miR-223-3p regulates cell growth and apoptosis via FBXW7 suggesting an oncogenic role in human testicular germ tumors

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Abstract. miR-223-3p is deregulated in several tumor types and plays an important role in tumorigenesis and progression. However, its role in the pathogenesis of testicular germ cell tumor (TGCT) remains uncharacterized. We previously demonstrated that miR-223-3p expression was increased in TGCTs compared with normal testes (NT), suggesting that miR-223-3p may have an oncogenic role in TGCT. Using published dataset and The Cancer Genome Atlas database, we validated higher miR-223-3p expression in TGCTs than NT, and found a negative correlation between miR-223-3p and FBXW7 mRNA expression levels. Using both gain- and loss-of-function experiments, we show that miR-223-3p regulates FBXW7 protein expression, cell growth and apoptosis in TGCT cell lines. Additionally, we demonstrate that ectopic expression of the full-length coding sequence of FBXW7 could rescue the cell growth and apoptotic effects mediated by miR-223-3p. Our findings suggest an oncogenic role for miR-223-3p in TGCT, which promotes cell growth and inhibits apoptosis through repression of FBXW7.

Introduction

Testicular germ cell tumor (TGCT) is the most frequent solid malignancy occurring in males between the ages of 15 and 34 years (1), with a steadily rising incidence for the past few decades in the United States and Europe (2). Histopathologically, ~55% of all TGCTs are classified as seminomas, and the remaining cases as non-seminomas (3). The vast majority of TGCTs have an excellent cure rate with cisplatin-based treatment. Nevertheless, a subset of patients develops cisplatin resistance resulting in tumor progression and reduced survival (4). Therefore, a better understanding of the molecular mechanisms of TGCT tumorigenesis is needed for identification of new therapeutic targets and treatment development.

MicroRNAs (miRNAs) are small non-coding RNAs of ~20-24 nucleotides in length, which play important roles in a broad range of cellular processes, including tumor development and drug response (5). Genome-wide miRNA profiling studies have provided evidence of miRNA deregulations in TGCT. For example, the miR-371-373 cluster is frequently overexpressed in malignant TGCTs of all histopathological subtypes (6,7). Other miRNAs, such as the miR-302 cluster and miR-301, are differentially expressed based on the cellular differentiation of the tumor (7,8). To date, very few miRNAs have been functionally characterized in TGCT. miR-372 and miR-373 have been shown to play oncogenic roles in TGCT by targeting the tumor suppressor LATS2 (9). However, the functional roles of other differentially expressed miRNAs in TGCT have yet to be characterized.

We previously identified a subset of miRNAs that were differentially expressed between TGCTs and normal testes (NT) using a deep sequencing approach (10). Among these, miR-223-3p expression was higher in TGCTs as compared to NT. miR-223-3p is known to be deregulated in a broad range of hematological malignancies and solid tumors (11,12). However, its role in TGCT remains uncharacterized. miR-223-3p has been shown to regulate multiple targets in different cancer types. Among them, F-box/WD repeat-containing protein (FBXW7) is the most common target, which has been reported in acute T-cell lymphoblastic leukemia, esophageal squamous cell carcinoma and gastric cancer (13-15). FBXW7 is the substrate-recognition component of the SCF-(SKP1, CUL1, F-box protein)-ubiquitin-ligase complex, which has been demonstrated to function as a tumor suppressor by promoting the degradation of several oncprotein substrates, including c-Myc, cyclin E, MCL-1, c-JUN, NFkB2 and Notch1 (16,17). Therefore, suppression of FBXW7 by miR-223-3p can promote tumor development and progression.

In this study, we investigated the expression and function of miR-223-3p and FBXW7 in TGCT clinical samples and cell lines. Our data show that miR-223-3p plays an oncogenic role in TGCT by promoting cell proliferation and inhibiting apoptosis via FBXW7.
Materials and methods

Clinical samples and cell lines. Fifteen frozen TGCTs and five NT were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute, USA. All samples were included in our previous small RNA-sequencing study (10). The study was approved by the Stanford Human Subjects Review Committee.

Two established TGCT cell lines were included in this study: the TCam-2 seminoma cell line and the 2102Ep non-seminoma cell line (18,19). TCam-2 was kindly provided by Dr Leendert H.J. Looijenga (Department of Pathology, Erasmus MC-University Medical Center Rotterdam, The Netherlands) and 2102Ep by Dr Peter Andrews (Department of Biomedical Science, University of Sheffield, UK). TCam-2 cells were grown in RPMI-1640 and 2102Ep cells were cultured in DMEM medium, supplemented with 10% fetal bovine serum. All cells were cultured at 37˚C with 5% CO₂. TCam-2 cells were grown in RPMI-1640 and 2102Ep cells were cultured in DMEM medium, supplemented with 10% fetal bovine serum. All cells were cultured at 37˚C with 5% CO₂. Authentication of the cell lines was verified by short tandem repeat profiling in our recent study (10).

Data extraction and analysis from published data and The Cancer Genome Atlas database. For comparison of miR-223-3p expression between TGCTs and NT, we extracted global TaqMan miRNA profiling data from the study of Gillis et al (7), which analyzed 61 germ cell tumors, three NT and five embryonal carcinoma cell lines. We excluded the 10 dyserminomas (ovarian germ cell tumors), one ovarian embryonal carcinoma, one ovarian yolk sac carcinoma and five cell lines, and re-analyzed the miR-223-3p expression by normalization to miR-16 in the 49 TGCTs and three NT.

For FBXW7 mRNA, we extracted the microarray gene expression data of 101 TCGTs and five NT from Gene Expression Omnibus (GEO accession no. GSE3218; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE3218).

For analysis of correlation between miR-223-3p expression and FBXW7 mRNA levels, we extracted miR-223-3p and FBXW7 mRNA data from The Cancer Genome Atlas (TCGA) testicular cancer database using the UCSC Xena browser (http://xena.ucsc.edu/). These miR-223-3p and FBXW7 expression data had been generated by miRNA expression Illumina HiSeq and exon expression RNAseq, respectively.

RNA extraction. Total RNA was extracted using the miVana miRNA isolation kit (AM1560; Ambion/Thermo Fisher Scientific, Waltham, MA, USA) and RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All RNA samples were stored at -80°C until further use.

TagMan reverse transcription quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed to evaluate the transfection efficiency of miR-223-3p overexpression or inhibition using the StepOnePlus Real-Time PCR system (Applied Biosystems/Thermo Fisher Scientific). cDNA was synthesized from 20 ng of total RNA and used to quantify miR-223-3p (ID 002295) and RNU48 (ID 001093). All reactions were performed in triplicate. The relative expression of miR-223-3p was normalized to RNU48, and the fold change of miR-223-3p in cells transfected with miR-223-3p mimic/inhibitor relative to their respective control was reported as 2-ΔΔCt.

Transfection. For miR-223 overexpression and inhibition, 2x10⁴ cells were transfected with 30 nM of miRNA inhibitor (anti-miR-223, AM12301 or anti-miR negative control no. 1, AM17010; Ambion) or 10 nM of miRNA mimic (pre-miR-223, PM12301 or pre-miR negative control no. 1, AM17110; Ambion) using siPORT NeoFX transfection agent (AM4511; Ambion).

For co-transfection of miR-223 mimic and FBXW7-expressing plasmid, 1.5x10⁴ cells were co-transfected with 500 ng of pCMV-Myc FBXW7 and 10 nM of pre-miR-223 or pre-miR-NC using Lipofectamine 2000 (no. 11668-019; Invitrogen/Thermo Fisher Scientific). Cells co-transfected with an empty vector and pre-miR-NC was used as a control. Cells were collected 48 h after transfection for subsequent analysis. The pCMV-Myc FBXW7 plasmid was obtained from Addgene (no. 16652; Cambridge, MA, USA; https://www.addgene.org/). The empty vector was prepared by cleavage of pCMV-Myc FBXW7 with BgIII and NotI to remove the full-length coding sequence of FBXW7.

Annexin V cell apoptosis and EdU (5-ethyl-2'-deoxyuridine) cell proliferation assays. Cell apoptosis and proliferation were evaluated in TCam-2 and 2102Ep cells 72 h after transfection using Annexin V FITC Apoptosis kit (PHN1018; Invitrogen) and Click-iT EdU Alexa Fluor 488 flow cytometry assay (C10425; Invitrogen), respectively. All experimental conditions were according to the manufacturer's instructions and analyzed by NovoCyte flow cytometer (ACEA Biosciences, San Diego, CA, USA). At least three independent experiments were performed in each cell line.

Trypan blue exclusion assay. Trypan blue exclusion assay was performed in TCam-2 and 2102Ep cells 48 or 72 h after transfection. Cells were stained with 0.4% trypan blue solution and counted by TC10™ Automated Cell Counter (Bio-Rad, Hercules, CA, USA).

WST-1 assay. Cell growth was measured by WST-1 colorimetric assay (no. 11644807001; Roche Diagnostics, Indianapolis, IN, USA) in TCam-2 and 2102Ep cells 72 h after transfection. Cells were plated into a 96-well plate at a concentration of 5x10³/well in 100 µl culture medium. At different time intervals (0, 24, 48 or 72 h after transfection), 10 µl of WST-1 reagent was added to each well and incubated for 3 h at 37°C. After incubation, absorbance values were detected at the wavelengths 450 nm (measurement) and 650 nm (reference) using the VERSAmax ELISA Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Each experimental group was performed in six replicates for each time-point and all experiments were repeated three times independently.

Western blotting. Total protein lysates were extracted using NP-40 cell lysis buffer (FNN0021; Invitrogen), supplemented with 1 mM of phenylmethylsulfonyl fluoride (P7626; Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitor (P8340; Sigma-Aldrich). Protein concentrations were determined using the Pierce™ BCA Protein assay kit (no. 23227;
Pierce Biotechnology, Thermo Fisher Scientific). Thirty micrograms of protein lysates were separated in NuPAGE Novex 4-12% Bis-Tris gels (NP0321BOX; Invitrogen) and transferred to 0.2 µm nitrocellulose membranes (no. 88024; Invitrogen). After blocking with 5% skim milk powder (no. 70166; Sigma-Aldrich) in Tris-buffered saline/0.05% Tween-20, membranes were incubated with anti-FBXW7 (NBP1-59631; Novus Biologicals, Littleton, CO, USA; 1:1,000 dilution), anti-cleaved PARP (ab32064; Abcam, Cambridge, UK; 1:1,000 dilution) or anti-Myc-Tag (no. 2276; Cell Signaling Technologies, Danvers, MA, USA; 1:50 dilution) overnight at 4˚C. Anti-rabbit IgG-HRP (no. 170-6515; Bio-Rad Laboratories; 1:3,000 dilution) or anti-mouse IgG-HRP (sc-2005; Santa Cruz Biotechnology, Dallas, TX, USA; 1:10,000 dilution) was used as secondary antibodies. For normalization purpose, the membrane was incubated with anti-GAPDH (sc-47724; Santa Cruz Biotechnology; 1:1,000 dilution). Signals were detected using the Novex ECL HRP chemiluminescent substrate reagent (WP20005; Invitrogen) and LAS-1000 image analyzer (Fujifilm, Tokyo, Japan).

Statistical analyses. All statistical analyses were performed using MS Office Excel 2007 or SPSS 22.0 (IBM Corp., Armonk, NY, USA). Comparisons between TGCT and NT were performed by Mann-Whitney U test, and the transfection experiments were assessed by Student's paired t-test. Correlation between miR-223-3p and FBXW7 mRNA expression levels was evaluated using Pearson's correlation analysis. All statistical tests were two-sided and P-values <0.05 were considered as statistically significant.

Results

Expression of miR-223-3p and FBXW7 in TGCTs and NT. To validate our previous observation of miR-223-3p overexpression in TGCTs, we re-analyzed miR-223-3p expression from the miRNA profiling data of Gillis et al (7), with inclusion of 49 TGCTs and 3 NT. In agreement with our previous finding (10) (Fig. 1A), miR-223-3p expression was quantified by RT-qPCR and miR-16 was used for normalization. (C) The graph shows the normalized expression data of FBXW7 mRNA in NT (n=5) and TGCTs (n=101), which were extracted from the microarray gene expression of GEO database accession no. GSE3218. (D) miR-223-3p expression and FBXW7 mRNA data were obtained from the TCGA database. Correlation was assessed using the Pearson's correlation analysis. (E) Western blot analysis of FBXW7 protein in NT (n=3) and TGCTs (n=14). GAPDH was used as a loading control. Data represent mean ± SD. All comparisons were evaluated using Mann-Whitney U test. *P<0.05; ***P<0.001.
Effect of miR-223-3p modulation on FBXW7 in TGCT cell lines. To further determine whether miR-223-3p could regulate FBXW7 in TGCT, we performed miR-223-3p overexpression and inhibition in two TGCT cell lines and evaluated the effect on FBXW7 protein expression using western blot analysis. As shown in Fig. 2A, cells transfected with anti-miR-223 showed significantly lower miR-223-3p expression than the anti-miR-NC-treated cells in both cell lines (P=0.018 for both). Similarly, miR-223-3p expression was significantly increased in cells transfected with pre-miR-223 relative to its negative control (TCam-2: P=0.014 and 2102Ep: P=0.003). The data support the efficiency of transfection.

Furthermore, inhibition of miR-223-3p led to a significant increase of FBXW7 expression in TCam-2 (1.9-fold; P=0.003) and 2102Ep (1.3-fold; P=0.015) cells. Similarly, overexpression of miR-223-3p significantly reduced FBXW7 expression in both cell lines (0.8-fold and P<0.01 for both) (Fig. 2B). The findings indicate that miR-223-3p suppresses FBXW7 expression in human TGCT cells.

Functional consequences of miR-223-3p regulation in TGCT cells. To explore the functional role of miR-223-3p on apoptosis, we investigated the effect using flow cytometric detection of Annexin V-stained cells as well as by western blot analysis of cleaved PARP (cPARP, 25 kDa), which is an apoptosis marker. For the Annexin V assay, we observed that inhibition of miR-223-3p in TCam-2 cells significantly increased apoptotic cells by 80% (P=0.018), while overexpression of miR-223-3p reduced apoptotic cells by 38% (P=0.038), relative to their respective negative controls (Fig. 3A). Similar effects were also observed in 2102Ep cells, however, the effect was less pronounced compared to TCam-2 cells (27% increase in the miR-223-3p inhibition, P=0.009; 34% decrease in the miR-223-3p overexpression, P=0.016; Fig. 3A).

For the cPARP detection, silencing of miR-223-3p led to a significant increase of cPARP expression in both TCam-2 (1.6-fold, P=0.003) and 2102Ep (1.3-fold, P=0.007) cells, while overexpressing miR-223-3p resulted in a significant decrease of cPARP expression (TCam-2: 0.7-fold, P=0.016; 2102Ep: 0.6-fold, P=0.016).
2102Ep: 0.8-fold, \( P=0.002 \) (Fig. 3B). These results indicate that \( \textit{miR-223-3p} \) inhibits apoptosis in TGCT cells.

For cell proliferation, we applied three different assays: Click-iT EdU, WST-1 and trypan blue exclusion. Using the EdU assay, we observed reduction of EdU-positive cells upon silencing of \( \textit{miR-223-3p} \) in both TCam-2 (50.3 vs. 30.8%, \( P=0.026 \)) and 2102Ep cells (46.5 vs. 30.0%, \( P=0.045 \)), and increase of EdU-positive cells upon overexpression of \( \textit{miR-223-3p} \) (TCam-2: 48.1 vs. 61.6%, \( P=0.003 \); 2102Ep: 47.4 vs. 57.7%, \( P=0.027 \)) (Fig. 4A). Similarly, the trypan blue exclusion assay revealed reduction of cell count upon silencing of \( \textit{miR-223-3p} \) (TCam-2: 0.8-fold, \( P=0.004 \); 2102Ep: 0.8-fold, \( P=0.016 \)) and increase of cell number upon overexpression of \( \textit{miR-223-3p} \) (TCam-2: 1.3-fold, \( P=0.006 \); 2102Ep: 1.2-fold, \( P=0.011 \)) (Fig. 4B). The WST-1 assay also showed that silencing of \( \textit{miR-223-3p} \) reduced cell growth at 72-h post-transfection in both TCam-2 (\( P=0.043 \)) and 2102Ep (\( P=0.041 \)) cells (Fig. 4C). Taken together, the results support that \( \textit{miR-223-3p} \) promotes cell proliferation in TGCT cell lines.

\( \textit{miR-223-3p} \) mediates regulation of cell growth and apoptosis through \( \textit{FBXW7} \) in TGCT. Given that \( \textit{FBXW7} \) expression is a well-characterized target of \( \textit{miR-223-3p} \), we tested whether ectopically expressed \( \textit{FBXW7} \) could rescue the \( \textit{miR-223-3p} \)-mediated apoptotic and proliferative effects. We co-transfected TCam-2 cells with pre-\( \textit{miR-223} \) together with a plasmid expressing the entire open reading frame of \( \textit{FBXW7} \) without the \( \textit{miR-223-3p} \) binding site (pCMV-Myc FBXW7) or...
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The effects on cell apoptosis and proliferation were determined using western blot analysis of cPARP and trypan blue exclusion assay, respectively. As shown in Fig. 5A, the endogenous FBXW7 was reduced in both cells co-transfected with pre-miR-223 and Myc-FBXW7 or vector control as compared with the negative control-transfected cells, indicating the suppression of endogenous FBXW7 by miR-223-3p overexpression.

Ectopic expression of miR-223-3p significantly reduced the abundance of cPARP (P=0.043) and increased the number of live cells (P=0.006) as compared to their respective controls; the effects were abolished by the ectopically expressed FBXW7 (Fig. 5). Together, our data indicate that miR-223-3p regulates cell growth and apoptosis in TGCT cells through FBXW7.

Discussion

miR-223-3p expression was found higher in TGCTs than NTs in our previous study (10), and here, we validated the findings in independent cohorts using previously published dataset (7). Deregulation of miR-223-3p has been observed in a variety of tumor types. Overexpression was found in T-cell acute lymphoblastic leukemia (11), oral (12), esophageal (14), gastric

Figure 4. Effect of miR-223-3p regulation on cell growth in TGCT cells. (A) Representative flow cytometric images of proliferating cells with EdU incorporation in anti-miR-223 or pre-miR-223 cells and their respective negative controls at 72 h post-transfection (upper). The graph shows the changes of EdU-positive cells in both cell lines upon silencing or overexpression of miR-223-3p from three independent experiments (lower). (B) Total live cells were counted using trypan blue dye exclusion assay (n=4). (C) Relative cell growth was examined at different time-points in both cell lines transfected with miR-223-3p inhibitor or negative control using WST-1 assay (n=3 for each time-point). Data represent mean ± SD. P-values were calculated by paired t-test. *P<0.05; **P<0.01.
miR-223-3p plays vital roles in a variety of tumor types, either as an oncogene or tumor suppressor depending on the cellular contexts. Consistent with its dual role, miR-223-3p has been shown to function as an oncogene in T-cell acute lymphoblastic leukemia, gastric and lung cancers (13,15,21,27,28), and as a tumor suppressor in cutaneous T-cell lymphoma and prostate cancer (29,30). Given its diverse function in different cancer types, we characterized the functional role of miR-223-3p in TGCT cells.

miR-223-3p was shown to promote cell proliferation in TGCT cell lines in all three methods applied and which are based on different principles: the Click-iT EdU assay allows the detection of the thymidine analog EdU incorporated into cellular DNA during replication; the WST-1 assay is based on the metabolic activity of cells for conversion of the tetrazolium salt WST-1 into a colored dye, and the trypan blue exclusion assay provides direct counting of the number of live cells. Our findings support its oncogenic role in TGCT by promoting cell growth and inhibiting apoptosis in TGCT cell lines. Additionally, miR-223-3p has been shown to modulate drug response in several cancer types (31-35). Importantly, miR-223-3p regulates cisplatin sensitivity in gastric and esophageal cancers (31,32). Given that most TGCTs are responsive to cisplatin treatment, it is intriguing to speculate that miR-223-3p may play an important role in cisplatin sensitivity in TGCT. Further investigations are warranted to evaluate the role of miR-223-3p in cisplatin response in TGCT.

FBXW7 has been demonstrated as a direct target of miR-223-3p using luciferase reporter assays (13,15). Here, we show that FBXW7 expression is lower in TGCTs than NT and inversely correlated with miR-223-3p, and miR-223-3p regulates FBXW7 protein expression using both gain- and loss-of-function studies. Most importantly, ectopic expression of the FBXW7 open reading frame can rescue the cell growth and apoptosis effects mediated by miR-223-3p. Together, our findings suggest that miR-223-3p regulates FBXW7 in TGCT and this regulatory pathway plays an important role in TGCT pathogenesis.

In conclusion, we report deregulation of miR-223-3p and FBXW7 in human TGCT. Our findings also reveal an oncogenic role of miR-223-3p through repression of the FBXW7 tumor suppressor, suggesting that this regulation is important for cell proliferation and apoptosis in TGCT. This study provides additional evidence of miRNA function in testicular germ cell tumorigenesis.
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