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Supplemental Information

miR-582-3p and miR-582-5p Suppress Prostate Cancer Metastasis to Bone by Repressing TGF-β Signaling

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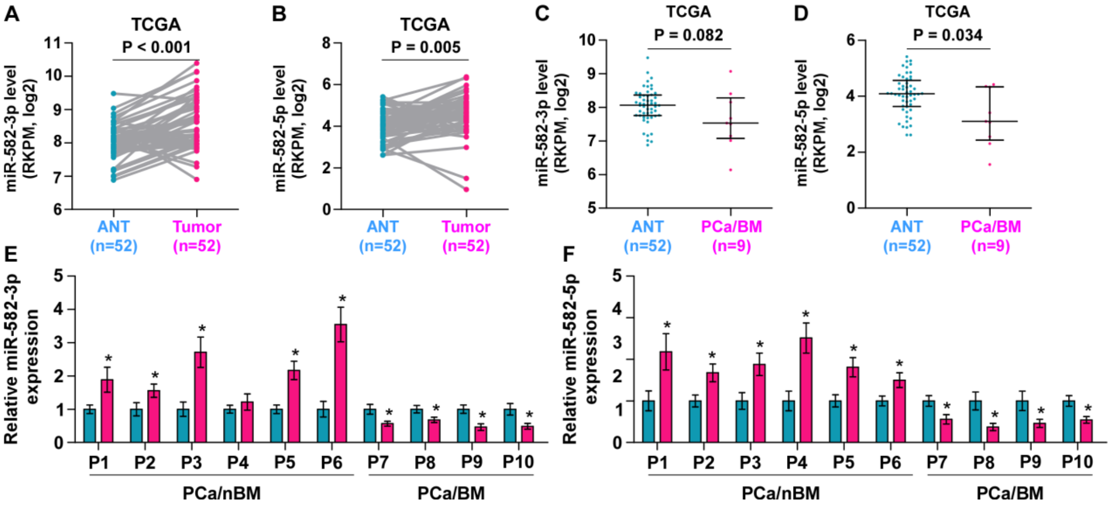
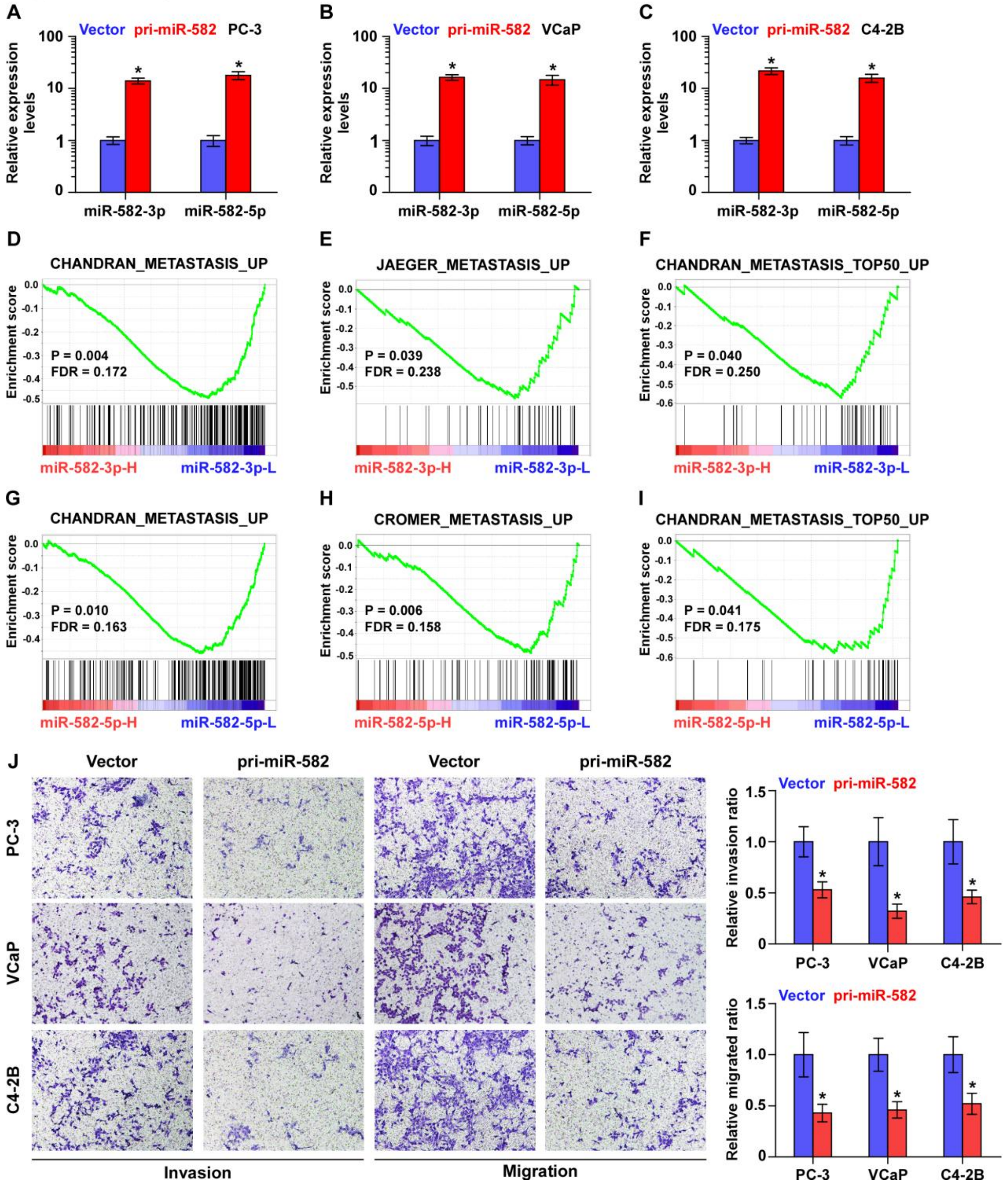
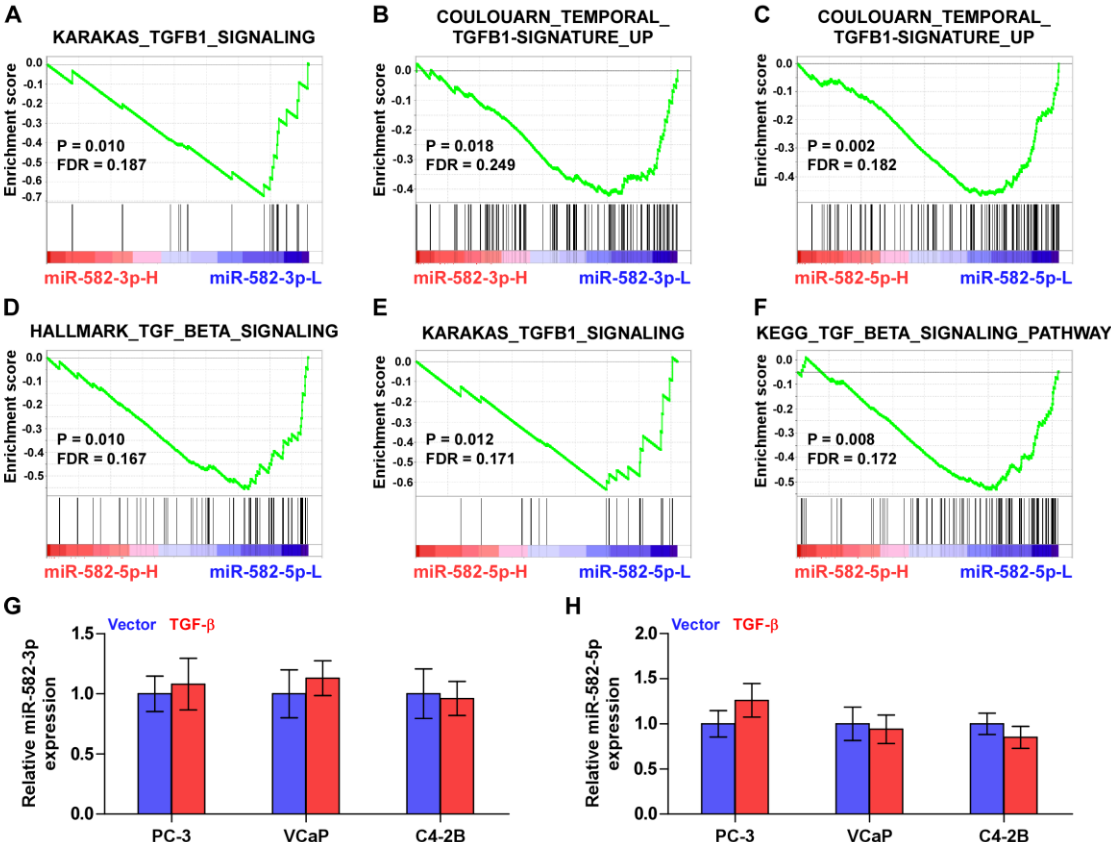
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



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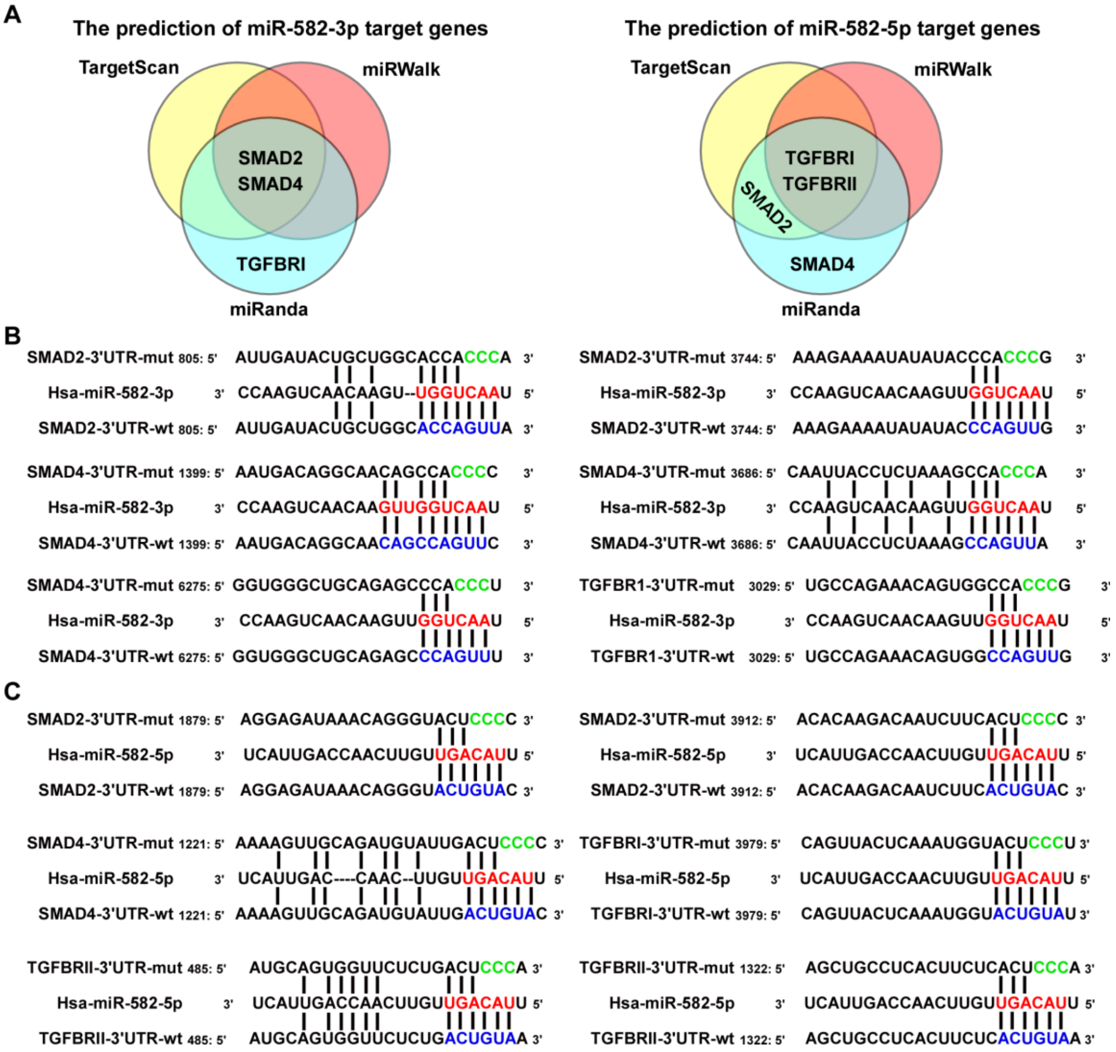

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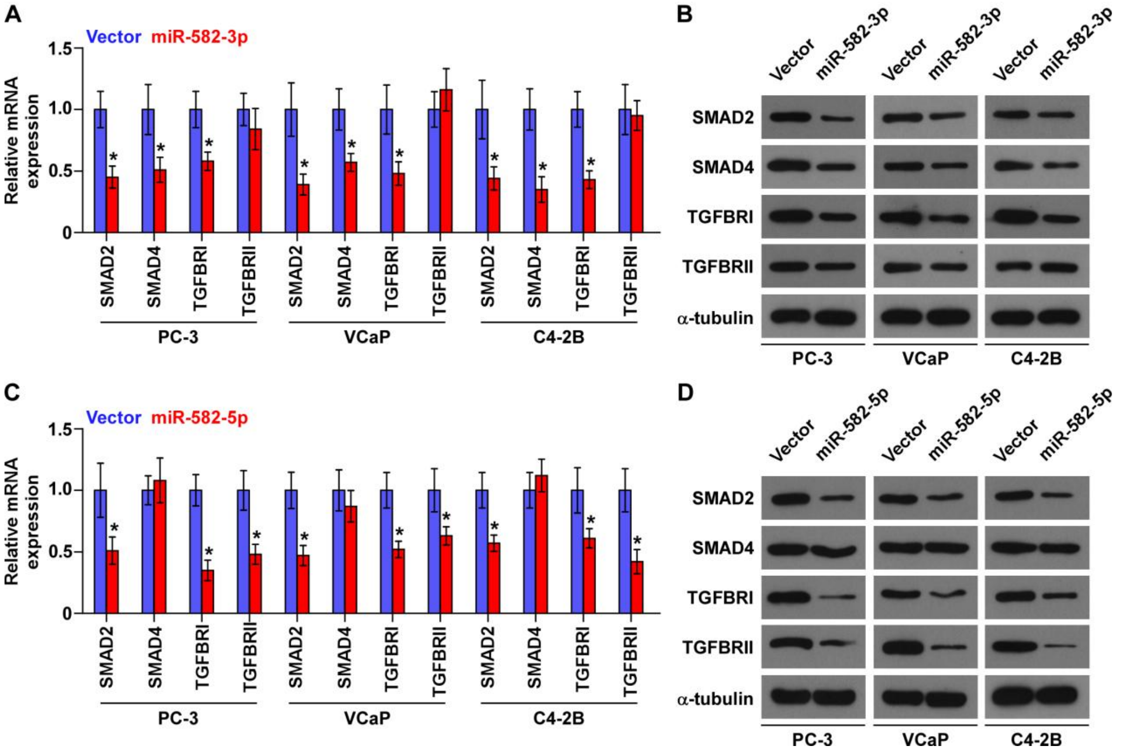

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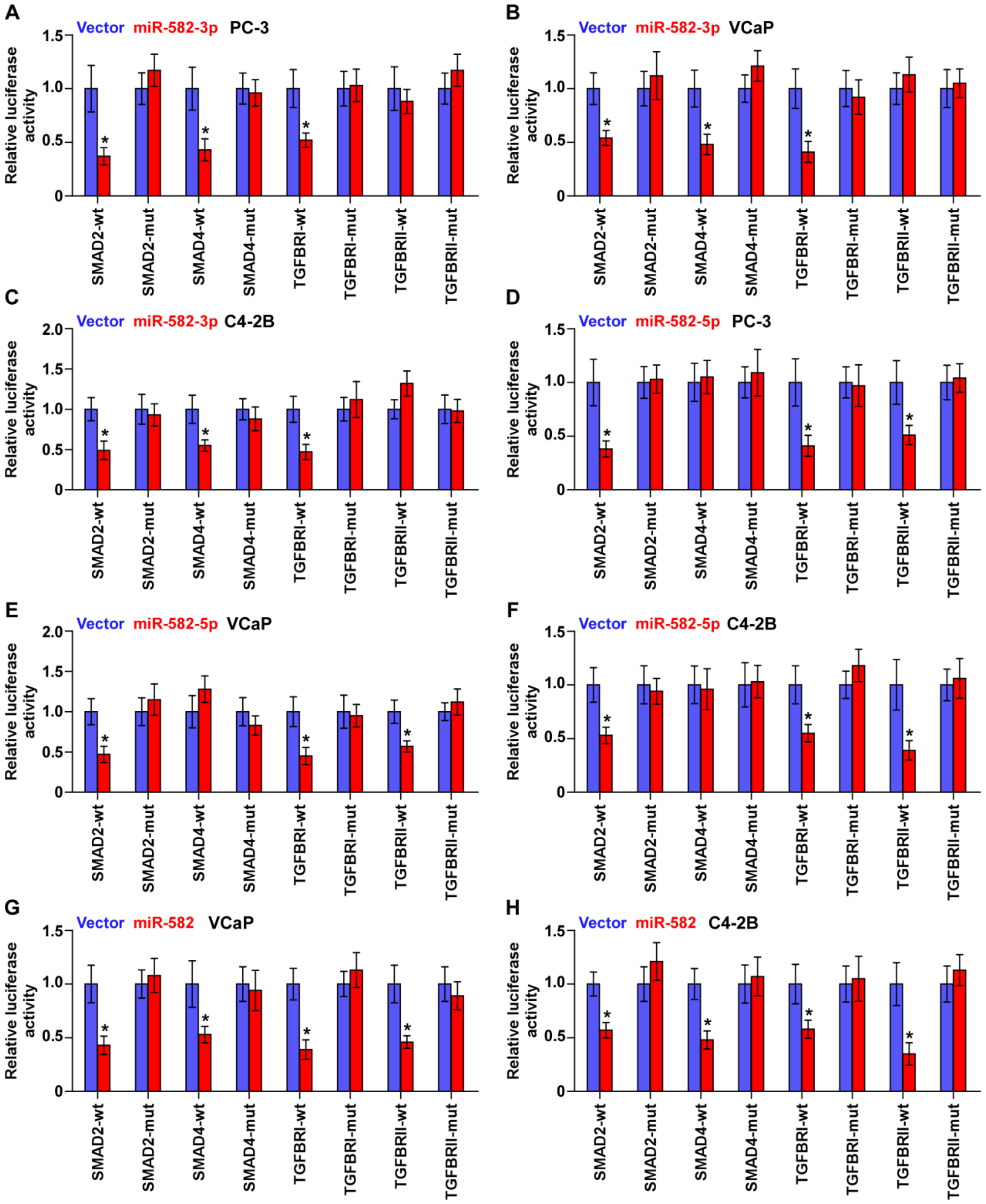

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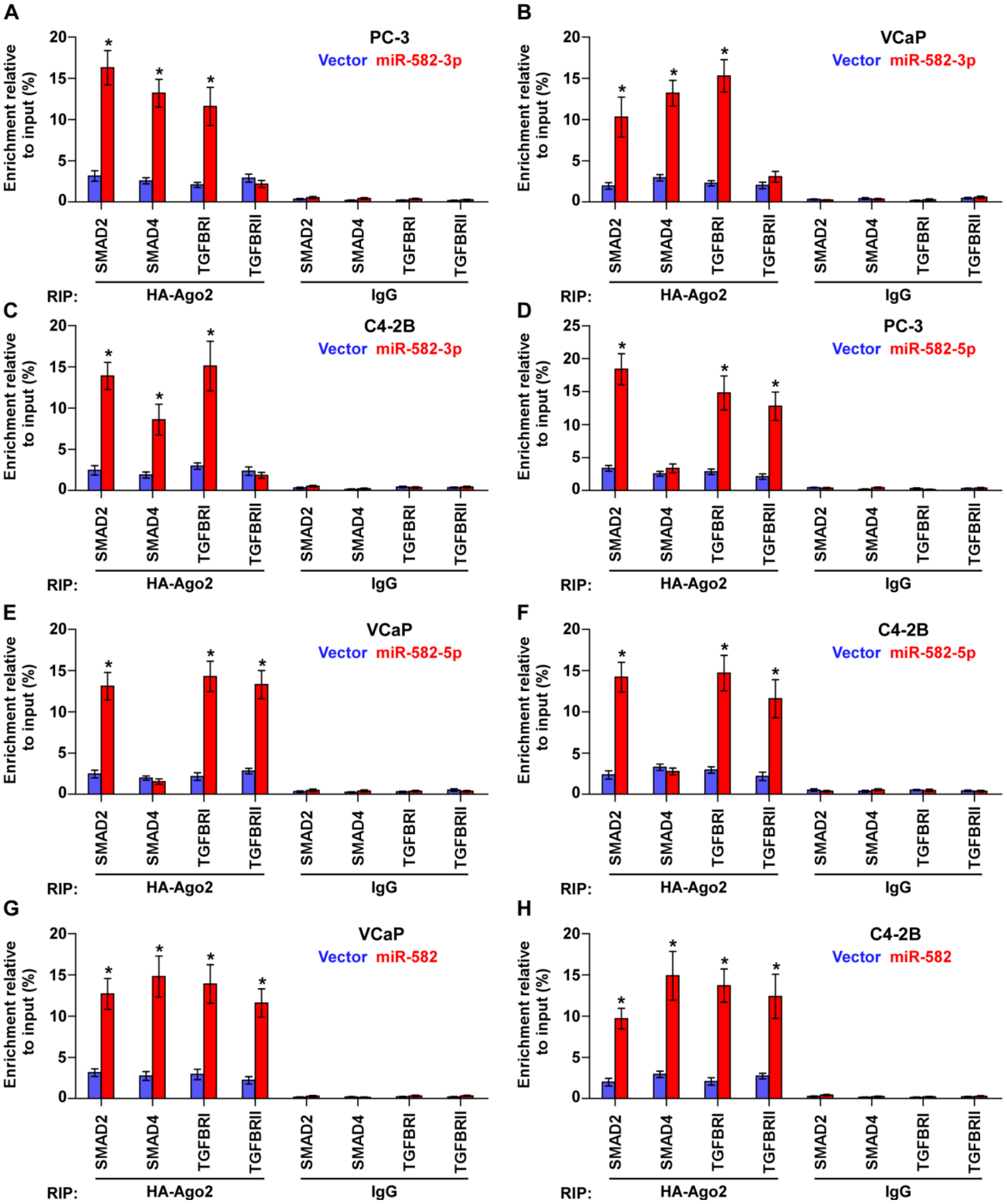
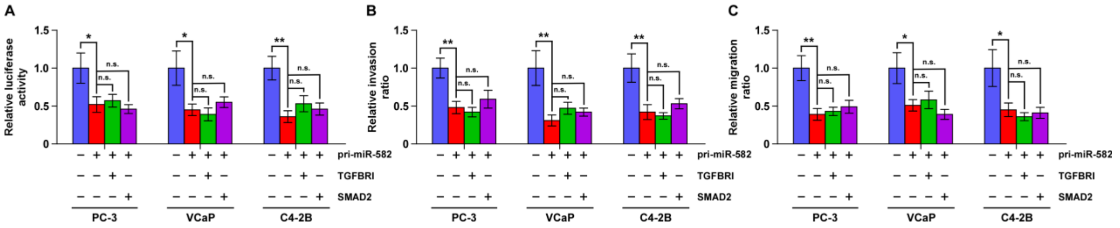
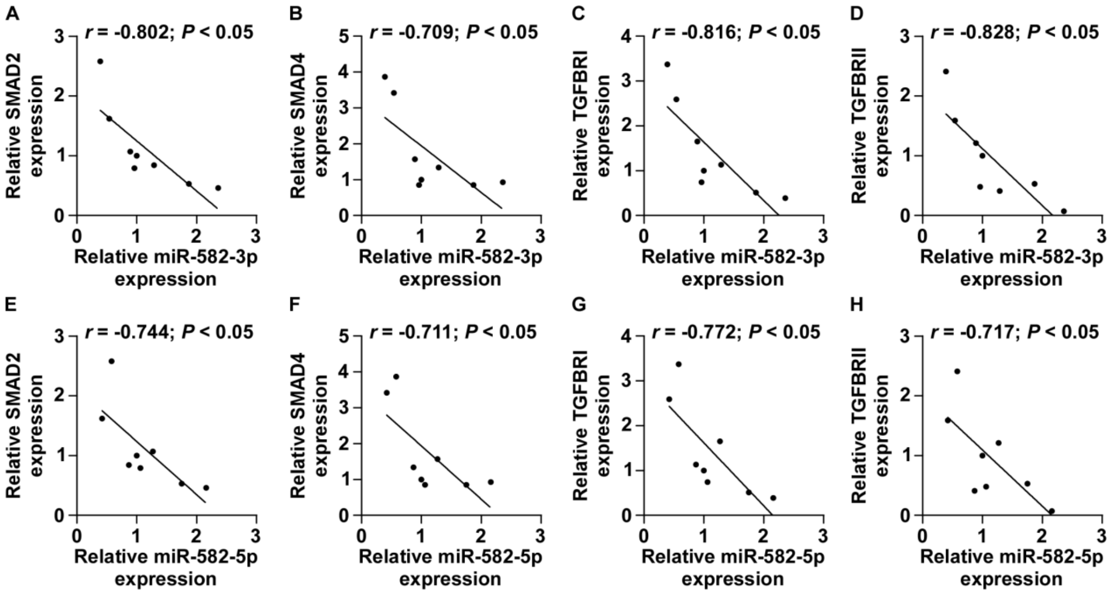


supplemental Figure 8



Supplemental Figure 9



Supplemental figure legends

Supplemental Figure 1. (A and B) miR-582-3p and -5p expression levels was elevated in paired PCa tissues compared with that in the matched adjacent normal tissues (ANT) as assessed by analyzing the PCa miRNA sequencing dataset from TCGA (n = 52). **(C and D)** miR-582-3p and -5p expression levels was reduced in bone metastatic PCa tissues (PCa/BM) compared with that in ANT as assessed by analyzing the PCa miRNA sequencing dataset from TCGA (ANT, n = 52; PCa/BM, n = 9). **(E and F)** Real-time PCR analysis of miR-582-3p and -5p expression in 6 paired non-bone metastatic and 4 paired bone metastatic PCa samples. Transcript levels were normalized to *U6* expression. *$P < 0.05$.

Supplemental Figure 2. (A-C) Real-time PCR analysis of miR-582-3p and -5p expression in PCa cells transduced with pri-miR-582 compared to the vector controls. Transcript levels were normalized by *U6* expression. Error bars represent the mean ± s.d. of three independent experiments. *$P < 0.05$. **(D-I)** Gene set enrichment analysis

(GSEA) revealed that low expression of miR-582-3p and -5p significantly and positively correlated with metastatic propensity. **(J)** Overexpression of miR-582-3p and -5p inhibited invasion and migration abilities in PCa cells. Error bars represent the mean ± S.D. of three independent experiments. *$P < 0.05$.

Supplemental Figure 3. (A-F) GSEA revealed that low expression of miR-582-3p and -5p significantly and positively correlated with TGF-β signaling. **(G and H)** Real-time PCR analysis of miR-582-3p and -5p expression in PCa cells treated with TGF-β (5 ng/ml for 48h). Transcript levels were normalized by U6 expression. Error bars represent the mean ± s.d. of three independent experiments.

Supplemental Figure 4. (A) Predictive target genes of miR-582-3p and -5p from TargetScan, miRanda and miRWalk. **(B and C)** Predicted miR-582-3p **(B)** or -5p **(C)** targeting wild-type sequences in 3'UTR s of SMAD2, SMAD4, TGFBRI and TGFBRII.

Supplemental Figure 5. (A and B) Real-time PCR and Western blotting analysis of SMAD2, SMAD4, TGFBRI and TGFBRII expression in the miR-582-3p mimics-transfecting or vector PCa cells. Transcript levels were normalized by *U6* expression. α-Tubulin served as the loading control. Error bars represent the mean ± s.d. of three independent experiments. *$P < 0.05$. **(C and D)** Real-time PCR and Western blotting analysis of SMAD2, SMAD4, TGFBRI and TGFBRII expression in the miR-582-5p mimics-transfecting or vector PCa cells. Transcript levels were normalized by *U6* expression. α-Tubulin served as the loading control. Error bars represent the mean ± s.d. of three independent experiments. *$P < 0.05$.

Supplemental Figure 6. (A-C) Luciferase assay of the cells transfected with pmirGLO-3'UTR reporter of SMAD2, SMAD4, TGFBRI and TGFBRII in the miR-582-3p mimics-transfecting or vector PCa cells. Error bars represent the mean ± S.D. of three independent experiments. *$P < 0.05$. **(D-F)** Luciferase assay of the cells transfected with pmirGLO-3'UTR reporter of SMAD2, SMAD4, TGFBRI and TGFBRII in the miR-582-5p mimics-transfecting or vector PCa cells. Error bars represent the mean ± S.D. of three independent experiments. *$P < 0.05$. **(G and H)** Luciferase assay of the cells transfected with pmirGLO-3'UTR reporter of SMAD2, SMAD4, TGFBRI and TGFBRII in the pri-miR-582-transfecting or vector VCaP and C4-2B cells. Error bars represent the mean ± S.D. of three independent experiments. *$P < 0.05$.

Supplemental Figure 7. (A-C) MiRNP IP assay showing the association between miR-582-3p and SMAD2, SMAD4, TGFBRI and TGFBRII 1transcripts in the miR-582-3p mimics-transfecting or vector PCa cells. Pulldown of IgG antibody served as the negative control. Error bars represent the mean ± S.D. of three independent experiments. *$P < 0.05$. **(D-F)** MiRNP IP assay showing the association between miR-582-5p and SMAD2, SMAD4, TGFBRI and TGFBRII 1transcripts in the miR-582-5p mimics-transfecting or vector PCa cells. Pulldown of IgG antibody served as the negative control. Error bars represent the mean ± S.D. of three independent experiments. *$P < 0.05$. **(G and H)** MiRNP IP assay showing the association between miR-582-3p and -5p and SMAD2, SMAD4, TGFBRI and TGFBRII 1transcripts in the pri-miR-582-transfecting or vector VCaP and C4-2B cells. Pulldown of IgG antibody served as the negative control. Error bars represent the mean ± S.D. of three independent experiments. *$P < 0.05$.

Supplemental Figure 8. TGF-β signaling activity restores invasion and migration abilities in miR-582-3p and -5p-overexpressing PCa cells. (A-C) Upregulating TGFBRI or SMAD2 didn't affect TGF-β signaling activity **(A)**, invasion **(B)** and migration **(C)** abilities in the pri-miR-582-transfecting PCa cells in the absence of TGF-β. *$P < 0.05$, and n.s. means no significance.

Supplemental Figure 9. Clinical relevance of miR-582-3p and -5p with SMAD2, SMAD4, TGFBRI and TGFBRII in human PCa tissues. (A-D) Correlation between miR-582-3p levels and SMAD2, SMAD4, TGFBRI and TGFBRII expression in PCa tissues.The expression levels of SMAD2, SMAD4, TGFBRI and TGFBRII were quantified by densitometry using Image J Software, and normalized to the levels of α-tubulin. The sample 1 was used as a standard. The relative expressions of miR-582-3p and these proteins were used to perform the correlation analysis. (**E-H**) Correlation between miR-582-5p levels and SMAD2, SMAD4, TGFBRI and TGFBRII expression in PCa tissues.The expression levels of SMAD2, SMAD4, TGFBRI and TGFBRII were quantified by densitometry using Image J Software, and normalized to the levels of α-tubulin. The sample 1 was used as a standard. The relative expressions of miR-582-5p and these proteins were used to perform the correlation analysis.

Supplemental Table 1. The relationship between miR-582-3p and clinicopathological characteristics in 157 patients with prostate cancer.

Parameters	Number of cases	miR-582-3p expression		*P* values
		Low	High	
Age (years)				
≤76	81	36	45	0.129
>76	76	43	33	
Differentiation				
Well/moderate	73	41	32	0.172
Poor	84	38	46	
Serum PSA				
<20.3	78	27	51	
>20.3	79	52	27	<0.001*
Gleason grade				
≤7	86	29	57	
>7	71	50	21	<0.001*
Operation				
TURP	65	35	30	
Needle biopsy	68	35	33	0.126
TURP+PP	5	4	1	
TURP+BO	11	2	9	
BO	8	3	5	
BM-status				
nBM	94	33	61	
BM	63	46	17	<0.001*

Abbreviation: PSA, prostate-specific antigen; TURP, Trans Urethral Resection Prostate; PP, Prior Prostatectomy; BO, Bilateral Orchiectomies; SD, Standard deviation; IHC, Immunological Histological Chemistry; BM, Bone Metastasis.

Supplemental Table 2. The relationship between miR-582-5p and clinicopathological characteristics in 157 patients with prostate cancer.

Parameters	Number of cases	miR-582-5p expression		*P* values
		Low	High	
Age (years)				
≤75	81	38	43	0.378
>75	76	41	35	
Differentiation				
Well/moderate	73	40	33	0.296
Poor	84	39	45	
Serum PSA				
<20.3	78	25	53	
>20.3	79	54	25	<0.001*
Gleason grade				
≤7	86	33	53	
>7	71	46	25	=0.001*
Operation				
TURP	65	34	31	
Needle biopsy	68	36	32	0.169
TURP+PP	5	4	1	
TURP+BO	11	3	8	
BO	8	2	6	
BM-status				
nBM	94	29	65	
BM	63	50	13	<0.001*

Abbreviation: PSA, prostate-specific antigen; TURP, Trans Urethral Resection Prostate; PP, Prior Prostatectomy; BO, Bilateral Orchiectomies; SD, Standard deviation; IHC, Immunological Histological Chemistry; BM, Bone Metastasis.

Supplemental Table 3. A list of primers used in the reactions for clone PCR.

Gene	Sequence (5` – 3`)	Product size
pri-miR-582-clone-F	CACTTCAGCCTTGAGGTACAAC	633 bp
pri-miR-582-clone-R	CTTCCCAGCTTTGCATCAGAG	
TGFBRI-3`UTR-2999-F	AGCAAGGTGGCTCCTGTG	1101 bp
TGFBRI-3`UTR-4099-R	GGCAGAGATTACACTGATAAAGCC	
TGFBRII-3`UTR-413-F	TTTATTGGAGAACTCCAGAACCAAGC	997 bp
TGFBRII-3`UTR-1409-R	CCTTGGTTAGGTGCAGATTTAATTC	
SMAD2-3`UTR-618-F	TGTTCAGTGGGGCTTAAACAGTC	272 bp
SMAD2-3`UTR-886-R	CCCAAAGACGGGCATAAGACAAAGGGACTTTC	
SMAD2-3`UTR-1787-F	CTTATGCCCGTCTTTGGGTAAAGGTGAACAAGAC	158 bp
SMAD2-3`UTR-1927-R	AAGTGAAATGAAGCATCCCATCTGTTATTCTCC	
SMAD2-3`UTR-3642-F	GGATGCTTCATTTCACTTTTTTTGTGCCCTTGTC	368 bp
SMAD2-3`UTR-4000-R	GAGGGAAGGCGCAAATTGG	
SMAD4-3`UTR-1184-F	TCAATTGGCAGTGACTTTGTATAGAG	321 bp
SMAD4-3`UTR-1495-R	AACGAAGGGACTCTACACATGATACACAATGTCC	
SMAD4-3`UTR-3588-F	GTGTAGAGTCCCTTCGTTATTGCCAACTTTAC	215 bp
SMAD4-3`UTR-3785-R	AACTCCATGACAAGATGGATTCTGCCTTTACG	
SMAD4-3`UTR-6175-F	CCATCTTGTCATGGAGTTGGCAAACTTTCTTC	236 bp
SMAD4-3`UTR-6401-R	TCTTCTCTGGCTTCTGGCATAG	

Supplemental Table 4. A list of primers used in the reactions for real-time RT-PCR.

Primer	
TGFBRI-up	CACAGAGTGGGAACAAAAAGGT
TGFBRI-dn	CCAATGGAACATCGTCGAGCA
TGFBRII-up	AAGATGACCGCTCTGACATCA
TGFBRII-dn	CTTATAGACCTCAGCAAAGCGAC
SMAD2-up	CACGCTAGGAAAACAGCCTC
SMAD2-dn	TCGGAAGAGGAAGGAACAAA
SMAD4-up	TTGATCCTTTGGAAACAGTGAA
SMAD4-dn	GCCTTCCCACTCCCCTC
NEDD9 -up	CTACAGGGTAAGGAGGAGTTT
NEDD9-dn	TGGGTCTCACATTGGTCAT
CTGF-up	GCTACCACATTTCCTACCTAGAAATCA
CTGF-dn	GACAGTCCGTCAAAACAGATTGTT
PTHRP-up	ACTCGCTCTGCCTGGTTAGA
PTHRP-dn	GGAGGTGTCAGACAGGTGGT
MMP13-up	AACATCCAAAAACGCCAGAC
MMP13-dn	GGAAGTTCTGGCCAAAATGA
ADAM19-up	TTTCTCAGAACAGCGGGACT
ADAM19-dn	TGTTGATCACCTTTCGCTTG
THBS1-up	TTGTCTTTGGAACCACACCA
THBS1-dn	TTGTCAAGGGTGAGGAGGAC
COL1A1-up	CCTGGATGCCATCAAAGTCT
COL1A1-dn	CGCCATACTCGAACTGGAAT
VEGFA-up	AAGGAGGAGGGCAGAATCAT
VEGFA-dn	CACACAGGATGGCTTGAAGA
IL11-up	TGAAGACTCGGCTGTGACC
IL11-dn	CCTCACGGAAGGACTGTCTC
GAPDH-up	ATTCCACCCATGGCAAATTC
GAPDH-dn	TGGGATTTCCATTGATGACAAG

Supplemental Table 5. The clinicopathological characteristics in 157 patients with prostate cancer

Parameters	Number of cases
Age (years)	
≤76	81
>76	76
Differentiation	
Well/moderate	73
Poor	84
Serum PSA at diagnosis, μg/mL	
<20.3	78
>20.3	79
Gleason grade	
≤7	86
>7	71
Operation	
TURP	65
Needle biopsy	68
TURP+PP	5
TURP+BO	11
BO	8
miR-582-3p expression	
≤4.04	79
>4.04	78
miR-582-5p expression	
≤3.88	79
>3.88	78
BM-status	
BM-free	94
BM	63

Abbreviation: PSA, prostate-specific antigen; TURP, Trans Urethral Resection Prostate; PP, Prior Prostatectomy; BO, Bilateral Orchiectomies; SD, Standard deviation; IHC, Immunological Histological Chemistry; BM, Bone Metastasis.