Tumor suppressive function of protein tyrosine phosphatase non-receptor type 23 in testicular germ cell tumors is lost upon overexpression of miR142-3p

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Running title: miR-142-3p inhibits PTPN23 expression in TGCTs

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Background: PTPN23 gene is a candidate tumor suppressor involved in the tumorigenesis of various organs.

Results: Expression of PTPN23 in testicular germ cell tumor cells is negatively regulated by miR-142-3p.

Conclusion: A lack of PTPN23 protein expression in human TGCTs inversely correlated with miR-142-3p expression.

Significance: Loss of PTPN23 expression mediated by miR-142-3p may be a key event in the pathogenesis of TGCTs.

SUMMARY
Protein tyrosine phosphatase non-receptor type 23 (PTPN23) is a candidate tumor suppressor involved in the tumorigenesis of various organs. However, its physiological role(s) and detailed expression profile(s) have not yet been elucidated. We investigated the function and regulation of PTPN23 in the formation of testicular germ cell tumors (TGCTs). Expression of PTPN23 in human TGCT cell lines was significantly lower than that in spermatogonial stem cells in mice. Overexpression of PTPN23 in NEC8, a human TGCT cell line, suppressed soft agar colony formation in vitro and tumor formation in nude mice in vivo. These data indicated that PTPN23 functions as a tumor suppressor in TGCTs. Multiple computational algorithms predicted that the 3' untranslated region (UTR) of human PTPN23 is a target for miR-142-3p. A luciferase reporter assay confirmed that miR-142-3p bound directly to the 3' UTR of PTPN23. Introduction of pre-miR-142 in PTPN23 transfectant of NEC8 led to suppressed expression of PTPN23 and increased soft agar colony formation. Quantitative RT-PCR data revealed significantly higher expression of miR-142-3p in human seminomas compared with normal testes. No difference in mRNA expression between seminoma and non-seminoma samples was detected by in situ hybridization. Both qRT-PCR and immunohistochemical analyses revealed that PTPN23 expression was significantly lower in TGCTs than in normal testicular tissues. Finally, a lack of PTPN23 protein expression in human TGCTs correlated with relatively higher miR-142-3p expression. These data suggest that PTPN23 is a tumor suppressor, and repression of PTPN23 expression by miR-142-3p plays an important role in the pathogenesis of TGCTs.

Phosphorylation of tyrosine residues plays a critical role in the regulation of cellular functions and is a central feature of the signaling cascades involved in oncogenesis. The regulation of tyrosine phosphorylation is coordinated by protein tyrosine kinases and phosphatases (PTPs). Perturbation of PTK signaling by overexpression, or by mutations that result in deregulated kinase activity, is often observed in cases of malignant transformation.

On the other hand, PTPs are predicted to
miR-142-3p inhibits PTPN23 expression in TGCTs

have tumor suppressive functions due to their antagonism of kinase activity. We have previously found that Ptpn23 gene is expressed in male germ line stem cells (1). PTPN23 (or HD-PTP) belongs to the non-receptor class subfamily of the PTP family, several members of which have been implicated in tumor suppression (2). For example, loss of PTPN13 in non-small cell lung cancer was shown to be associated with increased signaling through the epidermal growth factor receptor and HER2 tyrosine kinase receptors (3).

PTPN23 encodes a 1636 amino acid protein, the most striking feature of which is the sequence at the PTP active center (VHCSSG), which is distinct from the invariant sequence present in previously identified PTPs (VHCSAG). The gene encodes a BRO1-like protein (which plays a role in endosomal targeting), a histidine-rich domain, a PTP-like domain, and a protein-destabilizing sequence (PEST motif) (4). PTPN23 is highly evolutionarily conserved from yeast to human, and the Ptpn23 homozygous deletion mouse is embryonic lethal at around embryonic day 9.5, suggesting that PTPN23 is essential during the early stages of development (5).

Cao et al. showed that PTP-TD14, the rat homolog of PTPN23, inhibits activated H-ras-mediated transformation of NIH-3T3 cells (6). Later, a hemizygous missense mutation within the histidine-rich domain in the human PTPN23 gene was identified in a small cell lung cancer cell line (4). Several functions of PTPN23 have since been reported, including its role in the regulation of endothelial cell motility by modulating tyrosine phosphorylation of focal adhesion kinase, FAK (7) and its interaction with SRC (8). Furthermore, expression of PTPN23 reduced colony forming capacity of human renal cancer cells; a process independent of catalytic PTPase activity (9). In addition, a functional genomic screening using RNA interference identified PTPN23 as a gene involved in controlling ciliogenesis (10). Functional assays showed that silencing of PTPN23 markedly reduced the number of ciliated cells. Another functional screening using RNA interference showed that PTPN23 acts a negative regulator of SRC in breast cancer to modulate cell motility and invasion (11).

TGCTs are the most common malignancies in adolescent and adult males aged 14 to 40 years. TGCTs are a heterogeneous group of neoplasms classified as seminomas or non-seminomas (embryonal carcinomas, teratomas, chorionicarciomas, and yolk sac tumors). An isochromosome of the short arm of chromosome 12 is the most common and characteristic cytogenetic aberration in TGCTs.

In addition, molecular genetic changes in human TGCTs showed 3p allele loss, suggesting the presence of a tumor suppressor gene within this region (13-15). Notably, the PTPN23 gene is located within this region (chromosome 3p21.3), in an area of the genome frequently lost in breast (16), lung (17), nasopharyngeal (18), cervical (19) and kidney (20) carcinomas. However, deletion of the PTPN23 gene in TGCTs has not yet been reported.

Micro RNAs (miRNAs), a class of small RNA molecules that negatively regulate their mRNA targets in a sequence-specific manner, are frequently dysregulated in human cancers and can act as potent oncogenes and tumor suppressor genes. miRNA overexpression has been observed in various human tumors, and these molecules target important tumor suppressors. For example, miR-21, miR-17-92, miR-221 and 222 target PTEN (21-23), and miR-372 and miR-373 target LATS2 (24).

In this study, we show that colony forming capacity in soft agar and tumorigenicity of a human TGCT cell line are suppressed by overexpression of PTPN23. These data indicated that PTPN23 functions as a tumor suppressor in TGCTs. Furthermore, we found that miR-142-3p bound directly to the 3’ UTR of PTPN23, and the tumor suppressive activity of PTPN23 was decreased by overexpression of miR-142 precursor. In human samples, PTPN23 expression was significantly downregulated and negatively correlated with miR-142-3p expression in TGCTs.

EXPERIMENTAL PROCEDURES

Establishment of a PTPN23-overexpressing TGCT cell line – NEC8 and NEC14 (human embryonal carcinoma-derived TGCT cell lines), and GC-1 (mouse spermantogonia-derived cell line) were purchased from American Type Culture Collection (Manassas, VA, USA). For the constitutive expression of PTPN23, PTPN23 cDNA spanning exon 1 to exon 25 (total of 5107 bp, GenBank Accession number: AB025194),
miR-142-3p inhibits PTPN23 expression in TGCTs

which includes the miR-142-3p binding site, was inserted into the pMYs-ires-EGFP retrovirus vector. The construct was then transfected into a Plat-A cell line to produce recombinant retrovirus. NEC8 cells were infected with retroviral supernatant supplemented with polybrene (8 µg/ml). Infected EGFP positive cells were sorted using a FACS Aria (BD Biosciences, San Jose, CA, USA). Cell lines were cultured in Dulbecco’s modified Eagle’s minimal essential medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio., Kerrville, TX, USA) in 5% CO₂ at 37°C. For cell cycle analysis, cells grown in the 1% FBS-DMEM were incubated with 0.1% NP40-PBS and propidium iodide (2 µg/ml) at room temperature for 15min. DNA content and cell cycle phase were determined on a FACS Calibur (BD Biosciences).

Quantitative real-time PCR — Total RNAs and miRNAs were isolated using miRNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. Reverse transcription (RT) was performed at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min using the TaqMan MicroRNA RT kit (Life Technologies, Carlsbad, CA, USA). Quantitative real-time PCR (qRT-PCR) detection of mature miR-142-3p was performed using the TaqMan microRNA assay and Universal PCR Master Mix (Life Technologies). The reactions were performed at 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min using LightCycler 480 Real-Time PCR system (Roche Applied Science, Indianapolis, IN, USA).

Inhibitor of mir-142-3p (Anti-hsa-miR-142-3p: 5'-UGUAGUGUUUCCUACUUUAUGGA-3') and negative control RNA were purchased from QIAGEN. Anti-hsa-miR-142-3p or control RNA was transfected into NEC8 cells at a final concentration of 50 nM using siPORT NeoFX (Life Technologies) according to the manufacturer’s instructions. Two days after transfection, total and miRNAs were extracted and reverse transcribed into cDNA using the PrimeScript RT mix for RT-PCR (Takara, Otsu, Japan). Real-time PCR was performed with SYBR premix Ex Taq II (Takara) and following primers: human PTENB (85 bp), 5'-CCGACACTGTCAAGGAAACCTT-3' (forward) and 5'-CTGATGTCCTTCAGGGAAGC-3' (reverse), human GAPDH (66 bp), 5'-ATGTTCTCATGGGTGTGAA-3' (forward) and 5'-GGAGGCCATTGCTGATGATCT-3' (reverse), mouse Ptpn23 (192 bp), 5'-CTGACCACTCAGAGATGAG-3' (forward) and 5'-TCTGTAGTGTTGAGTTCAC-3' (reverse), mouse Gapdh (132 bp), 5'-AACTTTGACCTTGGAGAAAG-3' (forward) and 5'-GGATGAGGATGATGTTTCT-3' (reverse).

For the overexpression experiments, pre-miR-142-3p (Life Technologies) was transfected into GC-1 cells at a final concentration of 50 nM using siPORT NeoFX. The qRT-PCR results, recorded as threshold cycle number (Ct), were normalized against an internal control (RNU6B) and expressed as fold changes.

Western blot analysis — Protein was extracted from control/NEC8 and PTEN23/NEC8 cells using RIPA lysis buffer supplemented with a proteinase inhibitor cocktail (Roche Applied Science) and subjected to electrophoresis on a Super Sep 3-10% polyacrylamide gel (Wako, Osaka, Japan) in the presence of SDS under reducing conditions. Proteins transferred onto a Hybond-P PVDF membrane (GE Healthcare, UK) for overnight at 4°C. The blots were blocked by 5% skim milk (Difco, Detroit, MI, USA) and incubated with primary antibody. PTEN23 protein was detected with a polyclonal anti-PTPN23 antibody (1:200 dilution, #10472-1; ProteinTech Group, Chicago, USA) followed by incubation with HRP-conjugated secondary antibody (1:1000; GE Healthcare) for 1 hour at room temperature. As a loading control experiment, the membrane was re-blotted with an anti-α-tubulin antibody (1:500, T 9026; Sigma-Aldrich). Chemiluminescence detection was carried out using ECL Plus Detection reagent (GE Healthcare) and Luminescent Image Analyzer LAS-3000. (Fuji film, Tokyo, Japan).

In vitro colony assay and in vivo tumorigenesis assay in mice — Soft agar colony assay and tumor growth assay in nude mice were performed as previously described (25). Balb/c nu/nu male mice (6 weeks old) were purchased from Nihon SLC, Hamamatsu, Japan. All mice were maintained under a 12 hour light/12 hour dark cycle in a pathogen-free animal facility. All experimental procedures involving mice were pre-approved by the Ethical Committee for
miR-142-3p inhibits PTPN23 expression in TGCTs

Animal Experiments at Tokyo Metropolitan Institute of Medical Science, and were performed according to the guidelines for the Proper Conduct of Animal Experiments (http://www.scj.go.jp/en/animal/index.html).

Search for miRNA target sequences in the 3'UTR of PTPN23 – The candidate miRNA acting on PTPN23 gene was identified based on a conservative intersection of the following miRNA target prediction tools: TargetScan (http://genes.mit.edu/targetscan), miRANDA (http://www.microrna.org/), and PicTar (http://pictar.mdc-berlin.de/).

Luciferase reporter assay – To examine the role of miR-142-3p regulation in PTPN23 expression, a 132 bp fragment of the 3' UTR region of human PTPN23 containing the predicted miR-142-3p site was subcloned into the XbaI restriction site of pGL3 luciferase vector (Promega, Madison, WI, USA). Base pair change in the predicted miRNA seed sequence (ACACTACA to CAGTGCAG) was accomplished by site-directed mutagenesis using appropriate primers. NEC8 cells were transfected with 50 nM of pre-miR-142-3p or the negative control using siPORT NeoFX. After 6 hr, 50 ng of firefly luciferase reporter vector containing wild-type or the mutant PTPN23 3' UTR sequence and 20 ng of the pRL-CMV Renilla luciferase control vector (Promega) were transfected using Lipofectamine LTX (Life Technologies). Luciferase assays were performed 48 hr after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

Construction of a miR-142 precursor expression vector – Hsa-miR-142 precursor (287 bp) was amplified by PCR using following primers: 5'-TTGGGGGGATCTTAGGAAGC-3' and 5'-GGAGGGGGCGTAAGTGCTC-3'. It was then cloned into pEF6 vector (Invitrogen) and transfected into PTPN23/NEC8 cells followed by blasticidin selection.

Immunohistochemistry – Immunohistochemical staining was performed on formalin-fixed paraffin-embedded (FFPE) specimens. After dewaxing in xylene and graded ethanol, sections were incubated in 3% H2O2 solution for 15 minutes to block endogenous peroxidase activity. The sections were incubated with the anti-PTPN23 primary antibody (1:100) at 4°C overnight and processed using the DAB system (Vector, Burlingame, CA, USA). Paraffin sections of control/NEC8-derived solid tumors were evaluated using an anti-GFP (1:200, #A-6455; Invitrogen) in combination with Alexa Fluor 488 conjugated anti-rabbit IgG secondary antibody (#4412; Invitrogen).

In situ hybridization – To investigate the cell-specific distribution of miRNA in normal testes and TGCTs, in situ hybridization was performed using 5'- and 3'-digoxigenin-labeled LNA modified DNA oligonucleotides complementary to the mature miRNA (Exiqon A/S, Vedbaek, Denmark). Expression of miR-142-3p in a testis cancer tissue array (TE231; US Biomax, Rockville, MD) was also examined by in situ hybridization according to the manufacturer’s instructions.

Human patient samples – Following FFPE tissue sections were purchased from US Biomax. HuCAT381 Human Testis cancer (seminoma: age 45), HuFPT151 Normal Human Testis (age 74), and testis cancer tissue array with normal testis control tissues (TE231). Normal human Testis (T2234260: age 26) and Human Testis cancer (T2235260-3: seminoma, age 47) were purchased from BioChain (Newark, CA, USA). Patient specimens (S3: seminoma, age 36, S4: seminoma age 35, S5: seminoma age 43, S6: seminoma age 34) were taken at the time of surgery at Yokohama City University Medical Center (Yokohama, Japan) between 2002 and 2012. We obtained written informed consent from all the patients and the study were approved by the Ethical Committee of the Yokohama City University Medical Center and School of Medicine. Total RNAs and miRNAs from FFPE tissues were extracted using a RecoverAll total nucleic acid isolation kit (Life Technologies).

Statistical analyses – Statistical differences in the levels of mRNA expression, data in the colony assays, and reporter activities in the luciferase assays were determined using the Student’s t-test. A P-value of <0.01 was considered to be significant.

RESULTS

Establishment of a PTPN23-overexpressing TGCT cell line – We previously identified Ptpn23 as one of the spermatogonia specific genes. PTPN23 protein positive cells were found near the basement membrane of the seminiferous tubules in adult mouse testes, where spermatogonial stem cells reside (Fig. 1). As PTPN23 is a candidate tumor suppressor, we investigated the hypothesis that loss of PTPN23 function may play a role in the development of
TGCTs. First, we examined *PTPN23* mRNA expression in two TGCT cell lines and compared it with that in e-Kit+ spermatogonial stem cells isolated from 7-day old mice. Notably, the levels of *PTPN23* expression in both TGCT cell lines were approximately one-forth of the *Pttn23* level in spermatogonial stem cells (Fig. 2A).

Using retroviral infection, we generated an NEC8 transfectant (*PTPN23*/NEC8) overexpressing PTPN23 and green fluorescent protein (GFP). *PTPN23*/NEC8 cells was morphologically indistinguishable from control NEC8 cells (control/NEC8) (Fig. 2B), although *PTPN23* expression in *PTPN23*/NEC8 was higher than control/NEC8 at both mRNA and protein levels (Fig. 2C). The growth rate and maximum density of *PTPN23*/NEC8 cells in 10%FBS-DMEM was identical to that of control/NEC8 cells (Fig. 2D). The doubling time of these cells was calculated to be 24 hr from the exponential growth phase of the growth curve. However, in 1% FBS-DMEM, *PTPN23*/NEC8 grew more slowly than control/NEC8 cells (approximately 20% reduction of the growth rate) (Fig. 2D). Consistent with this data, percentage of cells in G1 phase slightly increased under this experimental condition (Fig. 2E).

*PTPN23* overexpression in TGCT cells suppresses their anchorage-independent growth in vitro and tumorigenicity in vivo – To determine whether *PTPN23* has a tumor suppressive function in TGCT cells, we performed soft agar colony formation assays, and transplantation experiments into athymic *nu/nu* mice. The colony number of *PTPN23*/NEC8 cells in soft agar was only 4% of that of control/NEC8 cells (Fig. 3A). We confirmed surrogate GFP expression in the control/NEC8-derived colonies in soft agar (Fig. 3A, upper panels). These data indicate a inhibitory role of *PTPN23* against anchorage-independent growth of tumor cells.

Next, we examined *in vivo* tumorigenicity by subcutaneously inoculating control/NEC8 or *PTPN23*/NEC8 cells (5 x 10⁶ cells) into nude mice. The control cells formed palpable tumors in all mice within 3 weeks of subcutaneous injection, and the tumors grew to volume of 2,000 mm³ after 6 weeks. However, none of the mice injected with *PTPN23*/NEC8 cells developed tumors (Fig. 3B), even at 3 months after inoculation (data not shown). Taken together, these data suggest that *PTPN23* is a key negative regulator for the transformed phenotypes of TGCTs. Immunohistochemical analysis of tumor xenografts excised from the mice injected with control/NEC8 cells revealed the GFP expression (Fig. 3B, upper panels), proving the donor origin of the tumors.

*PTPN23* is a direct target of miR-142-3p – miRNAs exert their function by regulating the expression of their downstream target gene(s). Multiple computational algorithms using TargetScan, miRanda and PicTar predicted that the 3' UTR of human *PTPN23* gene is a target for miR-142-3p. The 3' UTR of *PTPN23* mRNA contained a complementary nucleotide sequence for the seed region of miR-142-3p (Fig. 4A). This “seed” region of miR-142-3p was highly conserved among species, indicating for its regulatory importance (Fig. 4B).

To experimentally verify above possibility, the miR-142-3p binding sequence from the 3' UTR of *PTPN23* mRNA (WT-3'UTR), or its mutated version (Mut-3'UTR), was cloned downstream of the firefly luciferase reporter gene and then cotransfected into NEC8 cells along with pre-miR-142-3p or pre-miR-negative control. The reporter activity of the luciferase gene fused with WT-3'UTR, but not Mut-3'UTR, was repressed by co-expression of miR-142-3p (Fig. 4C), demonstrating that miR-142-3p targets the 3' UTR of *PTPN23* mRNA. When we compared expression levels of miR142-3p between *PTPN23*/NEC8 and control/NEC8 cells, it was decreased in the former cells (Fig. 4D). This is likely due to complex formation with the 3'UTR of exogenously expressed *PTPN23* mRNA.

Next, we introduced anti-miR-142-3p into NEC8 cells and found that miR-142-3p inhibition resulted in the increase of endogenous *PTPN23* mRNA (Fig. 4E). Moreover, overexpression of miR-142-3p in a mouse spermatogonia-derived cell line GC-1 reduced the expression of *Pttn23* mRNA (Fig. 4F). Thus, we demonstrated inverse correlation between miR-142-3p and *PTPN23/Pttn23* expressions in various male germ cell-derived cell lines.

Restoration of tumorigenicity by re-introduction of miR-142 in *PTPN23*/NEC8 cells – To further confirm that the miR-142-3p mediated reduction of *PTPN23* restores tumorigenicity, pre-miR-142 was stably transfected into *PTPN23*/NEC8 cells (miR-142/PTPN23/NEC8) followed by qRT-PCR and Western blot analysis. The expression of miR-142-3p mRNA was greatly increased in miR-142/PTPN23/NEC8 cells (Fig. 4G).
54). In these cells, PTPN23 mRNA was approximately 40% reduced (Fig. 5B) and PTPN23 protein was mostly abolished (Fig. 5C) when compared to control/PTPN23/NEC8 cells. We then examined anchorage-independent growth of miR-142/PTPN23/NEC8 cells. As expected, miR-142/PTPN23/NEC8 cells gave rise to 4 times more colonies in the soft agar culture than control/PTPN23/NEC8 cells (Fig. 5D).

Negative correlation between PTPN23 and miR-142-3p expressions in TGCTs and normal testes – Finally, we investigated expressions of miR-142-3p and PTPN23 in various histological subgroups of TGCTs and normal testes. Expression levels of miR-142-3p and PTPN23 in TGCTs (S1–S6) and normal testis sample (N1, N2) were validated by qRT-PCR. Expression of miR-142-3p in TGCTs was significantly higher than that in normal testicular sections (Fig. 6A). Remarkably, three of six cases displayed 5 times more expression of miR-142-3p. Thus, at least 50% of TGCTs express very high level of miR-142-3p. By contrast, PTPN23 expression was significantly lower in TGCTs than in normal testes (Fig. 6A). In situ hybridization and immunohistochemical analyses confirmed that miR-142-3p expression levels were inversely correlated with PTPN23 protein levels in both TGCTs and normal testes (Fig. 6, B and C). We did not observe any differences between histological subgroups of TGCTs.

**DISCUSSION**

The PTPN23 gene is localized at human chromosome 3p21, a region known to be a hot spot for deletion in various carcinomas. This implicates the possibility for PTPN23 to function as a tumor suppressor. In this report, we showed that PTPN23 expression was downregulated in human TGCT cell lines and primary tissues isolated from TGTC patients when compared with spermatogonial stem cells and normal testes samples.

We investigated roles of PTPN23 in the TGCT development. Because serum requirement was increased by PTPN23 overexpression in NEC8 cells, we performed anchorage-independent growth assays and in vivo tumorigenesis assays. The results revealed that PTPN23 expression results in the suppression of neoplastic phenotypes. It has been reported that suppression of PTPN23 enhances motility of endothelial cells and bladder carcinoma cells by modulating FAK (7,26). Furthermore, suppression of PTPN23 induced mammary epithelial cell invasion and increased SRC activity (11). Cell migration capacity is critical for invasion and metastasis of cancer cells. In this report, we showed for the first time that PTPN23 strongly suppresses the tumorigenic activity of TGCTs. Although most critical tumor suppressive functions of PTPN23 remains elusive, a higher serum requirement could contribute to the loss of tumorigenic activity in PTPN23/NEC8 cells.

Because tumor suppressor genes are recessive, both copies of a tumor suppressor gene should be inactivated by mutation or deletion to promote the tumor development. A mutational analysis of PTPs identified mis-sense, nonsense and frame-shift mutations in colorectal carcinomas (27). They examined the coding exons of 87 members of the PTP family including PTPN23, and detected 77 mutations in the PTPR, PTPRG, PTPRT, PTPN3, PTPN13 and PTPN14 genes. Korf et al. examined frame-shift mutations in the coding repeats of 16 PTPs in 54 primary colorectal carcinomas and found microsatellite instability. Frame-shift mutations in PTPN21 and PTPRS occurred with high frequency (16% and 12%, respectively). However, only one mutation was detected in a upstream region of the protein tyrosine phosphatase domain of PTPN23 in colorectal carcinoma (28). Mutational analysis of PTPN23 in COSMIC (Catalogue of Somatic Mutations in Cancer, Sanger Institute) identified 30 mutations in 5,277 carcinomas. Mutations were identified in lung (10 samples), large intestine (9 samples), skin (3 samples), breast (3 samples), prostate (2 samples), central nervous system (1 sample), oesophagus (1 sample), and upper aerodigestive tract (1 sample). However, no commonly-mutated hot spot codons were detected. In two TGCT cell lines (NEC8 and NEC14) used in this study, we did not detect any mutations in the coding regions of PTPN23 gene. Therefore, loss of PTPN23 expression in TGCTs may be caused by different mechanisms.

It was recently reported that oncogenic miRNAs suppressed tumor suppressor genes and promote T-cell acute lymphoblastic leukemia (29). We used multiple computational algorithms to search for miRNAs predicted to suppress PTPN23 expression, and identified conserved eight-nucleotides that matched the seed region of miR-142-3p within the 3’ UTR of PTPN23 gene. Reporter assays proved that PTPN23 is a target of miR-142-3p. Moreover,
ectopic expression of miR-142 in PTPN23/NEC8 cells—efficiently repressed PTPN23 expression, thereby colony forming capacity in soft agar being restored. Thus, higher level of miR-142-3p is supportive for TGCTs by way of the PTPN23 downregulation. This is a novel molecular interaction between miRNA and PTPs during tumorigenesis.

We next found significantly higher expression of miR-142-3p in TGCTs compared with normal testes. Differences in miR-142-3p expression between seminoma and non-seminoma samples were not detectable by in situ hybridization. In contrast, expression of PTPN23 in normal testicular tissues was significantly higher than that in TGCTs. miR-142-3p is an evolutionarily conserved miRNA that is aberrantly expressed in adult T-cell leukemia (30), head and neck squamous cell carcinomas (31,32), esophageal squamous cell carcinoma (33), lung adenocarcinoma (34), and hepatocellular carcinoma (35). These data suggest that miR-142-3p is a potential marker for the malignant progression of tumors and functions as an oncogenic miRNA in human cancers. In relation to this observation, a recent report indicated that miR-372 and miR-373 are highly expressed in TGCTs and inhibit the expression of LATS2 tumor suppressor gene (24). Further, comparison of miRNA expression in normal testis and seminomas identified miR-373 as the most downregulated miRNA among the 156 miRNAs present in normal testis. Interestingly, miR-142-3p was listed as the second most downregulated miRNA (35). RAC1 and TAB2 were identified as target genes for miR-142-3p in hepatocellular carcinoma cells and acute myeloid leukemia (36,37). RAC1, TAB2 and PTPN23 are in the high rank of 331 predicted targets (Target Scan Human Database, Release 6.2) and 102 conserved targets (PicTar Database) of miR-142-3p. These results strongly support PTPN23 as one of the target genes of miR-142-3p. MiR-142-3p was expressed at high levels in TGCTs but at low to undetectable levels in normal testes, indicating that miR-142-3p might possess an oncogenic function in the germ cell development. Many miRNAs are significantly overexpressed in various cancers. These oncogenic miRNA functions in tumor development via repressing the expression of genes having tumor suppressor activity (38,39).

However, the regulatory mechanisms that control the specific expression of miRNAs in human cancers are largely unknown, miR-142 is highly expressed in hematopoietic cells and directly targets the 3′UTR of IL-6 gene (40). Recently, it was reported that PU.1, C/EBPβ, CBPβ and Runx1 regulate the hematopoietic specific expression of miR-142 (41). The transcriptional start sites (TSS) of human miRNAs were reportedly identified, based on high-throughput sequencing data (42). A putative TSS for the hsa-miR-142 is located 1,200 bp upstream of the miR-142 precursor gene, and a putative TATA box is located 30 bp upstream of the TSS. We previously reported that a member of DEAD box protein family, Ddx1, is required for the testicular tumorigenesis, and acts partially through the transcriptional activation of 12p stem cell genes (25). Interestingly, candidate binding sites for Ddx1-mediated transcriptional activation are present 290-310, 505-525 and 885-905 bp upstream of the TSS of the miR-142 precursor gene. Therefore, Ddx1 might be involved in the upregulation of miR-142-3p in TGCTs. A future challenge will be to elucidate the miRNA-mediated regulatory networks in TGCTs.

In conclusion, we demonstrated that PTPN23 has a tumor suppressor activity in TGCTs. We also showed that miR-142-3p inhibits PTPN23 expression via a conserved miR-142-3p recognition motif located within the 3′ UTR of PTPN23 mRNA. Importantly, expression of miR-142-3p inversely correlated with PTPN23 level in TGCTs and normal testes. These data collectively revealed a novel miRNA-dependent downregulation of PTPN23 expression, that is important for the development of TGCTs.
miR-142-3p inhibits PTPN23 expression in TGCTs

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miR-142-3p inhibits PTPN23 expression in TGCTs

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FOOTNOTES

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The abbreviations used are: PTPN23, protein tyrosine phosphatase non-receptor type 23; TGCTs, testicular germ cell tumors; UTR, untranslated region; PTPs, protein tyrosine phosphatases; miRNA, microRNA; DMEM, Dulbecco’s modified Eagle’s medium; FFPE, formalin-fixed paraffin-embedded; GFP, green fluorescent protein; TSS, transcriptional start sites.

FIGURE LEGENDS

FIGURE 1. Expression of Ptpn23 protein in adult mouse testis. A, phase-contrast microphotograph of a section from adult mouse testis. B, Immunofluorescent staining of the testis section with an anti-Ptpn23 antibody. C, DAPI staining of the testis section. D, Merged image of B and C. Scale bar, 100 μm.

FIGURE 2. Establishment of a PTPN23-overexpressing TGCT cell line. A, Expression of Ptpn23 mRNA in c-Kit+ spermatogonial stem cells and PTPN23 mRNA in two human TGCT cell lines, NEC8 and NEC14. Expression levels were normalized against an internal control (Gapdh or GAPDH). Each value represents the mean ± s. e. m. (n = 3). *P < 0.01 compared with value of c-Kit+ spermatogonial stem cells. B, Morphology of NEC8 cells transfected with the control vector (control/NEC8) and NEC8 cells overexpressing PTPN23 (PTPN23/NEC8). Scale bar, 50 μm. C, Left: expressions of PTPN23 mRNA in control/NEC8 and PTPN23/NEC8 cells. Each reaction was performed in triplicate. *P < 0.01 compared with control/NEC8 cells. Right: total cell lysates from control/NEC8 and PTPN23/NEC8 cells were subjected to western blot analysis with anti-PTPN23 or α-tubulin (control) antibodies. D, Cell numbers in cultures of control/NEC8 or PTPN23/NEC8 cells in the presence of 1% or 10% FBS were counted every day. Each value represents the mean ± s. e. m. (n = 3). E, Cell cycle profiles of control/NEC8 or PTPN23/NEC8 cells grown in 1% FBS-DMEM by FACS.

FIGURE 3. PTPN23 overexpression in TGCT cells suppresses anchorage-independent growth in vitro and tumorigenicity in vivo. A, Control/NEC8 or PTPN23/NEC8 cells (1 × 10^6 cells) were inoculated into soft agar medium. After 2 weeks in culture, the number of colonies was counted. Each value represents the mean ± s. e. m. (n = 5) *P < 0.01 compared with control/NEC8 cells. Upper panel: bright-field image (left) and fluorescent image (right) of the control/NEC8-derived soft agar colonies. B, Control/NEC8 or PTPN23/NEC8 cells (5 × 10^6 cells) were injected subcutaneously into athymic nude mice and the tumor volume was periodically measured. Each value represents the mean ± s. e. m. (n = 3). Upper panel: hematoxylin and eosin (left) and immunohistochemical staining (right) of paraffin-embedded sections of control/NEC8 cell-derived solid tumors. Scale bar, 50 μm.

FIGURE 4. PTPN23 is a direct target of miR-142-3p. A, The miR-142-3p target site within the 3′UTR of human PTPN23 gene. B, Comparison of the miR-142-3p seed sequence in seven different species. C, Luciferase assays using reporter vectors containing the wild-type PTPN23 3′UTR seed sequence (WT-3′UTR), or the mutant construct, in which mutations were introduced into the seed sequence (Mut-3′UTR). NEC8 cells were transfected with pre-miR-142-3p expression vector or the control vector in combination with one of the reporter construct. Each value represents the mean ± s. e. m. (n = 3). *P < 0.01 compared with the control. D, Expression of miR-142-3p mRNA in control/NEC8 and PTPN23/NEC8 cells. Each value represents the mean ± s. e. m. (n = 3). *P < 0.01 compared with control/NEC8 cells. E, NEC8 cells were transfected with anti-miR-142-3p or negative
miR-142-3p inhibits PTPN23 expression in TGCTs

control. Total RNA was harvested 48 h after transfection and analyzed for PTPN23 mRNA expression using qRT-PCR. Each value represents the mean ± s. e. m. \((n = 3)\). \(*P < 0.01\) compared with control/NEC8 cells. F, GC-1 cells were transfected with pre-miR-142-3p or the pre-miR-negative control. RNA was harvested 72 h after transfection and analyzed for Ptpn23 expression. Each value represents the mean ± s. e. m. \((n = 3)\). \(*P < 0.01\) compared with control/GC-1 cells.

**FIGURE 5. Restoration of tumorigenicity by re-introduction of miR-142 in PTPN23/NEC8.**

A, Expression of miR-142-3p in the miR-142 precursor transfected PTPN23/NEC8 cells. Each value represents the mean ± s. e. m. \((n = 3)\). \(*P < 0.01\) compared with control/PTPN23/NEC8. B, Expression of PTPN23 in the miR-142 precursor transfected PTPN23/NEC8 cells. Each value represents the mean ± s. e. m. \((n = 3)\). \(*P < 0.01\) compared with control/PTPN23/NEC8. C, Cell lysates from control/PTPN23/NEC8 and miR-142/PTPN23/NEC8 cells were subjected to western blot analysis with anti-PTPN23 or α-tubulin (control) antibodies. D, Restoration of colony forming capacity in soft agar by miR-142 transfection into PTPN23/NEC8 cells. Control/PTPN23/NEC8 or miR-142/PTPN23/NEC8 cells were inoculated into soft agar medium. After 2 weeks in culture, the number of colonies was counted. Each value represents the mean ± s. e. m. \((n = 5)\) \(*P < 0.01\) compared with control/PTPN23/NEC8.

**FIGURE 6. PTPN23 is significantly downregulated and its expression levels negatively correlate with those of miR-142-3p in TGCTs and normal testes.**

A, Expression of miR-142-3p (top) and PTPN23 (bottom) RNAs in human normal testes and TGCTs. RNU6B or GAPDH was used to normalize the amount of template cDNA. Experiments were repeated three times and each value represents the mean ± s. e. m. B, Expression of miR-142-3p RNA and PTPN23 protein in human normal testis (N1) and TGCTs (S1, S3: seminoma; EC: embryonal carcinoma; YS: yolk sac tumor). Paraffin-embedded sections of human TGCTs and normal testicular regions were subjected to in situ hybridization with a digoxigenin-labeled miR-142-3p miRCURY LNA detection probe or to immunohistochemical analysis using an anti-PTPN23 antibody. Scale bar, 50 µm. Inset of left S1 panel: a high-magnification view of positive area. C, Expression of PTPN23 in human normal testes. Sections from human normal testicular tissues (TE803) were subjected to immunohistochemical analysis of PTPN23 expression. Scale bar, 100 µm.
Fig. 2

A

Relative PTPN23 level

spermatogonia
NEC8
NEC14

B

control/NEC8

PTPN23/NEC8

D

Relative PTPN23 level

control/NEC8
PTPN23/NEC8

PTPN23
α-tubulin

D

cell number (x10^5)
culture day

1
2
3
4
5

PTPN23/NEC8
10%FBS

PTPN23/NEC8
1%FBS

control/NEC8
10%FBS

control/NEC8
1%FBS

E

control/NEC8

G1:47.5%
S:35.3%
G2:15%

PTPN23/NEC8

G1:50.9%
S:34.9%
G2:12.4%
Fig. 3

A

B

colony number

Tumor volume (mm³)

Day after inoculation

control/NEC8

PTPN23/NEC8

control/NEC8

PTPN23/NEC8
Fig. 4

A

Position 25-32 of PTPN23 3' UTR

hsa-miR-142-3p

5'GCCUACCUGGUCCUUACACUACA...

| | | | | | |

3' AGGUAUUUCAUCCUUUGUGAUGU

B

H.sapiens: UGCCUACCUGGUCCUUACACUACA
P.troglodytes: CGCCUACCUGGUCCUUACACUACA
M. mulatta: CGCCUACCUGGUCCUUACACUACA
M.musculus: UGCCUAUCUGAUCCUUACACUACA
R.norvegicus: UGCCUGUCUGGUCCUUACACUACA
O.cuniculus: UGCCUGUCCUGGUCC--ACACUACA
C.familiaris: UGCCUGCCCGGUCCUUACACUACA

C

![Graph showing multiple bars representing different conditions with error bars.]

D

Relative miR-142-3p level

control/NEC8
PTPN23/NEC8

E

Relative PTPN23 level

control/NEC8
pre-miR-142-3p/NEC8

F

Relative ptpn23 level

control/GC-1
anti-miR-142-3p/GC-1
Fig. 5

A. Relative PTPN23 level

B. Relative miR-142 level

C. Western blot analysis of PTPN23 and α-Tubulin

D. Number of colonies
Fig. 6

A

Relative miR-142-3p level

B

miR-142-3p
PTPN23

C

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