus reflecting the essential roles of these miRNAs in mediating spermatogenesis. It has been demonstrated that miRNA expressions pattern differs between immature and mature testes in human [24], rhesus monkey [25] and pig [26]. In contrast, the microarray analyses showed that the most testicular miRNAs are preferentially expressed in the meiotic germ cells [27,28]. The expression of Mir214 is abundant in pachytene spermatocytes and this miRNA plays a key role in meiosis by targeting heat shock proteins [29]. The expression of the Mir449 cluster is strongly up-regulated upon meiotic initiation. The activity of these miRNAs is regulated by testes-specific transcription factors, CREMt and SOX5 [30]. The Mir34c is highly expressed in isolated pachytene spermatocytes and round spermatids [31,32]. The important spermatogenesis-related genes *Tgif2* and *Notch2* are the direct targets of miR34c [33]. The miR449 family modulates

Notch signaling pathway regulating germ cells differentiation [34]. Notably, the Mir6996 is located in the intron between the exons 22 and 23 of the mouse *Notch1* gene (gene ID:18 128) on the chromosome 2. It was reported that *Notch1* is expressed in the early spermatogonia and it may play a role in the maintenance of the spermatogonial stem cells [35]. More recent report demonstrated the expression of NOTCH1 in neonatal spermatogonia whereas in the adult mouse testis it was expressed in differentiating spermatocytes and spermatids [36]. The latest authors demonstrated also a role of *Notch1* in spermatogenesis. The expression profile of *Notch1* in adult testis corresponded with the expression profile of *Pxt1* and this suggests that the intron 22 of *Notch1* may be the source of Mir6996, allowing the processing and maturation of this miRNA to suppress *Pxt1* translation at the right time. Notch signaling has been also shown to regulate cells proliferation and apoptosis [37], both processes crucial for spermatogenesis. Moreover, another germ cell regulators of apoptosis such as *Bcl2* and *Atf1* are also regulated by miRNA [25,26,38]. The Mir6996 was reported to be upregulated in mouse macrophages during *Leishmania* infection [39]. Here we describe a novel role of this miRNA. The *Pxt1* expression starts with spermatocytes at pachytene stage of spermatogenesis [4] and the suppression of its translation seems to preserve normal spermatogenesis by preventing the PXT1 induced cell death. However, the mechanisms by which the *Pxt1* transcript is unleashed from the Mir6996 suppression in cells with DNA strand breaks remains yet unclear and it requires further studies.

The interaction of PXT1 and the protein encoded by *BCL2-associated athanogene 6* (*Bag6*) gene was previously reported and this interaction protected transfected cells from the PXT1 induced cell death [5]. Earlier studies discovered that the BAG6 (also known as *Bat3* or *Scythe*) is a multifunctional protein contributing to a number of cellular processes including the control of apoptosis in *Xenopus* and *Drosophila* [40–43]. *Bag6* is ubiquitously expressed with the highest expression level observed in the testis, thus it may play a role in the male germ cell differentiation [44,45]. BAG6 contains two C-terminal nuclear localization signals [46] and we previously demonstrated that the PXT1 bound by BAG6 had been relocated from cytoplasm to the nucleus [5]. Recently it has been demonstrated that human PXT1 can induce apoptosis by binding the antiapoptotic protein BCL-XL [47] and activating the apoptotic effector BAK [48]. Here we demonstrated that *Pxt1* mRNA is targeted by Mir6996-3p. Considering our previous reports and the current results we propose a model of the *Pxt1* activity control. As demonstrated in Fig. 4, we propose that in the first line the *Pxt1* transcript is targeted by Mir6996-3p (and possibly other putative miRNAs) that leads to its translation suppression. As this suppression may not be completed based on the ELISA assay applied in this work, thus the protein is detected at a relative low level. The resulting PXT1 protein then interacts with BAG6 and this complex relocates from cytoplasm to the nucleus. Previously published results support this hypothesis giving that the overexpressed PXT1, that cannot be completely bound by physiological level of BAG6, is located in cytoplasm and induces cell death in transiently transfected cells [5]. Moreover, upon *Bag6* gene knockout, the massive degeneration of the male germ cells leading to infertility is observed [47]. The phenotype of *Bag6* knockout mice was very similar to the phenotype of PXT-TR males [5,49]. We have previously demonstrated that *Pxt1* is expressed in testicular germ cells [4,5] but it is not expressed in caput, corpus nor cauda epididymis [6]. We now extend this analysis and verify that mRNA of *Pxt1* gene is not detectable also in sperm isolated from epididymis and vas deference (Supplementary Fig. 1). Therefore we conclude, that *Pxt1* must act in male germ cells in seminiferous tubuli, probably starting from the primary spermatocytes, as *Pxt1* expression is detectable for the first time at this spermatogenesis stage [4].
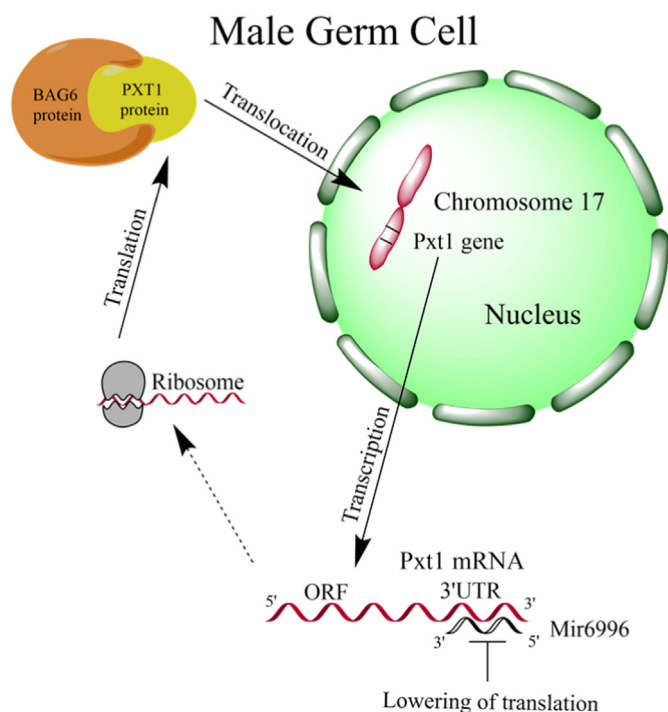
Fig. 4. Model of *Pxt1* activity control in mouse male germ cell.
Based on our previous [4—6] and current results we hypothesized that after transcription *Pxt1* mRNA is targeted by Mir6996 and the translation is strongly lowered. Weakly abundant PXT1 protein interacts with BAG6 and relocates from cytoplasm to the nucleus. We propose that in this way the PXT1-induced cell death is not initiated in developing healthy male germ cell.

In conclusion, we have demonstrated that, the Mir6996 regulates *Pxt1* gene and the former can suppress the latter translation. We suggest that *Pxt1* translation in mouse testis can be down-regulated by Mir6996 to a significant, although not complete extent confirmed by the ELISA detection of PXT1.

Author agreement

Herewith I declare that all authors have seen and approved the final version of the manuscript. The article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

CRediT authorship contribution statement

Igor Tomczyk: conceived and designed the experiments. **Mikołaj Rokicki:** performed the experiments. **Wioleta Sieńko:** performed the experiments. **Katarzyna Rożek:** performed the experiments. **Anna Nalepa:** performed the experiments. **Jasmin Wiench:** performed the experiments. **Paweł Grzmil:** conceived and designed the experiments, performed the experiments, managed the project, wrote the manuscript, performed the experiments.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2023.07.010.

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