

Original Article

Poly r(C) binding protein (PCBP) 1 is a negative regulator of thyroid carcinoma

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Abstract: Poly r(C) binding protein (PCBP) 1 or heterogeneous ribonucleoprotein (hnRNP) E1 is a RNA binding protein functional in multiple biological processes. PCBP1 has been shown to function as a tumor suppressor by negatively regulating translation of EMT inducer proteins in different cancers. Loss of PCBP1 expression or its Akt2-mediated phosphorylation at serine residue 43 has both been indicated to de-repress its regulation of EMT inducer proteins. However, its role in thyroid carcinoma has not been elucidated. Here we report that PCBP1 expression is significantly downregulated in thyroid carcinoma patients. *In vitro* kinase assay revealed that immunoprecipitated PCBP1 from transient or stably transfected thyroid carcinoma cells can be phosphorylated by recombinant Akt2 kinase. *In situ* analysis revealed that *PCBP1* is a putative target of miR-490-3p, which was further confirmed by *PCBP1* 3'UTR-based reporter assays using the wild-type or a miR-490 seed mutant 3'UTR. The endogenous regulation of the *PCBP1* 3'UTR reporter by miR-490-3p could be rescued by transfection of miR-490 antagomir in WRO and BCPAP cells. Stably overexpressing *PCBP1* BCPAP cells attenuated tumor formation completely as compared to empty vector overexpressing cells in xenograft assay. Cumulatively, our results indicate that PCBP1 functions as a tumor suppressor in thyroid carcinoma and that its expression is down regulated by high expression of the miR-490-3p observed in thyroid carcinoma patients.

Keywords: Poly r(C) binding protein, PCBP1, BCPAP, miR-490

Introduction

Among all endocrine malignancies, thyroid cancer is the most common, accounting for almost 90-95% of all endocrine tumors [1, 2]. It is also one of the cancers with a steady increase in the incidence rate, especially in men [1]. Importantly, nearly 30% of palpable nodules observed in thyroid disorders are ultimately diagnosed as malignant [3]. Thyroid cancer is classified as papillary, follicular, medullary, or anaplastic [4]. Papillary thyroid cancer is the most common and accounts for 70% of all thyroid cancers, whereas follicular thyroid cancer accounts for 10-15% of cases [5, 6]. Papillary thyroid cancers spread to lymph nodes in the neck in 50% of cases [7, 8]. However, the precise mechanism(s) involved in metastatic dissemination is not well understood [9].

It has been recently shown that the RNA binding protein, heterogeneous nuclear ribonucleo-

protein (hnRNP) E1 or poly r(C) binding protein (PCBP) 1 is central to maintenance of stemness in prostate cancer cells [10]. In LNCaP and DU145 prostate cancer cells, it was shown that after TGF- β treatment there is a progressive loss of PCBP1 protein expression, which in turn contributes to the enrichment and maintenance of CD44+CD24-CD133+ prostate stem cells [10]. In addition, it was shown that either loss of PCBP1 expression or phosphorylation-mediated post translational modification has a pro-tumorigenic and pro-metastatic effect in pancreatic, ovarian, breast, lung, gastric, and Burkitt lymphoma [11-17]. However, it is not clear if PCBP1 expression or its post translational modification participated in thyroid cancer pathogenesis.

In the current study, we show that loss of PCBP1 expression is commonly associated with papillary thyroid cancer and that this loss is perhaps the regulation of gene expression by miR-490-

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3p at the post-transcriptional level. Modulation of PCBP1 levels can affect *in vivo* tumorigenesis in thyroid cancer cell lines.

Materials and methods

Patient samples

Fresh-frozen and paraffin-embedded tissue specimens of papillary thyroid carcinoma and corresponding adjacent non-tumorous thyroid tissue samples were obtained from 10 Chinese patients at Tianjin Medical University General Hospital between 2014 and 2015. Only samples from those patients that did not undergo pre-operative local or systemic treatment were included in the current study. The study protocol was approved by the Institutional Review Board of Tianjin Medical University General Hospital, China. For freshly harvested samples, they were suspended in RNAlater (Life Technologies, Shanghai, China) before being snap frozen and stored in liquid nitrogen until use.

Immunohistochemistry

This part of the study was approved by the Institutional Review Board of Tianjin Medical University General Hospital. The tumor and adjacent normal tissue specimens obtained from 10 patients with papillary thyroid carcinoma were stained for PCBP1 expression (#ab-133421, Abcam, Waltham, MA, USA). The stained slides were scored as positive and negative by a pathologist blinded to tissue identity.

Cell and tissue lysis and western blot

Tissue specimens were homogenized in liquid nitrogen. Homogenized tissue specimens and cells were lysed in IP lysis buffer (Life Technologies, Shanghai, China) supplemented with Mini protease inhibitor cocktail (Roche Diagnostics, Indianapolis, USA). Whole cell lysates were resolved on a 4-20% NuPAGE gel (Life Technologies, Shanghai, China) and probed with PCBP1 antibody (#ab-133421, Abcam, Waltham, MA, USA). The blots were also probed with anti-GAPDH antibody (#ab-9485, Abcam, Waltham, MA, USA) to ensure equal loading across lanes.

Cell culture and treatment

WRO and BCAP cells were obtained from the cell and tissue bank at our center and cultured

in RPMI1640 medium, containing glutamine, 5% FBS (Lonza, Germany), and penicillin/streptomycin. Cells were maintained at 37°C under a humidified atmosphere of 5% carbon dioxide.

Gene construction

The pENTR-PCBP1 plasmid was obtained from Addgene and cloned into the p3XFLAG-CMV10 vector (Sigma-Aldrich, St. Louis, MO, USA). The *PCBP1* 3' UTR clone in pMirTarget was obtained from Origene. The *PCBP1* 3' UTR reporter was constructed by amplifying the endogenous *PCBP1* 3' UTR from the Origene. XhoI and ApaI sites were added to the 5' and 3' ends of the fragment during the preceding PCR reaction and cloned into the XhoI and ApaI site on the Rr-luc-6XCXCR4 Renilla luciferase vector (Addgene, Cambridge, MA, USA). To make the *PCBP1* 3'UTR mutant construct, site-directed mutagenesis was used to delete 77-83 region, corresponding to the hsa-miR-490-3p binding site. A firefly luciferase vector was used as transfection and normalization control in all luciferase assays. Constructs were sequence verified before being used for experiments.

Transfection

WRO and BCAP cells (4×10^4) were transiently transfected with FLAG-PCBP1 using Lipofectamine 3000 (Life Technologies, Shanghai, China). Stable clones were generated using G418 selection. For transient transfection, WRO cells were harvested 72 hours post-transfection. Stable BCAP clones overexpressing *GLB1* and *PCBP1* were also generated in cells that were initially transduced with firefly luciferase. For luciferase assay, BCAP and WRO cells (4×10^4) were transiently transfected with the indicated constructs using Lipofectamine 3000 (Life Technologies, Shanghai, China). Where indicated, cells were transfected with miR-490-3p antagomir (Life Technologies, Shanghai, China) along with the *PCBP1* 3'UTR constructs.

Luciferase assays

The dual-luciferase reporter assay system (Promega, Madison, Wisconsin, USA) was used to quantify renilla and firefly luciferase activities 96 hours post transfection. Each reporter plasmid was transfected at least twice in triplicate. Post-normalized data was represented as

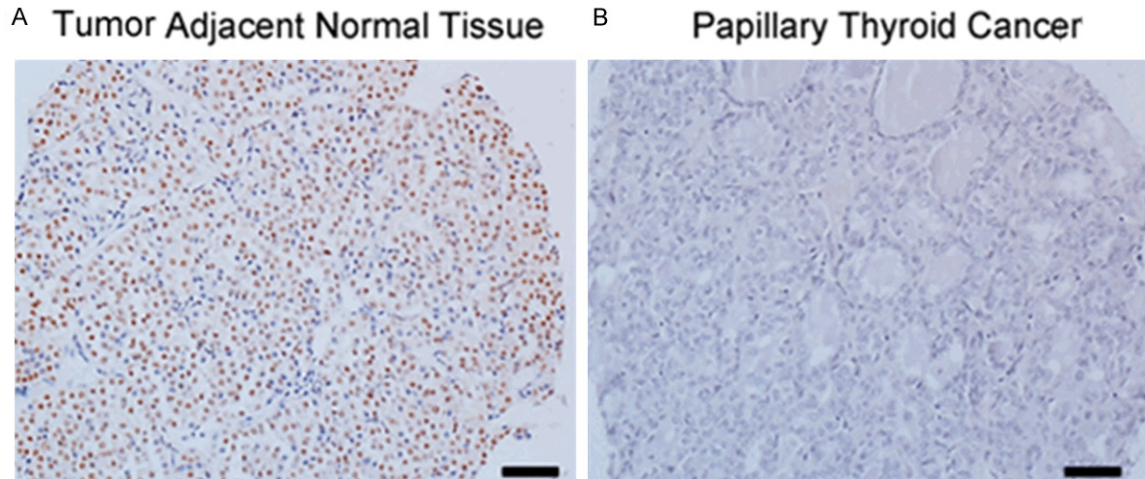


Figure 1. PCBP1 expression is suppressed in patients with papillary thyroid cancer. Representative immunohistochemistry images showing PCBP1 expression in papillary thyroid cancer and tumor adjacent normal tissues. Scale bar - 40 μ m.

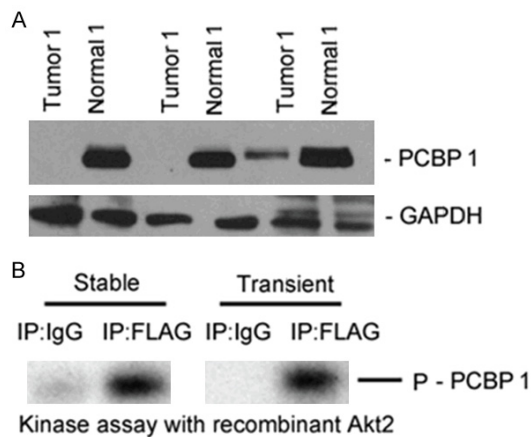


Figure 2. Downregulation of PCBP1 expression in papillary thyroid cancer patients seem to be widespread and its effect in thyroid cancer is perhaps independent of its post translational modification. A. Steady state expression of PCBP1 in papillary thyroid cancer and adjacent normal tissues from three different patients. The blots are representative of three independent experiments. B. *In vitro* kinase assay using immunoprecipitated PCBP1 from either transiently transfected or stably transfected WRO cells using recombinant Akt2 kinase showed robust PCBP1 phosphorylation.

relative fluorescence units (RFU) \pm standard deviation (SD).

In vitro kinase assay

In vitro kinase assays were performed using purified, active Akt2 enzyme (SignalChem, Richmond, Canada). Whole cell lysates for

immunoprecipitation (IP) from WRO cells were prepared in IP lysis buffer (Life Technologies, Shanghai, China) containing protease inhibitors and 1 mM Na_3VO_4 . Lysates were sonicated and clarified by centrifugation at 4°C for 10 minutes in a Beckman tabletop microcentrifuge at maximum speed. Cell lysates (1000 μ g) were incubated overnight at 4°C with 10 μ g of either mouse anti-FLAG antibody or mouse IgG (Sigma-Aldrich, St. Louis, MO, USA) crosslinked to protein A/G beads (Pierce Crosslink IP Kit, Life Technologies, Grand Island, NY, USA), made up to a total volume of 300 μ l with IP lysis buffer. The immune complexes were collected by centrifugation, washed five times with IP lysis buffer, and eluted with 10 μ l of 3 \times FLAG peptide. The eluant was diluted to 30 μ l with 1 \times kinase buffer (25 mM Tris, pH 7.5, 5 mM β -glycerolphosphate, 10 μ M ATP, 2 mM dithiothreitol, 0.1 mM Na_3VO_4 , 10 mM MgCl_2). The kinase reactions were initiated by adding 200 ng of Akt2 and 0.5 μ Ci of [γ - 32 P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences) in 20 μ l of kinase buffer. The reactions were stopped after 15-minute incubation at 30°C by adding SDS-PAGE loading buffer and heating to 95°C for 10 minutes. The samples were resolved on a 4-12% SDS-PAGE gel and analyzed by autoradiography.

Xenograft assay

The experiments were approved by the Institutional Animal Care and Use Committee of

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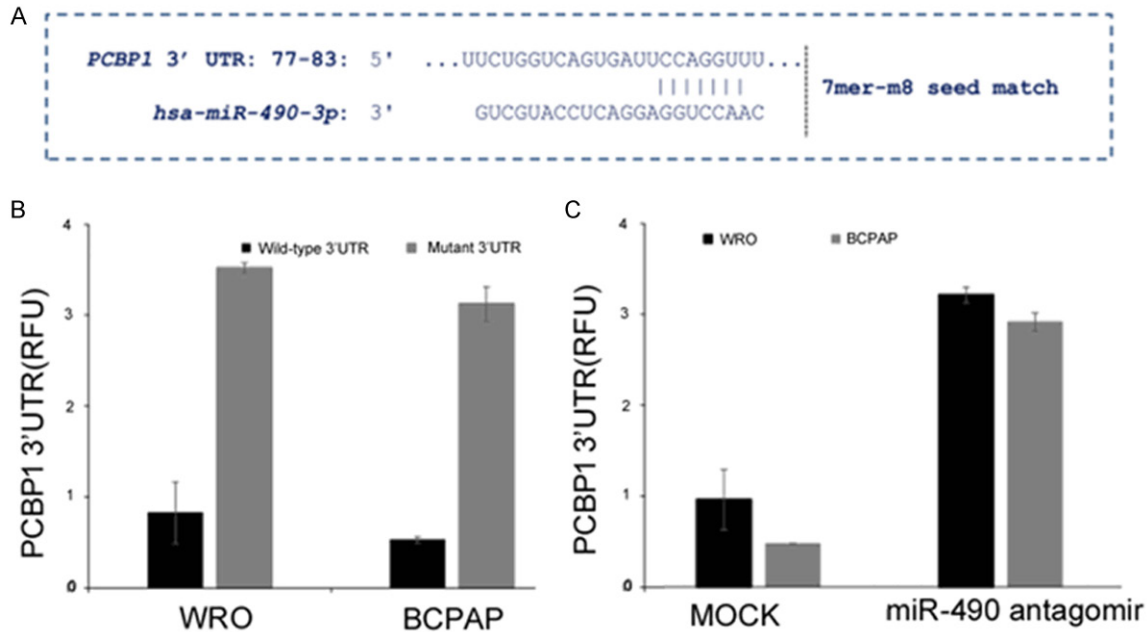


Figure 3. *PCBP1* is a bona-fide target of miR-490-3p in the thyroid cancer cell lines, WRO and BCPAP. **A.** *In situ* prediction of complementary 7mer-m8 seed match between miR-490-3p and the 3' UTR of *PCBP1* as predicted by TargetScan software. **B.** Relative luciferase activity of transiently transfected luciferase reporter constructs containing full-length or mutated (miR-490 binding site deleted) *PCBP1* 3' UTR in WRO and BCPAP cells. **C.** Relative luciferase activity of transiently transfected luciferase reporter constructs containing full-length *PCBP1* 3' UTR in WRO and BCPAP cells, either mock transfected or transfected with miR-490 antagonist.

Tianjin Medical University General Hospital. 10^6 BCAP cells stably overexpressing *PCBP1* or *GLB1* were injected subcutaneously in the hind flank of six-week-old female nude mice (n=5 per group). Mice were assessed for tumor formation weekly for 35 days using *in vivo* bioluminescence imaging with an IVIS Imaging System (IVIS imaging system 200, Xenogen Corporation, PerkinElmer, Waltham, USA) fitted with an ultra-sensitive CCD camera. Images at day 35 were obtained along with X-ray to confirm the presence or absence of tumor.

Statistical analyses

SPSS version 20.0 (IBM Corporation, NY, USA) was used for all statistical analysis. Results are expressed as means \pm SD. Statistical significance was based on two-tailed paired Student's t-test; $P < 0.05$ was considered to be significant.

Results

In order to evaluate *PCBP1* expression level in papillary thyroid cancer, we performed immunohistochemistry and western blot by a previ-

ously validated *PCBP1* antibody in 10 tumor and tumor-adjacent normal tissues. As shown in **Figures 1A** and **2A**, robust expression of *PCBP1* was detected in adjacent normal thyroid tissue. However, each tissue specimen of the 10 papillary thyroid cancers was scored as negative in the *PCBP1* staining (**Figure 1B**), suggesting that loss of *PCBP1* expression might be associated with thyroid cancer. In addition, none (2 out of 3 tested) or suppressed (1 out of 3 tested) *PCBP1* expression was observed by western blot in comparison with the corresponding adjacent normal tissue specimens (**Figure 2A**). Cumulatively this suggested that loss of *PCBP1* expression might be an underlying feature of thyroid cancer.

Given that it was shown that Akt2-kinase-mediated phosphorylation of *PCBP1* at serine residue 43 can induce translation of genes required for metastatic progression in the context of breast and lung cancer [12, 13, 15], we next tested if *PCBP1* was getting phosphorylated in the thyroid cancer cell line WRO. A FLAG-*PCBP1* plasmid was ectopically overexpressed in a transient or stable manner and FLAG immu-

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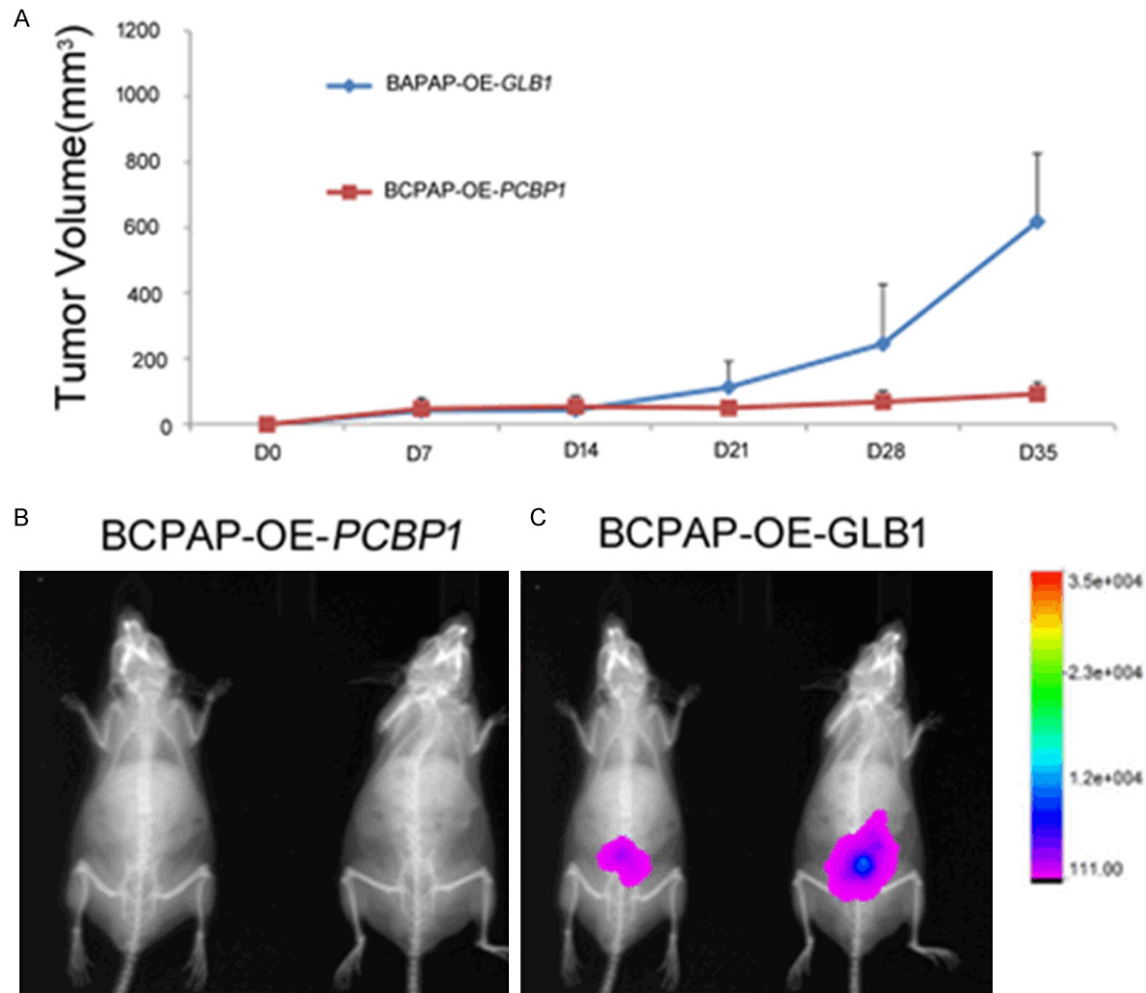


Figure 4. PCBP1 functions as a tumor suppressor in thyroid cancer. Luciferase expressing BCPAP stable transfectants, overexpressing either *GLB1* (encoding beta galactosidase) or *PCBP1*, were injected sub cutaneously into nude mice. Tumor formation in the BCPAP-PCBP1 overexpressing cells-injected mice was largely attenuated as quantified by (A) tumor volume over 35 days post-injection, and (B) overlay of X-ray and *in vivo* luciferase imaging in representative animals in the two experimental groups.

noprecipitates from corresponding cell lysates were used as substrates in an *in vitro* kinase reaction using recombinant Akt2 as the kinase. PCBP1 showed robust *in vitro* Akt2-mediated phosphorylation in both transiently and stably transfected WRO cells (**Figure 2B**), thus indicating that post-translational modifications of PCBP1 might not be responsible for its loss in thyroid cancer tissue specimens.

To explore if the loss of PCBP1 expression was due to being targeted by miRNA, we used *in situ* platforms, TargetScan and microCosm, to predict putative miRNAs targeting PCBP1. One of the miRNAs that came up in both platforms was miR-490-3p (**Figure 3A** and *data not*

shown). The miR-490-3p seed region in the 3'UTR of *PCBP1* was conserved across different species (*data not shown*).

We next determined if *PCBP1* is a bona fide target of miR-490-3p. To test this putative interaction, luciferase reporter constructs containing the wild-type *PCBP1* 3'UTR or a miR-490-3p binding mutant of the *PCBP1* 3'UTR reporter were transfected in WRO and BCAP cells (**Figure 3B**). Wild-type *PCBP1* 3'UTR containing reporter were inhibited 3.8 ± 0.02 folds ($P=0.004$) and 3.2 ± 0.05 folds ($P=0.003$) in WRO and BCPAP cells, respectively, compared to the miR-490-3p binding mutant reporter, which did not show any difference in the relative luciferase

ase activity in these two cell lines (**Figure 3B**), confirming that *PCBP1* mRNA was being targeted by the miR-490-3p in these thyroid cancer cell lines. Co-transfection of miR-490-3p antagomir also rescued the inhibition in both WRO and BCPAP cells further confirming *PCBP1* as a target of miRNA-490-3p (**Figure 3C**).

In order to understand the *in vivo* role of *PCBP1* in thyroid cancer formation, we finally did xenograft assays with BCPAP cells stably expressing firefly luciferase, and stably expressing either *GLB1* or *PCBP1*; whereas, BCPAP/*GLB1* cells formed steadily growing tumors by Day 35 (**Figure 4A, 4B**), the BCPAP/*PCBP1* cells did not form slowly regressing tumors ($P < 0.05$) (**Figure 4A, 4C**). Cumulatively, this indicated that loss of *PCBP1* expression function as a tumor suppressor in thyroid cancer, as revealed by lack of potent tumorigenic activity post-over-expression of *PCBP1*.

Discussion

Our results cumulatively show that miR-490-3p targets *PCBP1* in thyroid cancer resulting in its loss of expression. It remains to be determined if miR-490-3p expression is upregulated in thyroid cancer and if so by what mechanism. In addition, it also needs to be determined if loss of *PCBP1* expression is an underlying feature of other types of thyroid cancers, too. Our experiments suggest a causative role of loss of *PCBP1* expression in thyroid tumor formation and it will be important to determine how exactly loss of *PCBP1* contributes to thyroid cancer pathogenesis.

PCBP1 has been previously shown to regulate the stability of the pro-oncogenic p63 transcript in pancreatic, ovarian and breast cancer cell line [11]. In addition, repression of *PCBP1* has been shown to upregulate translation of genes and long non-coding RNA required for epithelial to mesenchymal transition and metastasis in different cancers, including breast, lung, and gastric cancer [12-16]. In addition, inactivating mutations in *PCBP1* has been identified in Burkitt lymphoma [17]. Thus, it might be possible that downregulation of *PCBP1* represents a central point, inhibition of which is a common mechanism to increase stemness and mesenchymal cell formation in a context dependent fashion, as observed by us in the current study and the others [11-17].

MiRNAs are evolutionarily conserved 21-23 nucleotides RNAs that regulate post-transcriptional gene expression either by blocking translation or degrading target messenger RNAs (mRNAs) and have been increasingly shown to function as tumor suppressors or oncogenes [18, 19]. MiRNAs can function in both normal and transformed cells and have even been shown to play a role in metastasis [20-23].

Our findings are in line with previous experiments showing a positive role of miR-490-3p in hepatocellular carcinoma [24]. However, miR-490-3p expression has been shown to be downregulated in Ewing's sarcoma and colon cancer [25, 26]. Hence, it would appear that whether miR-490-3p functions as a tumor suppressor or oncomir is context dependent.

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Disclosure conflict of interest

None.

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