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Long noncoding RNA PVT1 predicts poor prognosis and promotes the progression of colorectal cancer through the miR-24-3p/NRP1 axis in zebrafish xenografts

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Long noncoding RNAs (lncRNAs) play important roles in the progression of human cancer. It is reported that lncRNA plasmacytoma variant translocation 1 (PVT1) is involved in colorectal cancer (CRC), however, the underlying mechanism remains to be explored deeply, especially by *in vivo* models. In the present study, bioinformatics analysis showed that the expression level of PVT1 was upregulated in CRC tissues and highly associated with poor prognosis of CRC patients. In cultured CRC cells, knockdown of PVT1 inhibited cell proliferation and migration of CRC cells, while overexpression of PVT1 promoted the progression of CRC cells. In zebrafish xenografts, the silencing of PVT1 also suppressed the growth and metastasis of CRC cells. For mechanism studies, the binding relationships among PVT1, miR-24-3p, and Neuropilin 1 (NRP1) were predicted by starBase firstly. The luciferase reporter assays verified that PVT1 and NRP1 could bind with miR-24-3p directly. Further studies showed miR-24-3p negatively regulated the progression of CRC cells, the inhibition of miR-24-3p counteracted the repression effects of CRC progression when knocking down PVT1. In addition, the expression of NRP1 was regulated by PVT1, and NRP1 overexpression could partially rescue the inhibition effects of CRC progression when knocking down PVT1 *in vitro* and *in vivo*. Our study reveals that PVT1 promotes the proliferation and metastasis of CRC via regulating the miR-24-3p/NRP1 axis, which provides a prognosis biomarker and a potential therapeutic target for CRC patients.

Key words: lncRNA PVT1; miR-24-3p; NRP1; zebrafish xenograft; colorectal cancer

Colorectal cancer (CRC) is one of the common malignant cancers with high morbidity and mortality, it is the second cause of cancer death in the world [1, 2]. In recent years, although early diagnosis and new therapeutic approaches for CRC patients are developed rapidly, their poor prognosis and overall survival (OS) rate have not been improved significantly [3–5]. Therefore, understanding the molecular mechanisms in the development and progression of CRC is critical for changing such situations.

Long non-coding RNAs (lncRNAs), a class of non-coding transcripts more than 200 nucleotides in length, have been thought to be transcriptional "noise" before [6–8]. In recent years, it is proved that lncRNAs can regulate gene expression both at transcriptional and post-transcriptional levels [9, 10]. Many lncRNAs also have been identified to play important roles in human diseases, especially in cancer [11–16]. It is reported that lncRNAs regulate tumor proliferation, apoptosis, migration, and invasion, some of them have been identified as a potential prognostic biomarker of cancer [17–19]. The lncRNA plasmacytoma variant translocation 1 (PVT1) is located on human chromosome 8q24, which is the star genomic region for human cancer studies. The oncogene c-Myc locates in this region [20, 21] and it has been verified to play oncogenic roles in various human cancers, including lung cancer, breast cancer, and colorectal cancer [22]. Similar to c-Myc, PVT1 is also involved in multiple tumors and correlated with a poor prognosis [21, 23, 24]. However, the precise molecular mechanism of PVT1 in CRC needs to be investigated furtherly.

Many of lncRNAs, including PVT1, act as competing endogenous RNAs (ceRNAs) which competitively absorb miRNAs and indirectly regulate the expression of miRNA-targeting genes [25–27]. It has been reported that PVT1 can participate in the progression of CRC via ceRNA mechanisms, such as PVT1/miR-455/RUNX2 axis, PVT1/miR-106b-5p/FJX1 axis, and PVT1/miR-216a-5p/YBX1 axis [28]. PVT1 also can act as a sponge of miR-24-3p in LPS-induced cardiac fibroblasts and ischemic stroke [29, 30], while such regulation in tumor progression is still unclear.

Due to the difficulty, most studies of the ceRNA network have limited *in vivo* experiments, especially for tumor metastasis. The zebrafish xenograft model has been developed as a rapid and promising model for human cancer in recent years [31–33]. So far, more and more human cell line-derived zebrafish xenograft (zCDX) models have been established successfully, such as colorectal cancer, breast cancer, prostate cancer, lung cancer [32, 34], and they also have been applied to study the roles of lncRNA in human cancers [35]. Compared to mouse xenograft models, zebrafish xenografts can be used to evaluate the proliferation and metastasis of tumor cells simultaneously by direct observation *in vivo* [32, 36–38]. Taking these advantages, zebrafish xenograft provides an easy *in vivo* animal model for studying the ceRNA network.

In this study, we found that PVT1 was upregulated in CRC tissues and correlated with a poor prognosis by analyzing the public database of patients. Functional experiments showed that PVT1 promoted the proliferation and metastasis of CRC cells *in vitro* and *in vivo*. Furthermore, molecular mechanism studies showed for the first time that PVT1 upregulated the expression of NRP1 by competitively sponging miR-24-3p, and inhibition of miR-24-3p or overexpression of NRP1 could partially rescue the suppression effects of CRC cells when knocking down PVT1 *in vitro* and *in vivo*.

Patients and methods

TCGA and GEO database analysis. The expression levels of lncRNA PVT1 in tumor tissue samples were analyzed through the Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/) using The Cancer Genome Atlas (TCGA) database, which contains 275 colon adenocarcinoma tissue samples and 349 normal tissue samples, 92 rectum adenocarcinoma tissue samples and 318 normal tissue samples. The dataset GSE18105 from the Gene Expression Omnibus (GEO) database was used to analyze the expression levels of PVT1 in CRC tissues by the lnCAR software (https://lncar.renlab.org/), which contains 94 tumor samples and 17 normal samples. The dataset GSE41258 was used to compare the expression levels of PVT1 in primary and metastasis CRC tissues, which includes 67 metastasis tumors and 186 primary tumor samples by the lnCAR software. The dataset GSE28702 was also used to compare the expression levels of PVT1 in primary and metastasis CRC tissues, which includes 27 metastasis tumors and 56 primary tumor samples by the lnCAR software. The OS of CRC patients was analyzed by Kaplan-Meier plotter (https://kmplot.com/analysis/).

Cell culture. The human CRC cell lines were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Science (Shanghai, China). HCT116, SW620, and FHC cells were cultured in 1640 medium, SW480 cells were cultured in the DMEM medium, and LoVo cells were cultured in the F-12K medium. All culture media were supplemented with 10% fetal bovine serum (FBS), and all cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

RNA exaction and quantitative real-time PCR. Total RNA was exacted from cultured cells using TRIzol reagent (Invitrogen, CA, USA). Then the total RNA was reverse-transcribed to cDNA using random primers according to instructions of a PrimeScript RT kit (Takara, Dalian, China). The expression levels of different genes were detected by quantitative real-time PCR (qRT-PCR) using a SYBR Green Master Mix kit (Takara, Dalian, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control. The specific primers were used for the reverse-transcription of miRNA, and the sequences were: 5'-GTTGGCTCTGGTGCAGGGTCCGAG-GTATTCGCACCAGAGCCAACCTGTTC-3' (miR-24-3p), 5'-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCAC-CAGAGCCAACCGATGT-3' (miR-455-5p), 5'-GTTGGCTC-TGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAA-CAGGAGA-3' (miR-605-5p), 5'-GTTGGCTCTGGTGCAGG-GTCCGAGGTATTCGCACCAGAGCCAACGAGCTA-3' (miR-143-3p), 5'-GTTGGCTCTGGTGCAGGGTCCGAGG-TATTCGCACCAGAGCCAACACAAAC-3' (miR-497-5p). U6 was used as an internal control for detecting miRNA expression. All data were analyzed by the $2^{-\Delta\Delta Ct}$ methods. The primer sequences for qRT-PCR are listed in Table 1.

Cell transfection. A small interfering RNA (siRNA) targeting PVT1 (si-PVT1) (5'-CAGCCATCATGATGGT-ACT-3'), negative control (NC) siRNA (5'-TTCTCCGAA-CGTGTCACGT-3'), miR-24-3p mimics (5'-TGGCTCAGT-TCAGCAGGAACAG-3') or inhibitor (5'-CTGTTCCTGC-TGAACTGAGCCA-3') were all purchased from General Biosystems (China). The control plasmid (pcDNA3.1) and overexpression plasmids (pcDNA3.1-PVT1 or pcDNA3.1-NRP1) were synthesized by General Biosystems (China). The cells were cultured in the six-well plates, and the siRNAs or plasmids were transfected into the cells using Lipofectamine 2000 reagents (Invitrogen, USA) according to the manufacturer's protocol. After 24 h, the silencing efficiencies were examined by qRT-PCR.

Cell counting kit-8 assay. Cell proliferation was evaluated using Cell Counting Kit-8 (CCK-8, DOJINDO, Japan). The transfected cells were seeded at a density of 2,000 cells /well in the 96-well plates. 10 μl CCK-8 reagent containing 100 μl medium was added into each well and then cultured for 2 h. Cell proliferation was quantified by measuring the optional

Table 1. Primer sequences for qRT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
PVT1	TTGGCACATACAGCCATCAT	CAGTAAAAGGGGAACACCA
NRP1	CGGGACCCATTCAGGATCAC	GCTGATCGTACTCCTCTGGC
GAPDH	GGGAGCCAAAAGGGTCAT	GAGTCCTTCCACGATACCAA
miR-24-3p	GTTTGGCTCAGTTCAGCAG	GTGCAGGGTCCGAGGT
miR-455-5p	GGGTATGTGCCTTTGGACT	GTGCAGGGTCCGAGGT
miR-605-5p	GGTAAATCCCATGGTGCCT	GTGCAGGGTCCGAGGT
miR-143-3p	GTGGTGAGATGAAGCACTG	GTGCAGGGTCCGAGGT
miR-497-5p	GTTTCAGCAGCACACTGTG	GTGCAGGGTCCGAGGT
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT

density at 450 nm on a microplate reader (BioTek Elx800, USA) every 24 h from 0 to 96 h according to the manufacturer's instructions.

Colony formation assay. The transfected cells were seeded into six-well plates with a density of 600 cells/well. The culture media, containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, were replaced every three days. After 2 weeks, the colonies were washed twice with PBS, fixed with methanol for 10 min, and then stained with 0.1% crystal violet for 10 min. The stained colonies were photographed and counted.

Transwell assay. Cell migration was evaluated by Transwell assay using 24-well plates with 8 μm pore size chamber inserts. The transfected cells of 5×10^4 diluted with 200 μl serum-free medium were seeded into the upper chambers of transwell plates. Then the upper chambers were placed into the lower chambers in 24-well plates containing 800 μl medium with 10% FBS. After 24 h, the cells were fixed using methanol for 10 min and stained using 0.1% crystal violet for 10 min. After that, the cells were imaged under the inverted microscope (10×).

Western blot. The transfected cells were lysed by the Radio Immunoprecipitation Assay Lysis buffer (RIPA, Beyotime, China) for total protein extraction. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electro-transferred on polyvinylidene fluoride membranes (Millipore, Schwalbach, Germany), and then were blocked with 5% skim milk. Subsequently, the membranes were incubated with primary antibody (BOSTER, China) for neuropilin-1 (NRP1, 1:500, BM4125) or GAPDH (1:10000, BM1623) at 4 °C overnight. Then the membranes were put into the secondary antibody (1:5000, BA1054/BA1050, BOSTER, China) for 1 h at room temperature. After washing, the proteins were visualized with a BeyoECL plus kit (Beyotime, China).

Luciferase reporter assay. The potential binding site of PVT1 or NRP1 3' UTR with miR-24-3p was predicted by starBase (https://starbase.sysu.edu.cn/), miRDB (http://mirdb.org/), and DIANA (http://diana.imis.athena-innovation.gr/). The wild-type and mutant-type fragments of PVT1 and NRP1 3' UTR were synthesized and cloned into the pGL3-basic vector. The reporter plasmids were co-transfected with miR-24-3p mimics or NC mimics into HEK-293T cells using Lipofectamine 2000 reagent. After 48 h, the luciferase activity was detected by a luciferase assay kit (Promega, USA) according to the manufacturer's protocols.

Zebrafish xenograft models. The adult Tg(fli1a:EGFP) zebrafish were maintained at 28 °C and 14 h-10 h light-dark cycle in a fish auto culture system (Haishen, China). The embryos of zebrafish were cultured into 10% Hank's solution, which was composed of (in mM) 140 NaCl, 5.4 KCl, 0.25 Na_2HPO_4, 0.44 KH_2PO_4, 1.3 $CaCl_2$, 1.0 $MgSO_4$ and 4.2 $NaHCO_3$ (pH 7.2). The 48-hpf (hours post-fertilization) Tg(fli1a:EGFP) zebrafish embryos were used for cancer cell transplantation experiments in our study [39]. Zebrafish handling procedures were approved by the Institutional Animal Care and Use Committee of The Lianshui County People's Hospital, Kangda College of Nanjing Medical University (Huai'an, China).

Before injection, the tumor cells, transfected with siRNA or/and plasmid, were harvested and stained by CM-DiI (Invitrogen, USA) [35]. The 48-hpf zebrafish larvae were mounted with 1.2% low-melting agarose (Promega, USA), then approximately 400 labeled cells were injected into the perivitelline space (PVS) of zebrafish larvae under a microinjector (Picosprizer III, USA). Then the injected zebrafish larvae were cultured at 34 °C in a light incubator. To exclude the samples of unsuccessful transplantation, obvious more cell transplantation and less cell transplantation, the larvae with similar sizes of fluorescence area were selected for further research at 1 day post injection (dpi) and cultured at 34 °C until the end of experiments (Supplementary Figure S1). At 4 dpi, the zebrafish larvae were mounted with 1.2% low-melting agarose, then the images were acquired by stereotype microscope (MVX10, Olympus, Japan) or confocal microscope using 20x objective lens (Fluoview 3000, Olympus, Japan). The resolution of the images was 1600×1200 (MVX10) or 1024×1024 pixels (Fluoview 3000). After that, the images were used for evaluating the growth and metastasis of tumor cells by ImageJ software. The images were first segmented based on the fluorescence signal by ImageJ software, and the region of the yolk or trunk was chosen manually, then the total area with a positive signal in the yolk or trunk was calculated by ImageJ software. The total

area with a positive signal in the yolk or trunk was used to quantify the cell growth or metastasis, respectively (Supplementary Figure S2).

Statistical analysis. All the statistical data were analyzed using unpaired Student's t-test or Tukey's multiple comparisons tests after One-way ANOVA, and the figures were generated by GraphPad Prism 8.0. The values of $p<0.05$ were considered to be statistically significant. All results were presented as the mean ± SD.

Results

PVT1 is upregulated in CRC tissues and associated with poor prognosis of CRC patients. Firstly, by analyzing TCGA database, we found the expression of PVT1 was significantly upregulated both in colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ) compared to the normal tissues (Figure 1A). The data of the GEO dataset GSE18105 also showed that the expression of PVT1 was upregulated in

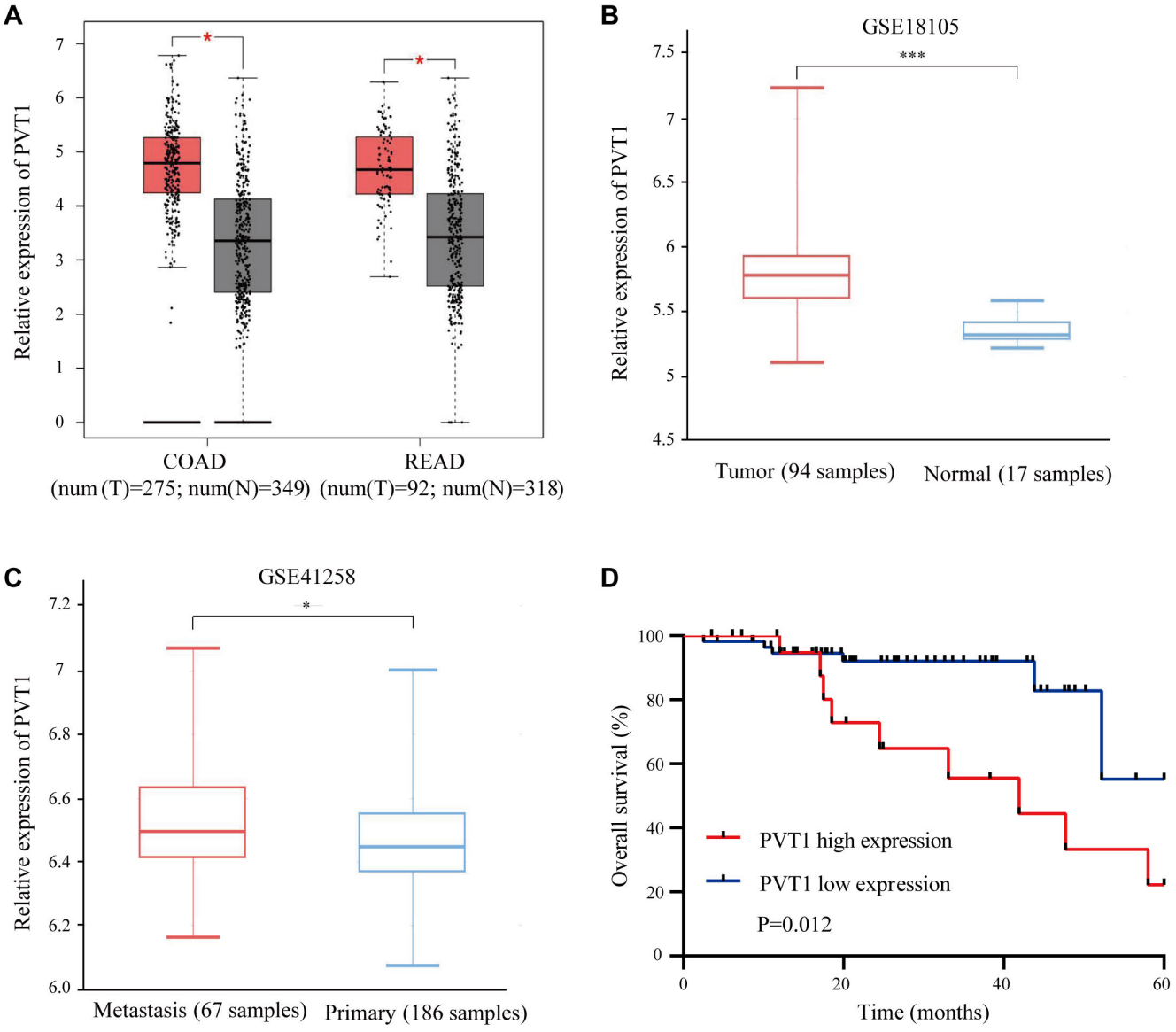

Figure 1. PVT1 is upregulated in CRC tissues and associated with a poor prognosis. A) The expression of PVT1 in CRC tissues (COAD and READ, T) was analyzed by TCGA database compared with normal tissues (N). B) The expression of PVT1 in CRC tissues was analyzed by the GEO dataset GSE18105 compared with normal tissues. C) The expression of PVT1 in metastasis CRC tissues was analyzed by GSE41258 compared with primary CRC tissues. D) OS of CRC patients with high PVT1 expression was analyzed by Kaplan-Meier plotter compared with that with low PVT1 expression. *p<0.05, ***p<0.001

CRC tissues (Figure 1B). To study the relationship between PVT1 and the metastasis of CRC, we then analyzed it in the lnCAR website and found the expression of PVT1 in metastasis tumors was a little higher than that in primary tumors of CRC patients in two GSE datasets (GSE41258 and GSE28702, Figure 1C, Supplementary Figure S3). Furthermore, the OS of CRC patients was also analyzed and showed that a high PVT1 expression level was correlated with a low survival rate of CRC patients (Figure 1D). These results present that the expression of PVT1 is upregulated in CRC tissues, and is also associated with poor prognosis of CRC patients.

PVT1 promotes the proliferation and migration of CRC cells *in vitro*. We examined the expression levels of PVT1 in four CRC cell lines (HCT116, SW480, SW620, and LoVo) and human normal colon epithelial cells (FHC). Compared with FHC, we found that PVT1 was highly expressed in CRC cells, especially in HCT116 and SW480 cell lines (Figure 2A). To verify the biological functions of PVT1 in CRC cells, we transfected siRNA or overexpression plasmid to downregulate or upregulate the expression of PVT1 in CRC cells. Compared with negative control siRNA (NC), PVT1 siRNA (si-PVT1) transfection efficiently decreased the expression of PVT1, with the knockdown efficiency of 79.7% and 89.4% in HCT116 and SW480 cells, respectively (Figure 2B). Overexpression plasmid (pcDNA3.1-PVT1) transfection increased the expression level of PVT1 by 128 times in LoVo and 56 times in SW620 cells respectively, which were compared to control (pcDNA3.1 empty vector transfection, Figure 2C, Supplementary Figure S4A). Then we used CCK-8 to assess the proliferation of CRC cells and found that PVT1 knockdown inhibited the proliferation of HCT116 (Figure 2D) and SW480 cells (Figure 2E) but the overexpression of PVT1 increased the proliferation of LoVo cells (Figure 2F) and SW620 cells (Supplementary Figure S4B). And the colony formation assays also verified the proliferation role of PVT1 in CRC (Figures 2G–2I). Subsequently, we used Transwell assays to assess the migration of CRC cells and found that the silencing of PVT1 significantly suppressed the cell migration in HCT116 (Figure 2J) and SW480 cells (Figure 2K). On the contrary, the migration of LoVo and SW620 cells was enhanced when overexpressing PVT1 (Figure 2L, Supplementary Figure S4C). Taken together, these data show that PVT1 promotes the proliferation and migration of CRC cells *in vitro*.

Knockdown of PVT1 inhibits the growth and metastasis of CRC cells *in vivo*. To verify the functional roles of PVT1 in CRC cells *in vivo*, the zebrafish xenograft models were used. HCT116 cells, transfected with si-PVT1 or NC and labeled by CM-DiI, were transplanted into the PVS of 48-hpf (hours post fertilization) Tg(fli1a: EGFP) transgenic zebrafish larvae, which had the vascular endothelial cells labeled by EGFP. At 4 days post injection (dpi), we quantified the area of CM-DiI positive signals in the yolk representing the cell growth (Figures 3A, 3B), and found silencing PVT1 inhibited the growth of HCT116 cells compared with the NC groups in zebrafish xenograft models (Figure 3C). We then quantified the area of CM-DiI positive in the trunk (Figures 3D, 3E) representing cell metastasis, and found that PVT1 knockdown also inhibited the metastasis of HCT116 cells in zebrafish xenograft models (Figure 3F). Similarly, the knockdown of PVT1 also inhibited the growth and metastasis of SW480 cells in zebrafish xenograft models (Figures 3G–3L). These results demonstrate that PVT1 regulates the growth and metastasis of CRC cells *in vivo*.

PVT1 negatively regulates the expression of miR-24-3p as a sponge in CRC cells. Previous studies reveal that PVT1 can act as a miRNA sponge to regulate the progression of various tumors [22, 40]. We used three online tools to predict candidate miRNAs, which could bind with PVT1, including starBase, miRDB, and DIANA Tools. By cross-comparison, we found 13 miRNAs predicted by all three online tools (Figure 4A). Then we examined the expressions of miR-24-3p, miR-143-3p, miR-455-5p, miR-497-5p, and miR-605-5p when knocking down PVT1, and found three miRNAs (miR-24-3p, miR-455-5p, miR-605-5p) whose expression levels were upregulated when knocking down in SW480 cells (Figure 4B). It is reported that miR-24-3p plays regulation roles in the proliferation, migration, and invasion of CRC cells [41] and our analysis also showed its potential association with the poor prognosis of CRC (Figure 4C). Therefore, we chose miR-24-3p for further studies. According to the predicted binding site between PVT1 and miR-24-3p by starBase (Figure 4D), the luciferase-reporter assays were performed for verification *in vitro*. Compared with control, we found that the luciferase activity was suppressed when co-transfecting PVT1 reporter plasmid and miR-24-3p mimics in HEK-293T cells (Figure 4E), while such regulation was totally blocked when co-transfecting the mutant PVT1 reporter plasmid and miR-24-3p mimics (Figures 4D, 4E). These results suggest that PVT1 negatively regulates the expression of miR-24-3p through binding with miR-24-3p directly in CRC cells.

Knockdown of miR-24-3p promotes the proliferation and metastasis of CRC cells *in vitro* and *in vivo*. To study the roles of miR-24-3p in CRC, we efficiently silenced the expression of miR-24-3p by transfecting miR-24-3p inhibitor (Figure 4F). CCK-8 and Transwell assays showed that miR-24-3p inhibition promoted the proliferation and migration of SW480 cells *in vitro* (Figures 4G, 4H). By using zebrafish xenograft models, the knockdown of miR-24-3p also promoted the growth and metastasis of CRC cells *in vivo* (Figures 4I, 4J). These data show that miR-24-3p negatively regulates the progression of CRC cells *in vitro* and *in vivo*.

Knockdown of miR-24-3p rescues the progression of CRC cells, which is repressed by PVT1 silencing. To determine whether miR-24-3p mediated the regulation of PVT1 in CRC progression, we studied it by co-transfecting si-PVT1 and miR-24-3p inhibitor into SW480 cells simultaneously. Similar to our previous results, the knockdown of PVT1 suppressed the proliferation of CRC cells, but miR-24-3p

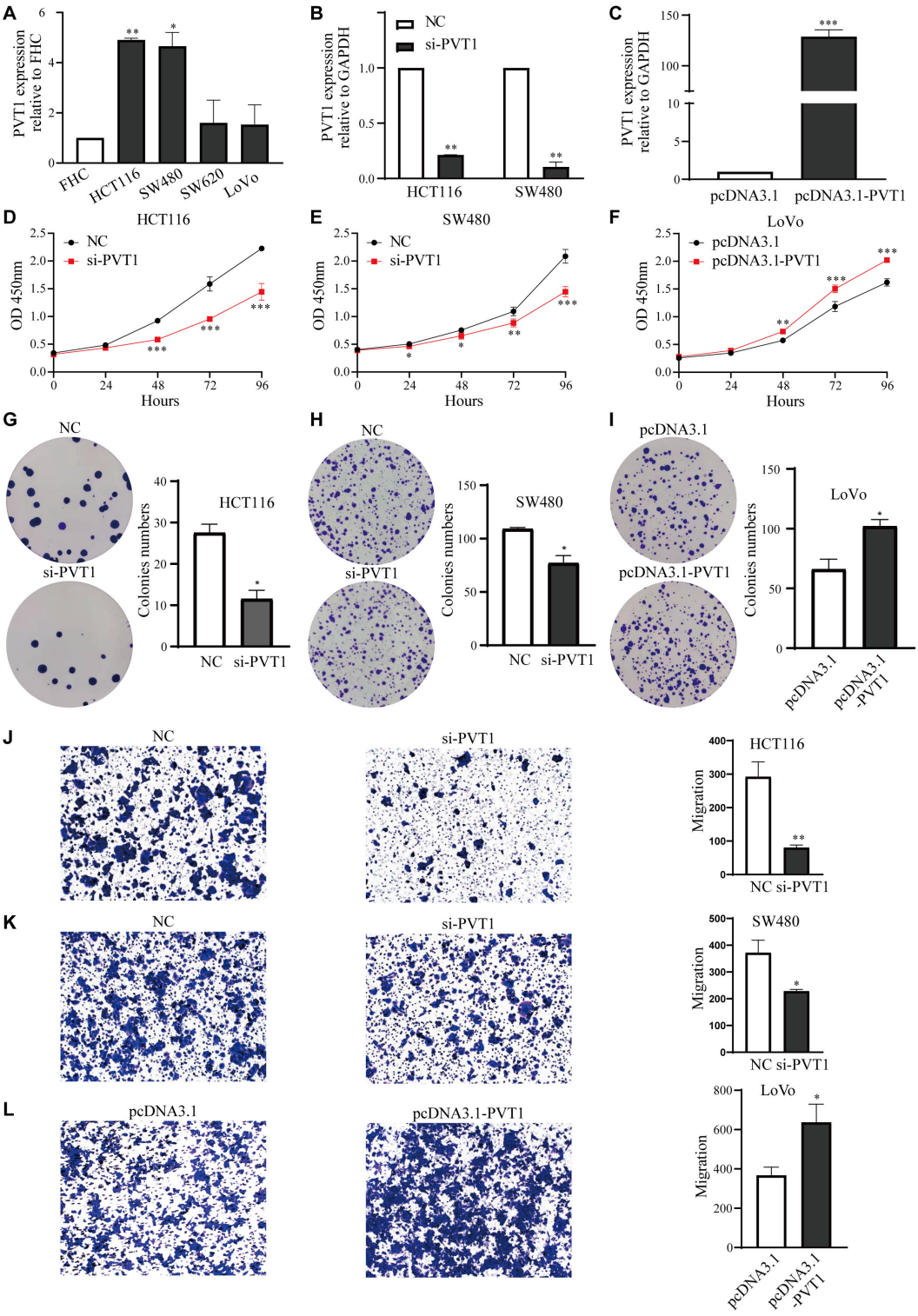

Figure 2. PVT1 promotes the proliferation and migration of CRC cells *in vitro*. A) The expression level of PVT1 was measured by qRT-PCR in human CRC cell lines (HCT116, SW480, SW620, LoVo) compared with the normal colon epithelial cells (FHC). B) The knockdown efficiency of PVT1 siRNA was examined by qRT-PCR in HCT116 and SW480 cells. C) The overexpression efficiency of PVT1 plasmid was examined by qRT-PCR in LoVo cells. D–F) CCK-8 assays were performed to evaluate the cell proliferation of HCT116 (D), SW480 (E), and LoVo cells (F) when downregulating or upregulating the expression of PVT1. G–I) Colony formation assays were performed to evaluate the cell proliferation in HCT116 (G), SW480 (H), and LoVo cells (I) when downregulating or upregulating the expression of PVT1. J–L) Transwell assays were performed to evaluate the cell migration in HCT116 (J), SW480 (K), and LoVo cells (L) when downregulating or upregulating the expression of PVT1. *p<0.05, **p<0.01, ***p<0.001

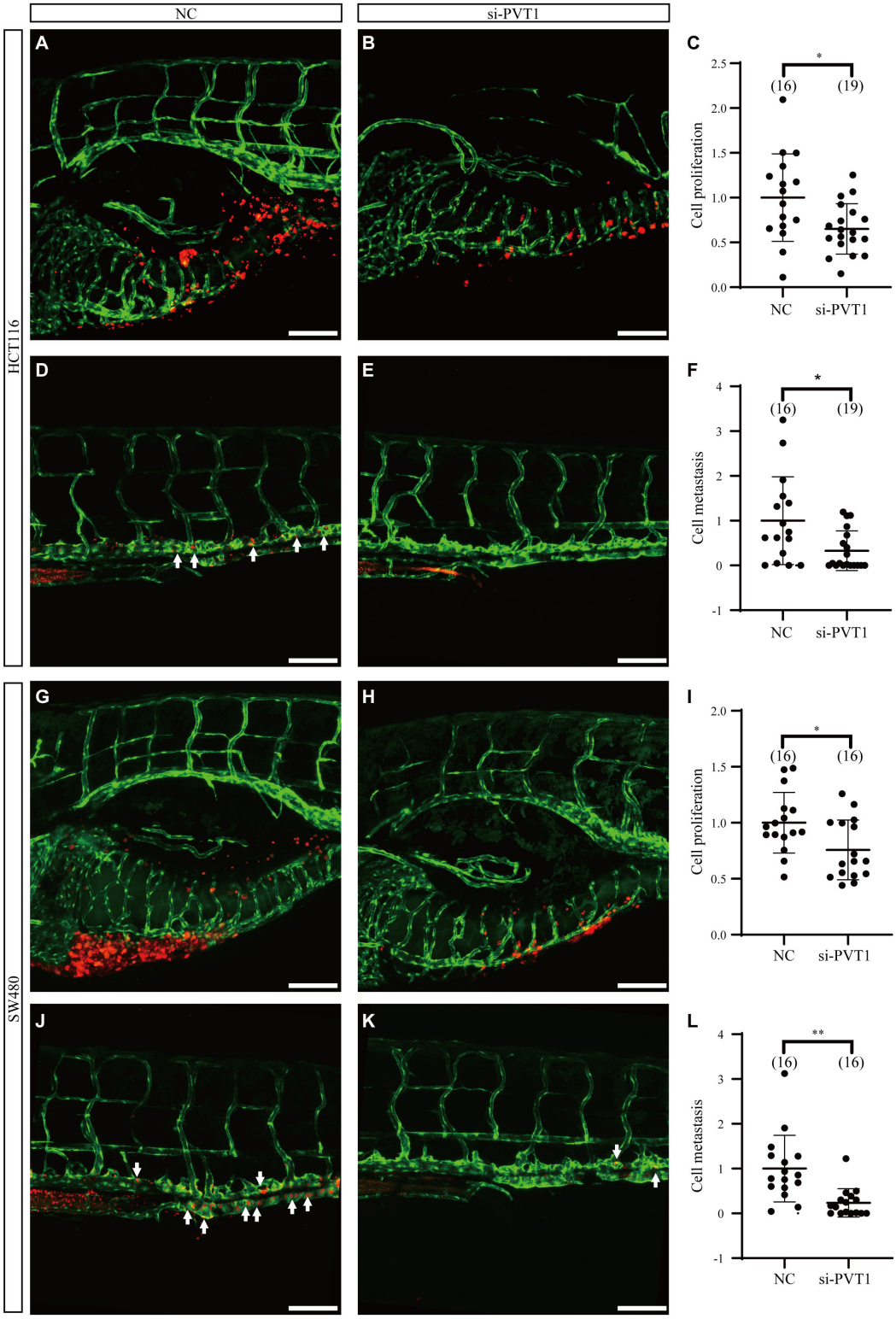

Figure 3. PVT1 knockdown inhibits the growth and metastasis of CRC cells in zebrafish xenografts. A, B) The CM-DiI-positive signals in the yolk of zebrafish represented the proliferation of HCT116 when transfecting NC (A) or si-PVT1 (B). C) Quantification of cell proliferation in zebrafish xenograft when transfecting NC or si-PVT1 in HCT116 cells. D, E) The CM-DiI-positive signals in the truck of zebrafish represented the metastasis of HCT116 when transfecting NC (D) or si-PVT1 (E). F) Quantification of cell metastasis in zebrafish xenograft when transfecting NC or si-PVT1 in HCT116 cells. G–L) Cell proliferation (G–I) and metastasis (J–L) in zebrafish xenograft when transfecting NC or si-PVT1 in SW480 cells. Arrows represent the metastasis CRC cells. Scale: 100 μm. *p<0.05, **p<0.01

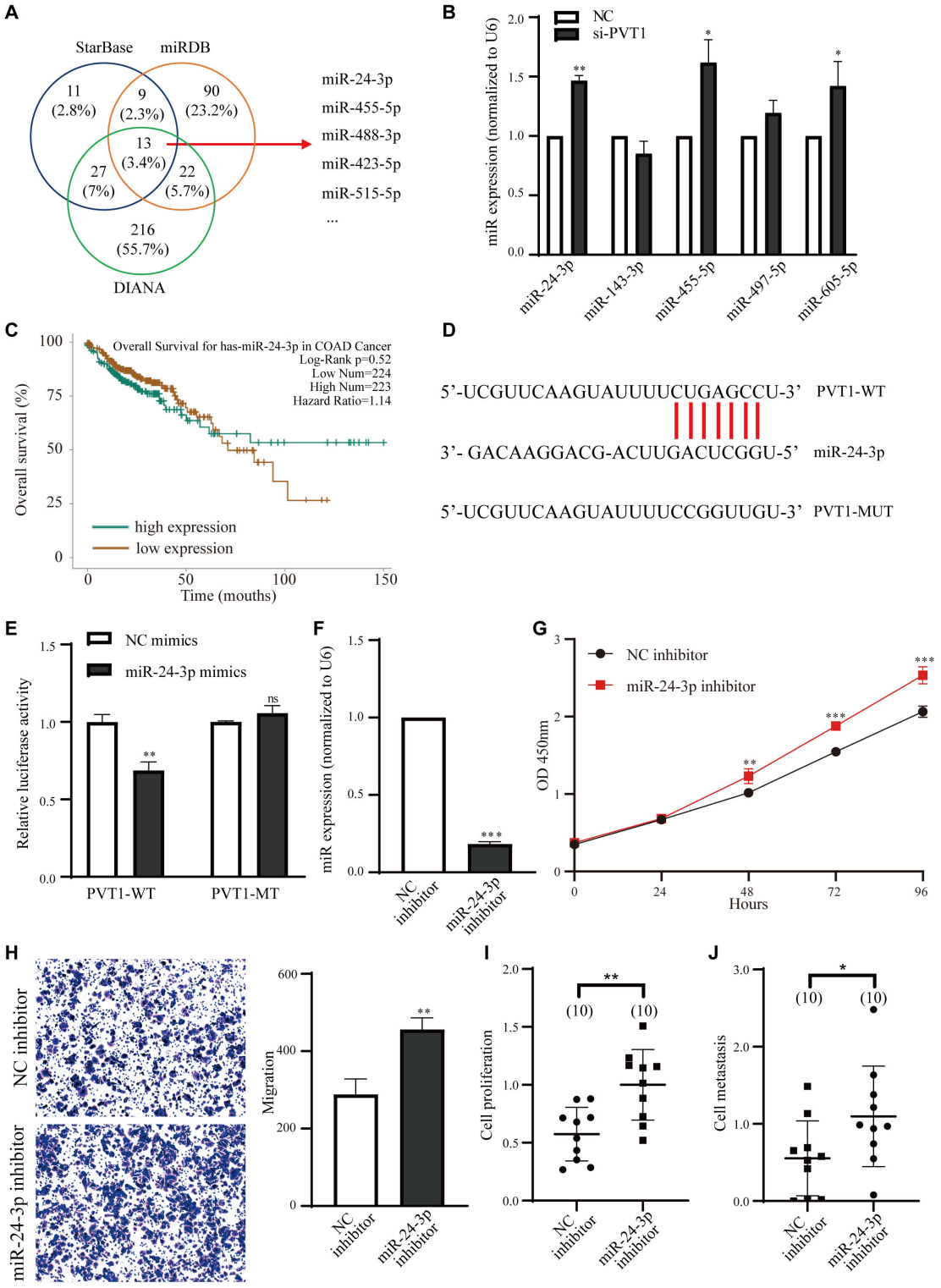

Figure 4. miR-24-3p binds with PVT1 and negatively regulates CRC progression *in vitro* and *in vivo*. A) Venn diagram for miRNA candidates that might bind with PVT1. B) The expression levels of five miRNAs were examined when knocking down PVT1. C) The correlation between the expression levels of miR-24-3p and OS of CRC patients. D) The schematic diagram of the potential binding site between miR-24-3p and PVT1 which was predicted by starBase. E) Luciferase activities of PVT1-WT or PVT1-MT were examined when co-transfecting with miR-24-3p mimic. F) The knockdown efficiency of miR-24-3p when transfecting its inhibitor. G, H) The proliferation and migration of CRC cells when silencing miR-24-3p. I, J) Quantification of the proliferation and metastasis of CRC cells when silencing miR-24-3p by zebrafish xenografts. *p<0.05, **p<0.01, ***p<0.001

inhibition counteracted the suppression effects, which were caused by PVT1 knockdown (Figure 5A). Transwell assay also showed that the silencing of miR-24-3p partly restored cell migration of CRC cells which was suppressed by PVT1 knockdown (Figure 5B). These results indicate that PVT1 promotes the progression of CRC cells through miR-24-3p.

NRP1 is a direct target of miR-24-3p in CRC cells. To find the direct downstream target genes of miR-24-3p, we also used starBase, miRDB, and DIANA tools for prediction. A serious of tumor-related genes were predicted by cross-comparison, such as NRP1, PAK4, MMP14, KLF4, FSCN1 (Figure 6A). We selected NRP1 for further research because it was reported that NRP1 was involved in the progression of CRC [42]. Then we overexpressed miR-24-3p by transfecting its mimics into SW480 cells, and found that the expression of NRP1 was decreased both on transcriptional and translational expression levels (Figures 6B, 6C). According to the binding site between 3'UTR of NRP1 and miR-24-3p, which was predicted by the starBase tool (Figure 6D), we did the luciferase reporter assays and found that miR-24-3p could bind with 3'UTR of NRP1 directly (Figure 6E). These data show that miR-24-3p can downregulate the expression of NRP1 in CRC cells by binding the 3'UTR of NRP1 directly.

Overexpression NRP1 rescues the progression of CRC cells, which is suppressed by miR-24-3p overexpression. To determine whether NRP1 mediated the regulation of miR-24-3p in CRC progression, we performed similar experiments by co-transfection NRP1-overexpressing plasmid (pcDNA3.1-NRP1) and miR-24-3p mimic into SW480 cells simultaneously. First, we transfected pcDNA3.1-NRP1 into SW480 cells and examined the overexpression efficiency by qRT-PCR and western blot. We found NRP1-overexpressing plasmid transfection could efficiently upregulate the expression level of NRP1 (Figures 6F, 6G). NRP1 overexpression not only promoted the proliferation of CRC cells but also rescued the CRC proliferation, which was suppressed by miR-24-3p overexpression (Figure 6H). Transwell assays also showed similar rescue roles when overexpressing NRP1 in the miR-24-3p-overexpressing group (Figure 6I). These results suggest that miR-24-3p regulates the progression of CRC cells via targeting NRP1.

Knockdown of PVT1 suppresses the growth and metastasis of CRC cells by regulating NRP1 expression *in vitro* and *in vivo*. To investigate whether PVT1 functioned through the NRP1 pathway in CRC cells, we first detected the expression level of NRP1 when knocking down PVT1 in SW480 cells. We found that the PVT1 knockdown reduced the expression level of NRP1 (Figures 7A, 7B). Then we co-transfected si-PVT1 and pcDNA3.1-NRP1 in CRC cells, and we also found the suppression effects, which were caused by the PVT1 knockdown were partially counteracted by NRP1 overexpression both in cell proliferation and migration (Figures 7C, 7D). NRP1-related OS analysis in CRC patients also indicated its clinical significance (Figure 7E), and we then examined the PVT1/NRP1 regulatory pathway in zebrafish xenografts. Zebrafish xenograft experiments also showed that NRP1 overexpression could rescue the CRC progression which was suppressed by PVT1 knockdown (Figures 7F, 7G). The results indicate that PVT1 regulates the progression of CRC cells through the NRP1 pathway *in vitro* and *in vivo*.

Discussion

It has been reported that the expression of PVT1 is elevated in multiple cancers, such as gastric cancer, breast cancer, and lung cancer [43]. PVT1 has been identified to play oncogenic roles through various pathways and to associate with poor prognosis in CRC patients [23]. In the present study, we found that PVT1 was upregulated in CRC tissues and predicted the poor prognosis by analyzing a public database. Functional studies demonstrated that PVT1 could promote the proliferation and metastasis of CRC cells *in vitro* and *in vivo*. These results verify the oncogenic roles of PVT1 in CRC.

PVT1 has been identified that can function as a miRNA sponge to regulate miRNA-targeting genes in CRC cells [22, 26, 30, 43]. In this study, we screened the miRNAs which could be absorbed by PVT1 in CRC cells. We found miR-24-3p could bind with PVT1 directly, and its expression level was regulated by PVT1. It is also reported that miR-24-3p functions as a tumor suppressor, which is downregulated in CRC tissues and inhibits the proliferation, migration, and invasion of CRC cells [41]. In the previous study, PVT1 has been proven that can sponge miR-24-3p in LPS-induced cardiac fibroblasts and ischemic stroke, while thus regulation has not been proved in cancers. In this study, bioinformatics analysis indicated that the expression level of miR-24-3p and OS of CRC patients were negatively correlated. Moreover, we also found that miR-24-3p knockdown not only promoted the progression of CRC but also prevented the inhibition effects of PVT1 silencing. These data demonstrate that PVT1 can function as an oncogene by sponging miR-24-3p in CRC.

NRP1 was proven to promote cell viability, migration, and invasion in CRC [42], and it was also a target gene of miR-24-3p [44, 45]. Bioinformatics analysis indicated that the expression level of NRP1 predicted the poor prognosis of CRC (Figure 7E). Based on it, we further studied whether the miR-24-3p/NRP1 axis played roles in the progression of CRC cells. Our data showed that miR-24-3p could directly bind with the 3'UTR of NRP1 and regulate the proliferation and migration of CRC cells through NRP1. Then we investigated whether PVT1 functioned through NRP1 in CRC. We found that the PVT1 knockdown decreased the expression of NRP1. NRP1 overexpression could rescue the tumor progression, which was inhibited by PVT1 silencing in CRC cells both *in vitro* and *in vivo*. These results reveal for the first time that PVT1 can function as a ceRNA by regulating the miR-24-3p/NRP1 axis in CRC cells.

Mouse xenograft models are widely applied in human cancer research, and they are also used for verification of

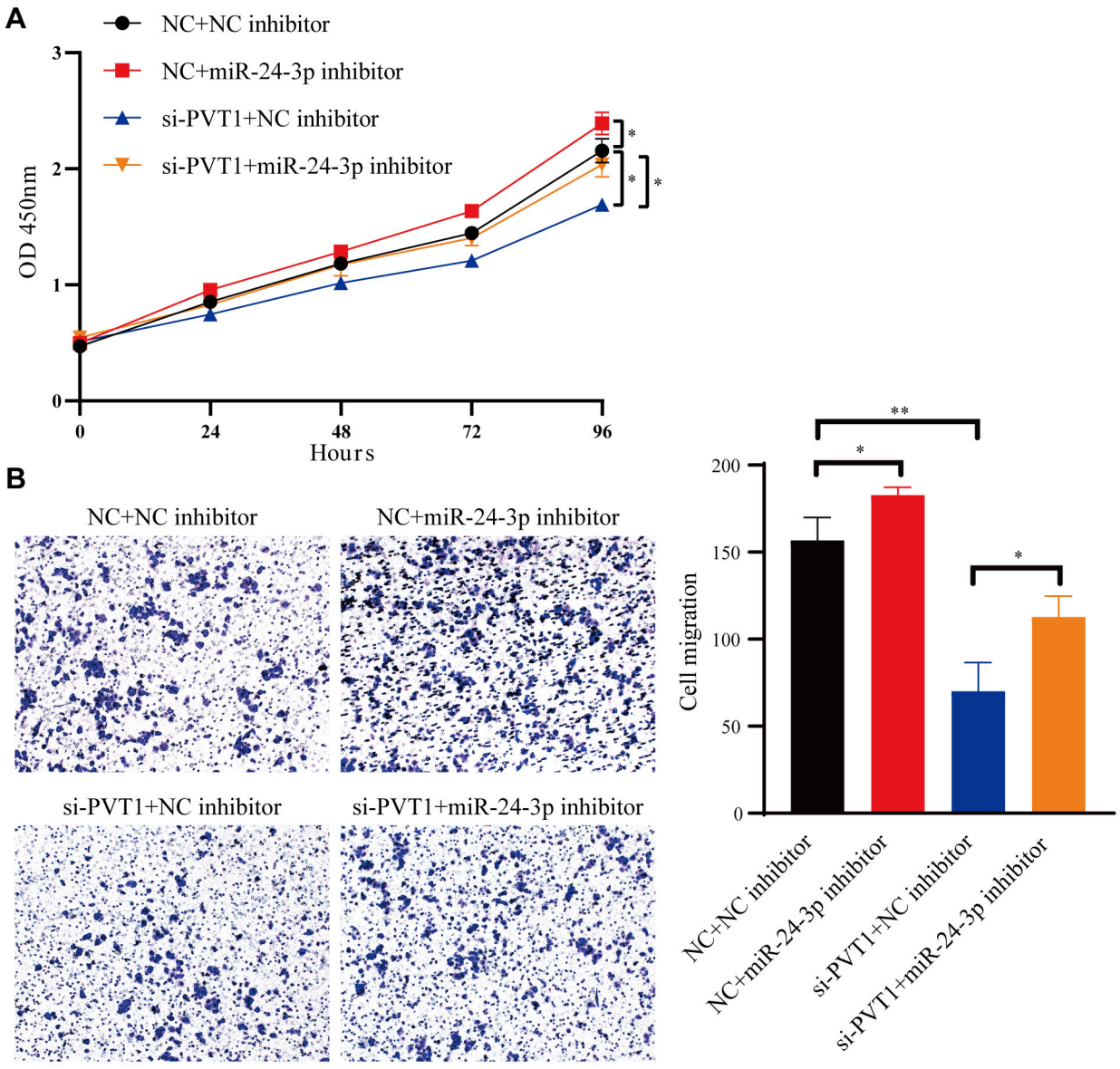

Figure 5. Knockdown of miR-24-3p rescues the progression of CRC cells, which is suppressed by PVT1 silence. A) The cell proliferation was evaluated by the CCK-8 assay when co-transfected miR-24-3p inhibitor and si-PVT1. B) The cell migration was evaluated by Transwell assay when co-transfected miR-24-3p inhibitor and si-PVT1. *p<0.05, **p<0.01

PVT1 in the proliferation of CRC, however, the metastasis mouse model for studying the function of PVT1 is very few [46, 47]. Compared with mouse models, zebrafish xenografts have multiple advantages in tumor biology. First, we can access the proliferation and metastasis of tumor cells within 96 hours after transplantation. Second, the proliferation and metastasis can be analyzed simultaneously by monitoring the behavior of tumor cells *in vivo* due to the transparent zebrafish larvae. Third, the genetic operation in cancer cells was relatively easy in zebrafish xenografts. Instead of shRNA plasmid transfection, siRNA transfected tumor cells can be used for the functional verification in 4 days. Using mouse models, He and his colleagues reported that PVT1 overexpression promoted the metastasis of CRC cells from spleen parenchyma to liver during 28 days post-injection [46]. In this study, we showed that silencing PVT1 by siRNA transfection also inhibited the proliferation and metastasis of CRC cells at 4 dpi in zebrafish xenografts, which was consistent with that in mouse models. By taking advantage of zebrafish xenografts, we furtherly not only verified the relationship between miR-24-3p and NRP1 in CRC progression but also demonstrated the regulatory mechanism between PVT1 and

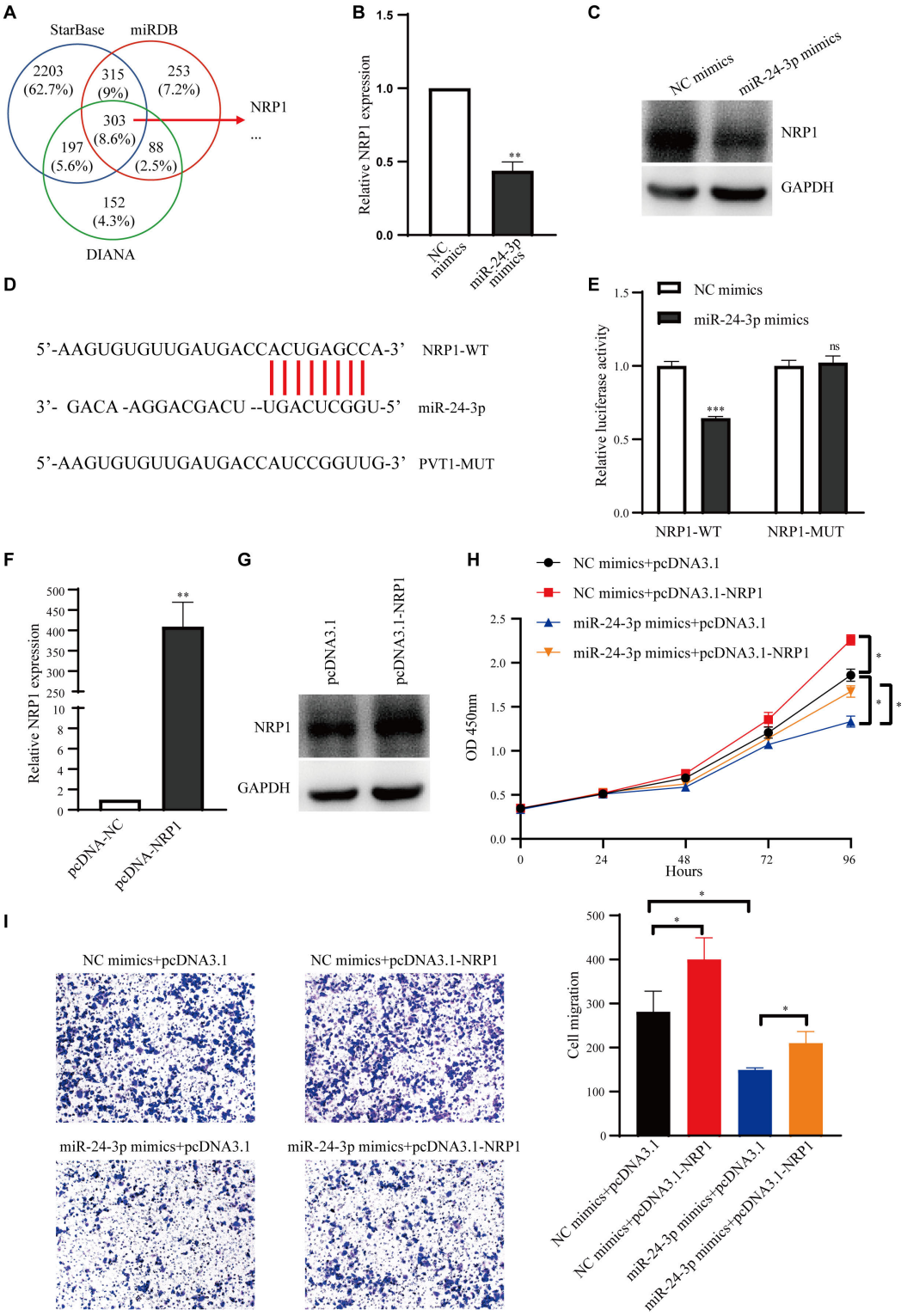

Figure 6. miR-24-3p negatively regulates CRC progression via targeting NRP1 directly. A) Venn diagram for the potential target genes of miR-24-3p. B, C) The expression of NRP1 was examined by qRT-PCR and western blot when overexpressing miR-24-3p in CRC cells. D) The schematic diagram of the potential binding site between miR-24-3p and NRP1. E) Luciferase activities of NRP1-WT and NRP1-MUT were examined when co-transfected with miR-24-3p mimic. F, G) The expression of NRP1 was measured by qRT-PCR and western blot when transfecting NRP1 overexpression plasmid. H, I) The proliferation (H) and migration (I) of CRC cells were examined when co-transfecting miR-24-3p mimic and NRP1 overexpression plasmid. *p<0.05, **p<0.01, ***p<0.001

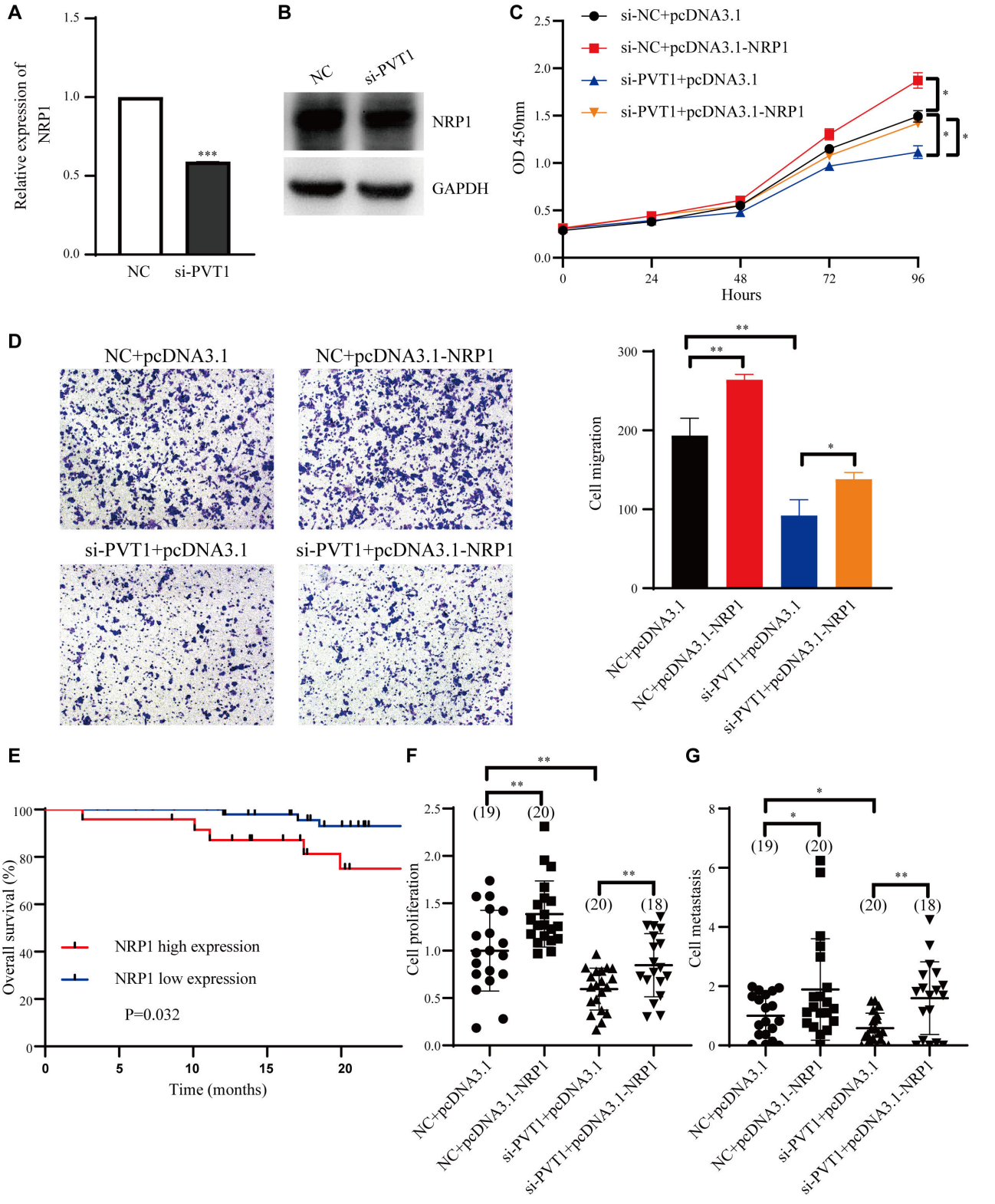

Figure 7. PVT1 regulates the progression of CRC cells via NRP1 *in vitro* and *in vivo*. A, B) The expression of NRP1 was examined when silencing PVT1 in CRC cells by qRT-PCR (A) and western blot (B). C, D) The proliferation (C) and migration (D) of CRC cells when co-transfected si-PVT1 and NRP1 overexpression plasmid. E) The correlation between the expression levels of NRP1 and OS of CRC patients. F, G) Quantification of the proliferation and metastasis of CRC cells when co-transfected si-PVT1 and NRP1 overexpression plasmid by zebrafish xenografts. *p<0.05, **p<0.01, ***p<0.001

NRP1 *in vivo*. These data suggest that zebrafish xenograft could be a reliable and feasible model for human cancer research, especially for ceRNA mechanism verification *in vivo*.

In summary, our results demonstrated the oncogenic roles of PVT1 in CRC cell lines. Additionally, we revealed a novel signaling pathway of PVT1 in CRC, which functioned as a ceRNA by regulating the miR-24-3p/NRP1 axis. Our results indicate that PVT1 could be a molecular marker of diagnosis and prognosis and also a potential treatment target for CRC patients. A preprint has previously been published [48].

Supplementary information is available in the online version of the paper.

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Long noncoding RNA PVT1 predicts poor prognosis and promotes the progression of colorectal cancer through the miR-24-3p/NRP1 axis in zebrafish xenografts

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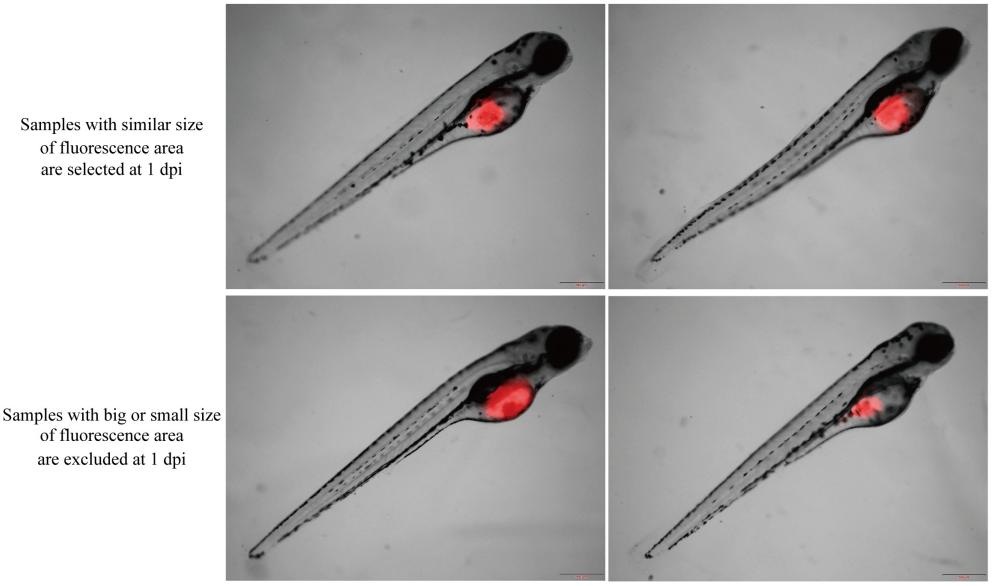
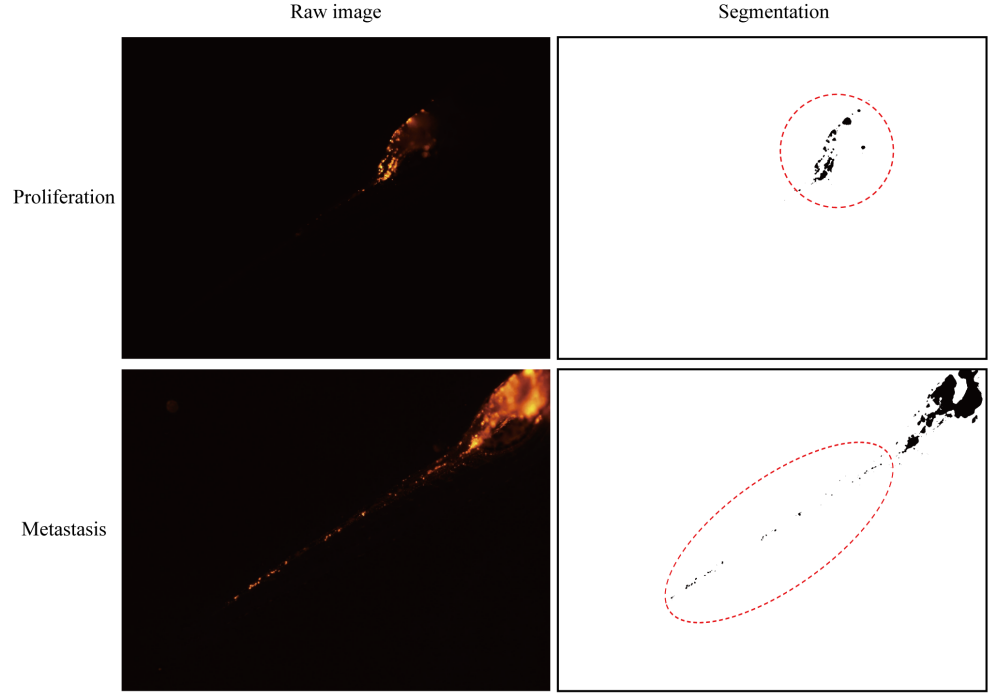
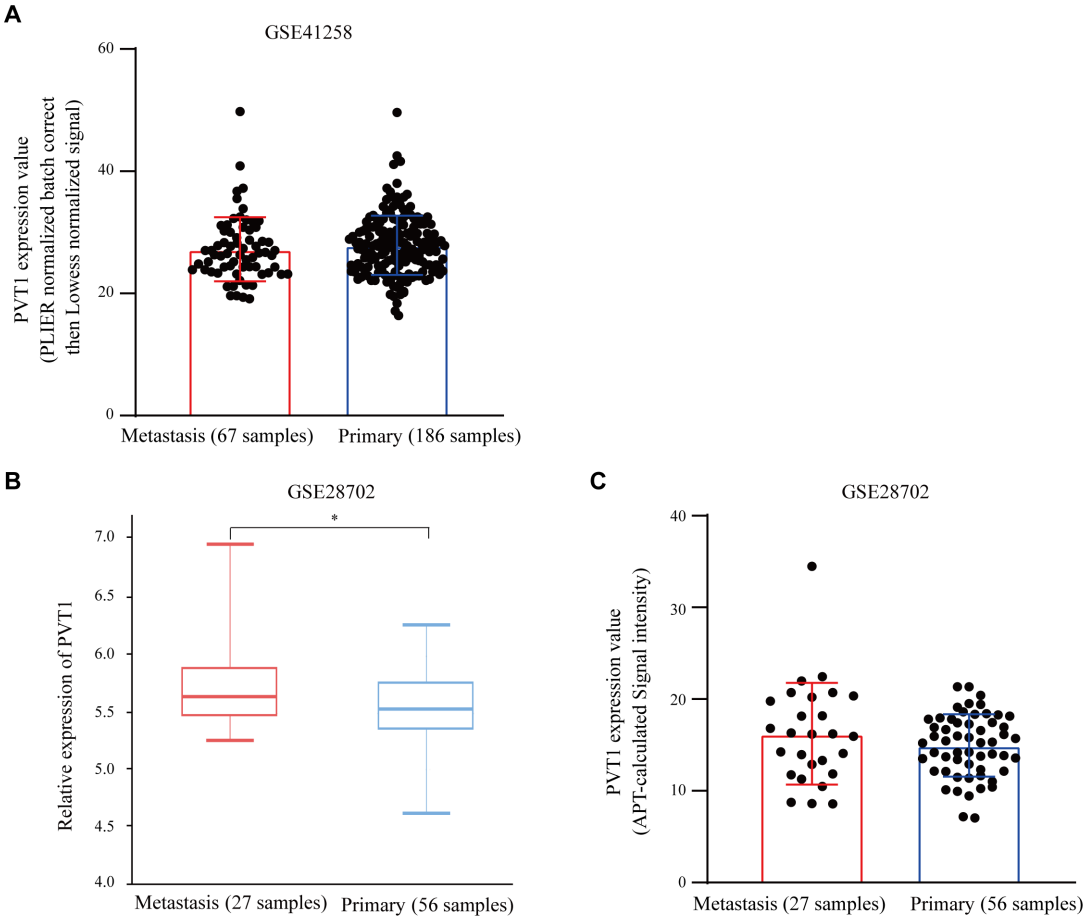
Supplementary Information



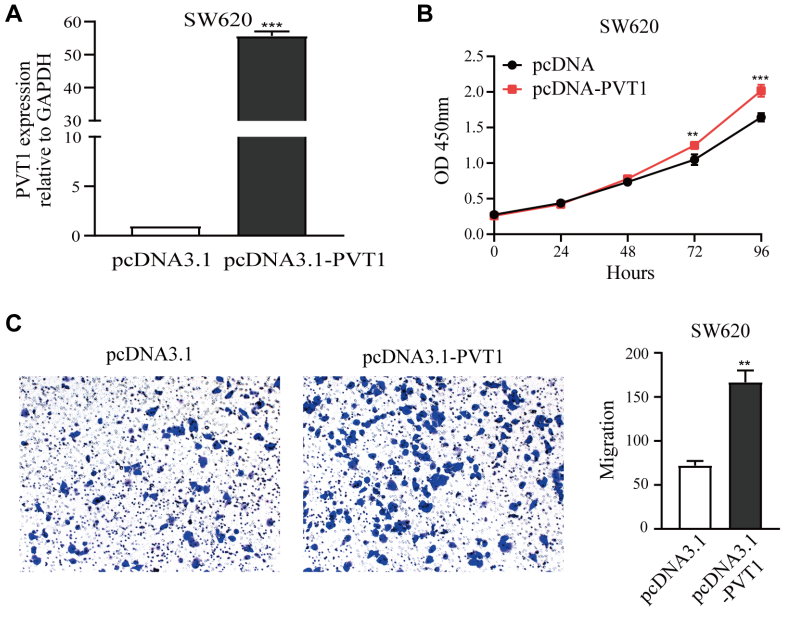
Supplementary Figure S1. The selection criteria of transplanted zebrafish samples at 1 dpi.



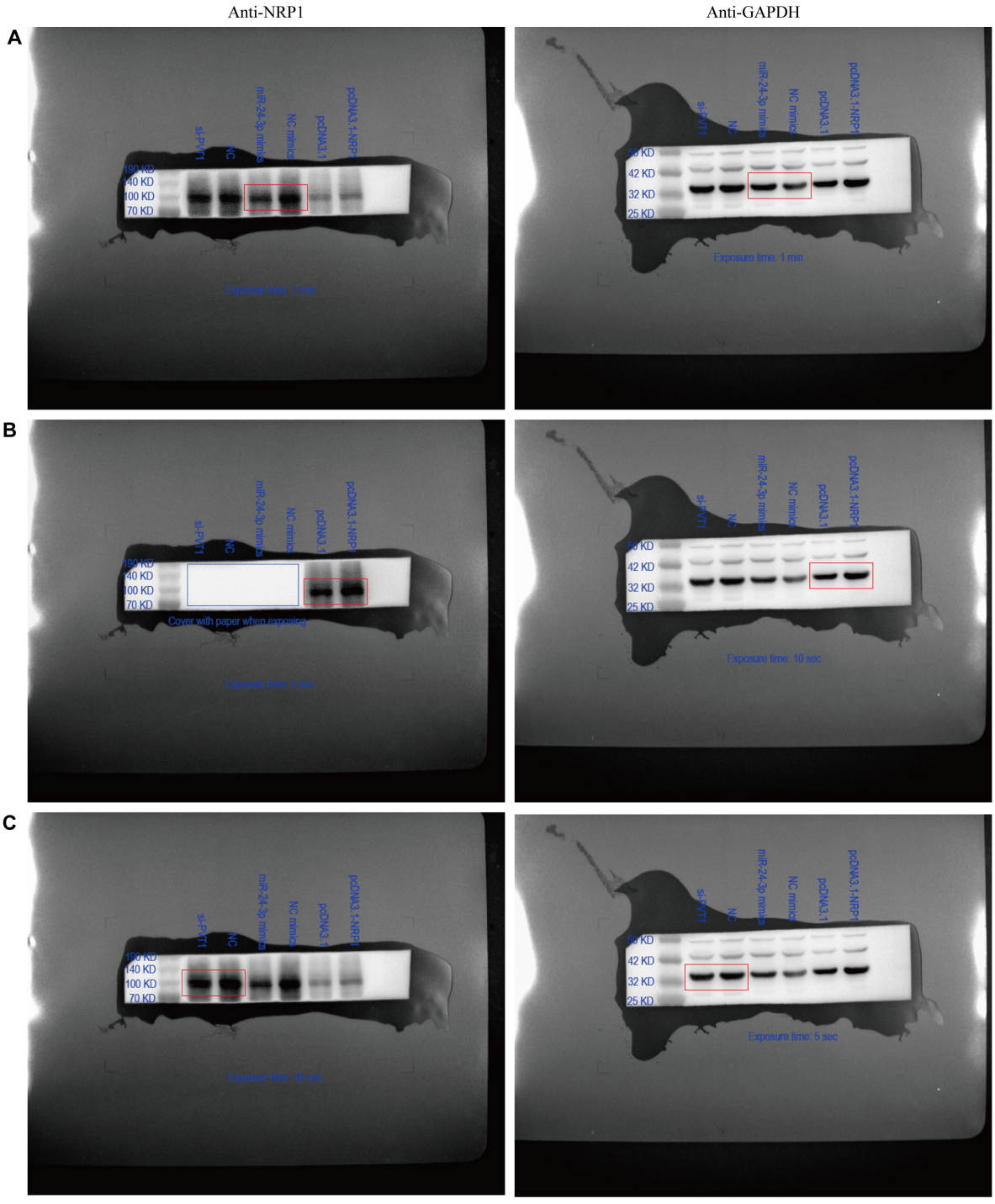
Supplementary Figure S2. The quantification method for assessing the growth and metastasis of tumor cells based on the fluorescence images.



Supplementary Figure S3. The comparison of PVT1 expression between metastasis CRC tissues and primary CRC tissues in GSE41258 or GSE28702. A) The SWARM plot of PVT1 expression in metastasis CRC tissues and primary CRC tissues in GSE41258 dataset based on the data of expression value (Series Matrix File(s)) from GEO datasets in NCBI website. B) The comparison of PVT1 expression between metastasis CRC tissues and primary CRC tissues in GSE28702 which is analyzed in lnCAR website. C) The SWARM plot of PVT1 expression in metastasis CRC tissues and primary CRC tissues in GSE28702 dataset based on the data of expression value (Series Matrix File(s)) from GEO datasets in NCBI website.



Supplementary Figure S4. PVT1 overexpression promotes the proliferation and migration of SW620 cells. A) The overexpression efficiency of PVT1 plasmid was examined by qRT-PCR in SW620 cells. B) CCK-8 assay was performed to evaluate the cell proliferation of SW620 cells when upregulating the expression of PVT1. C) Transwell assay was performed to evaluate the cell migration in SW620 cells when upregulating the expression of PVT1. **p<0.01, ***p<0.001



Supplementary Figure S5. Full Western blot images of anti-NRP1 and anti-GAPDH. A) Full Western blot images of miR-24-3p overexpression, which is supplementary to Figure 6C. B) Full Western blot images of NRP1 overexpression, which is supplementary to Figure 6G. C) Full Western blot images of PVT1 knockdown, which is supplementary to Figure 7B.