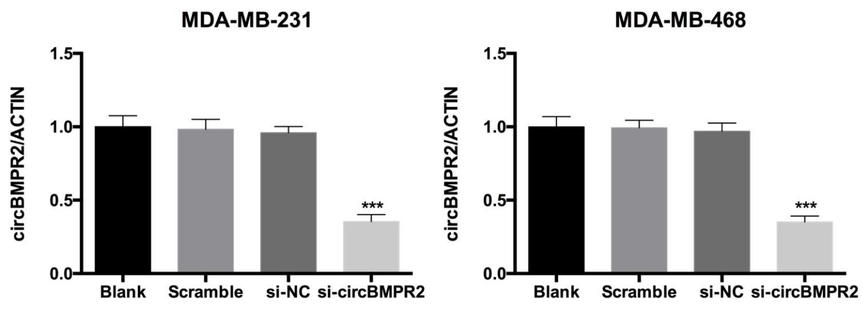
OMTN, Volume *17*

Supplemental Information

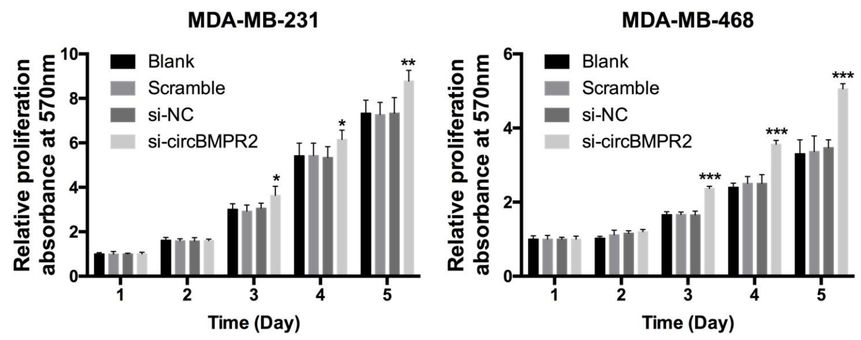
Targeting the circBMPR2/miR-553/USP4 Axis as a Potent Therapeutic Approach for Breast Cancer

Yiran Liang, Xiaojin Song, Yaming Li, Tingting Ma, Peng Su, Renbo Guo, Bing Chen, Hanwen Zhang, Yuting Sang, Ying Liu, Yi Duan, Ning Zhang, Xiaoyan Li, Wenjing Zhao, Lijuan Wang, and Qifeng Yang

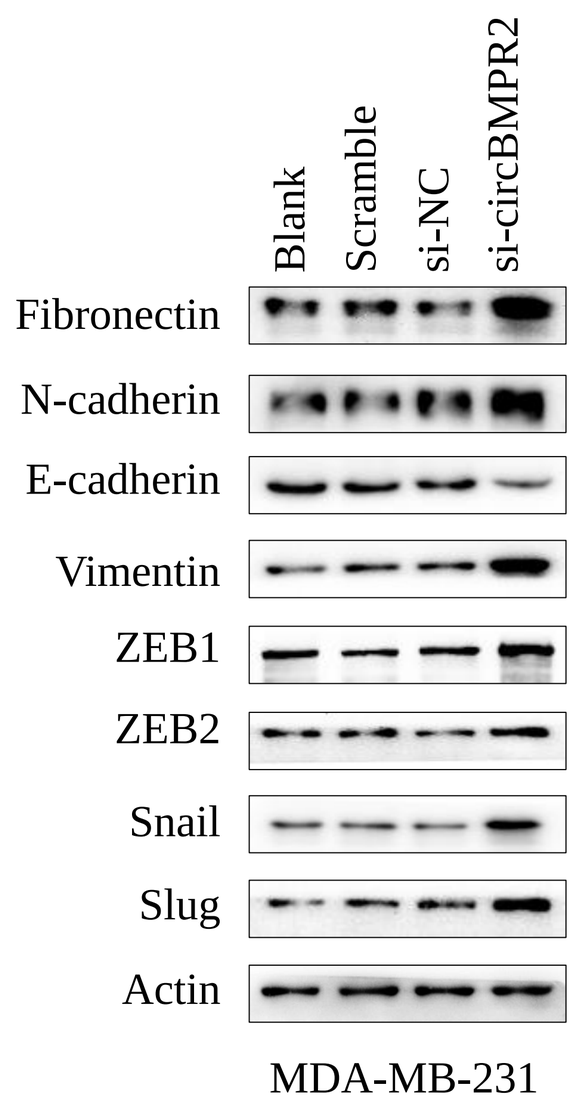
A


MDA-MB-231
circBMPR2/ACTIN
Blank
Scramble
si-NC
si-circBMPR2
***
MDA-MB-468
circBMPR2/ACTIN
Blank
Scramble
si-NC
si-circBMPR2
***

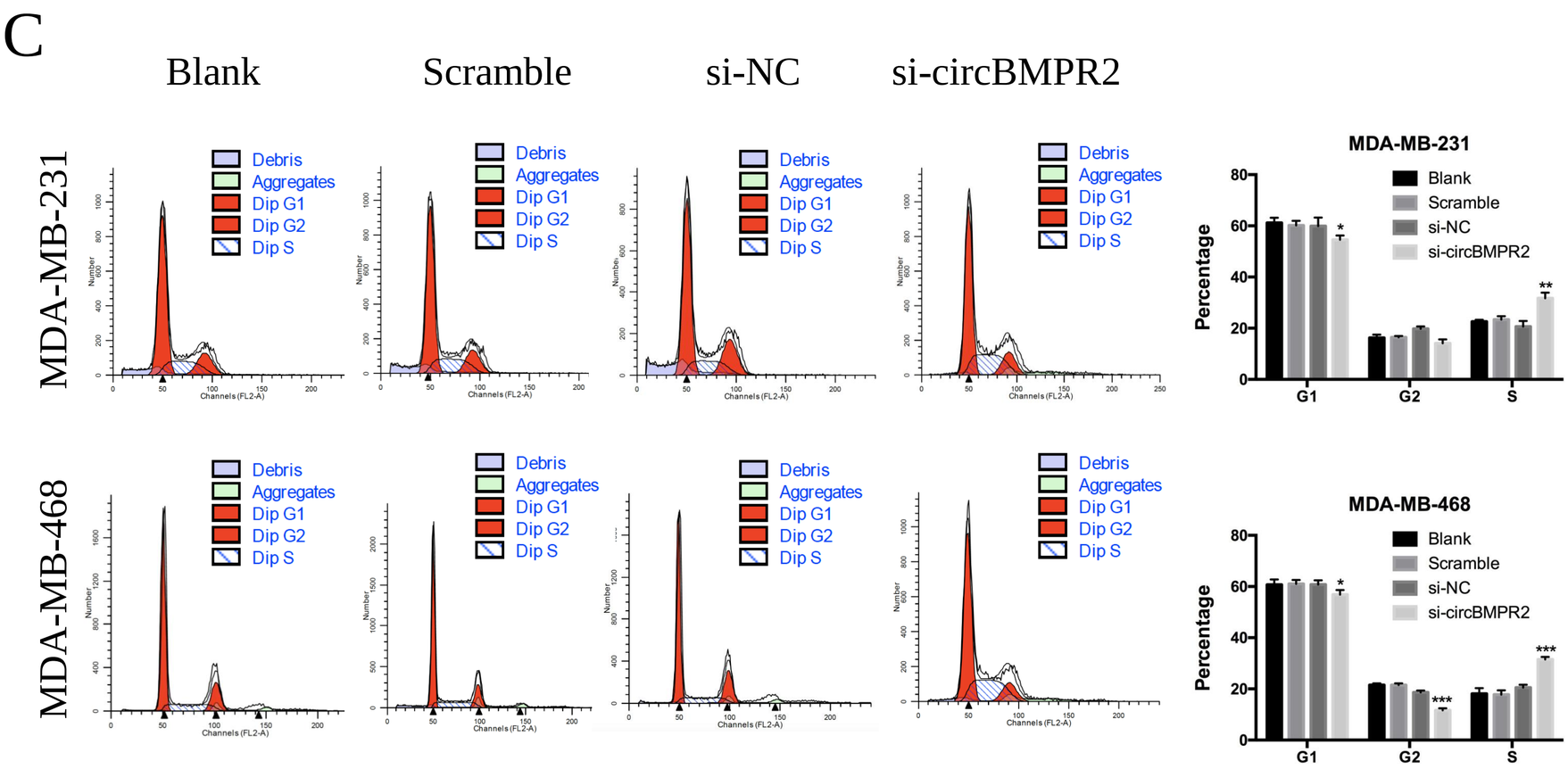
B


MDA-MB-231
Relative proliferation absorbance at 570nm
Blank
Scramble
si-NC
si-circBMPR2
Time (Day)
MDA-MB-468
Relative proliferation absorbance at 570nm
Blank
Scramble
si-NC
si-circBMPR2
Time (Day)

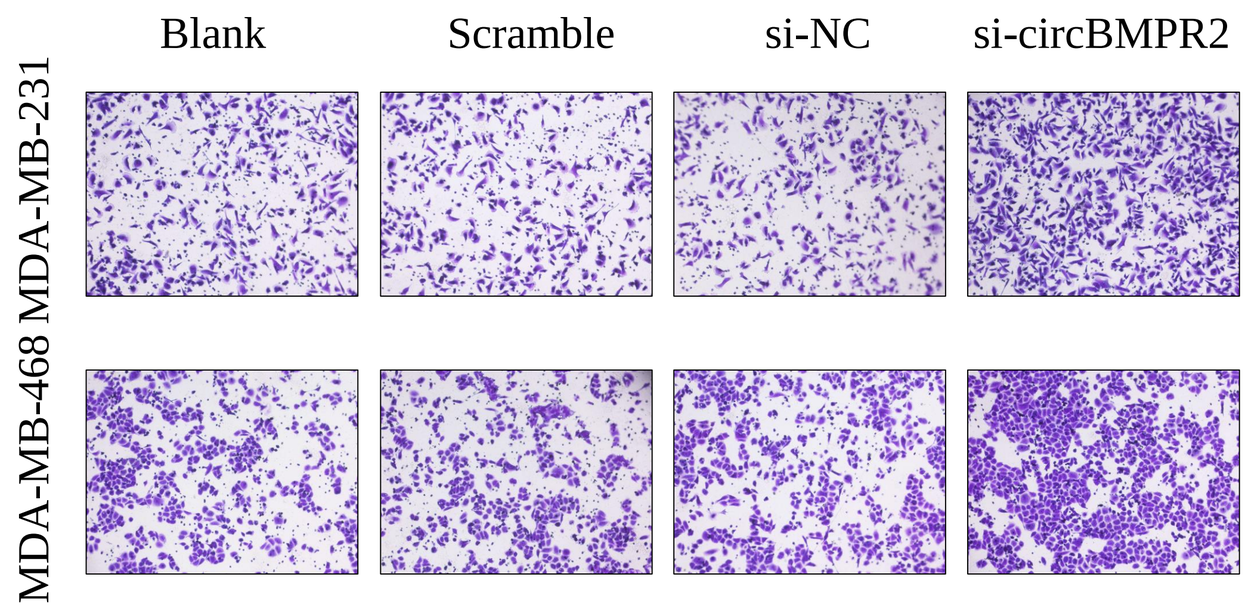
E

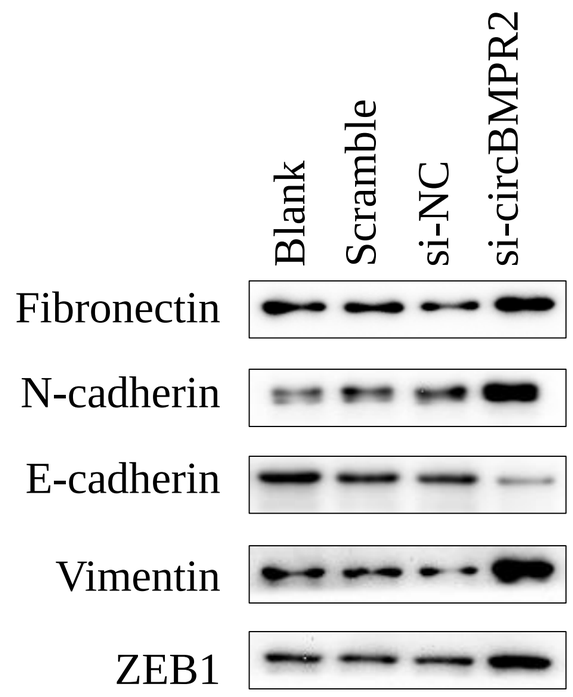

Blank
Scramble
si-NC
si-circBMPR2
Fibronectin
N-cadherin
E-cadherin
Vimentin
ZEB1
ZEB2
Snail
Slug
Actin
MDA-MB-231

C

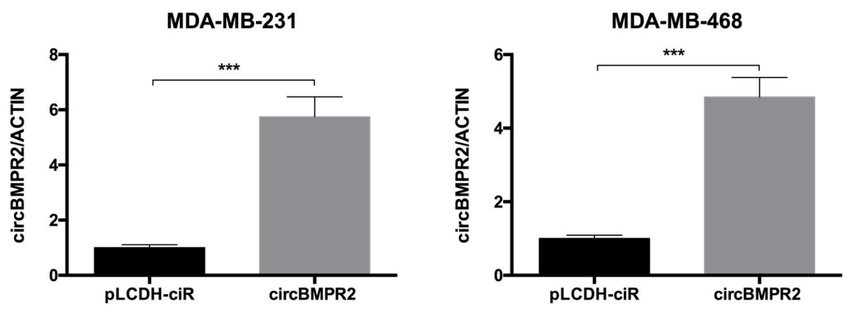

Blank
Scramble
si-NC
si-circBMPR2
MDA-MB-231
MDA-MB-468
Debris
Aggregates
Dip G1
Dip G2
Dip S
Channels (FL2-A)
MDA-MB-231
Percentage
Blank
Scramble
si-NC
si-circBMPR2
G1
G2
S
MDA-MB-468
Percentage
Blank
Scramble
si-NC
si-circBMPR2
G1
G2
S

D

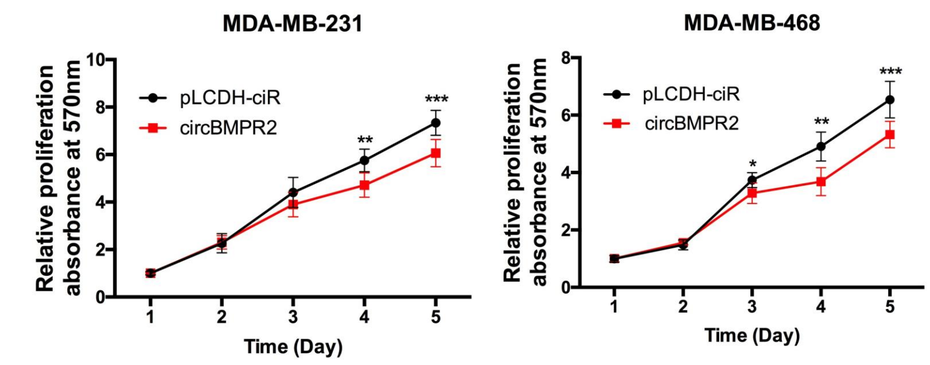

Blank
Scramble
si-NC
si-circBMPR2
MDA-MB-231
MDA-MB-468
MDA-MB-231
Relative Cell Number
Blank
Scramble
si-NC
si-circBMPR2
MDA-MB-468
Relative Cell Number
Blank
Scramble
si-NC
si-circBMPR2


Blank
Scramble
si-NC
si-circBMPR2
Fibronectin
N-cadherin
E-cadherin
Vimentin
ZEB1
ZEB2
Snail
Slug
Actin
MDA-MB-468

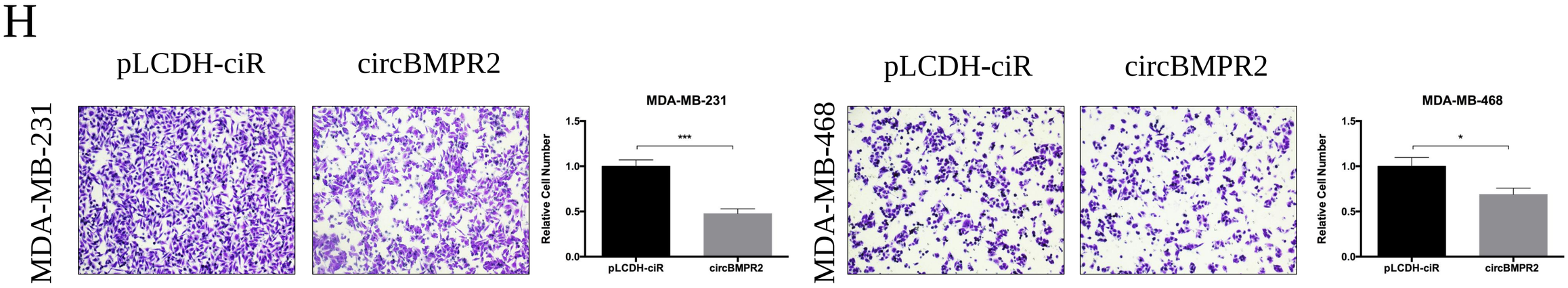
F


MDA-MB-231
circBMPR2/ACTIN
pLCDH-ciR
circBMPR2
MDA-MB-468
circBMPR2/ACTIN
pLCDH-ciR
circBMPR2

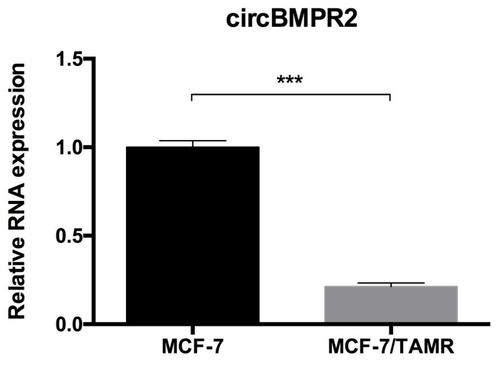
G


MDA-MB-231
Relative proliferation absorbance at 570nm
pLCDH-ciR
circBMPR2
Time (Day)
MDA-MB-468
Relative proliferation absorbance at 570nm
pLCDH-ciR
circBMPR2
Time (Day)

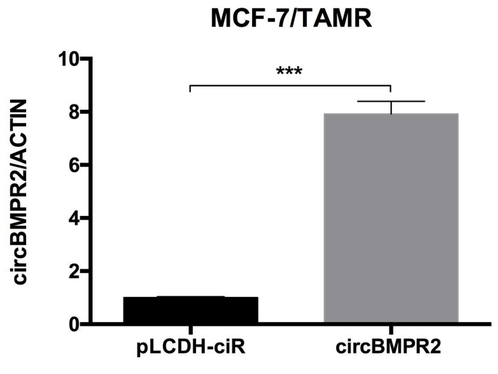
H


pLCDH-ciR
circBMPR2
pLCDH-ciR
circBMPR2
MDA-MB-231
MDA-MB-468
MDA-MB-231
Relative Cell Number
pLCDH-ciR
circBMPR2
MDA-MB-468
Relative Cell Number
pLCDH-ciR
circBMPR2

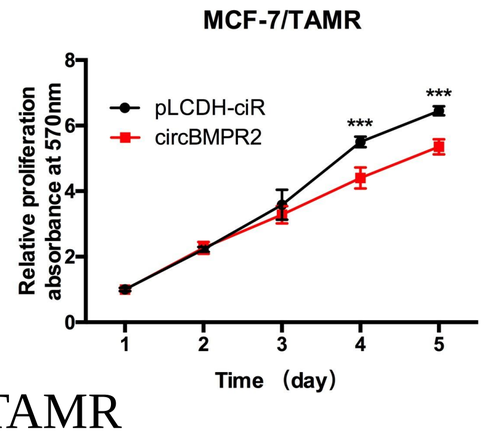
A


circBMPR2
Relative RNA expression
1.5
1.0
0.5
0.0
***
MCF-7
MCF-7/TAMR

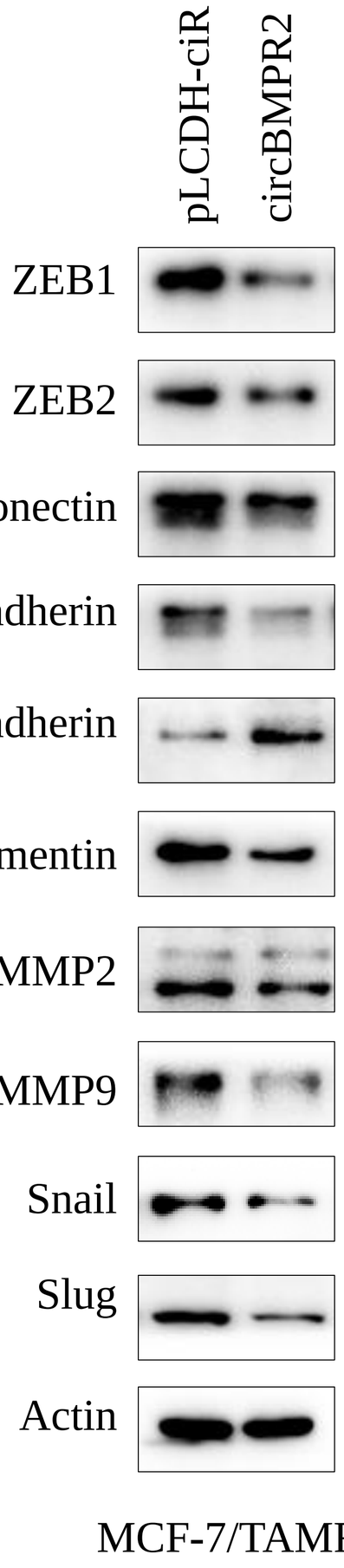
B


MCF-7/TAMR
circBMPR2/ACTIN
10
8
6
4
2
0
***
pLCDH-ciR
circBMPR2

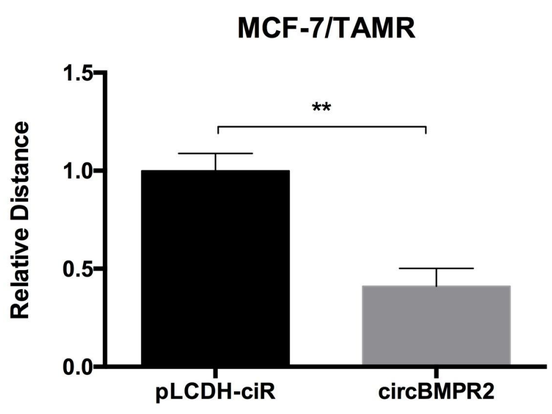
C

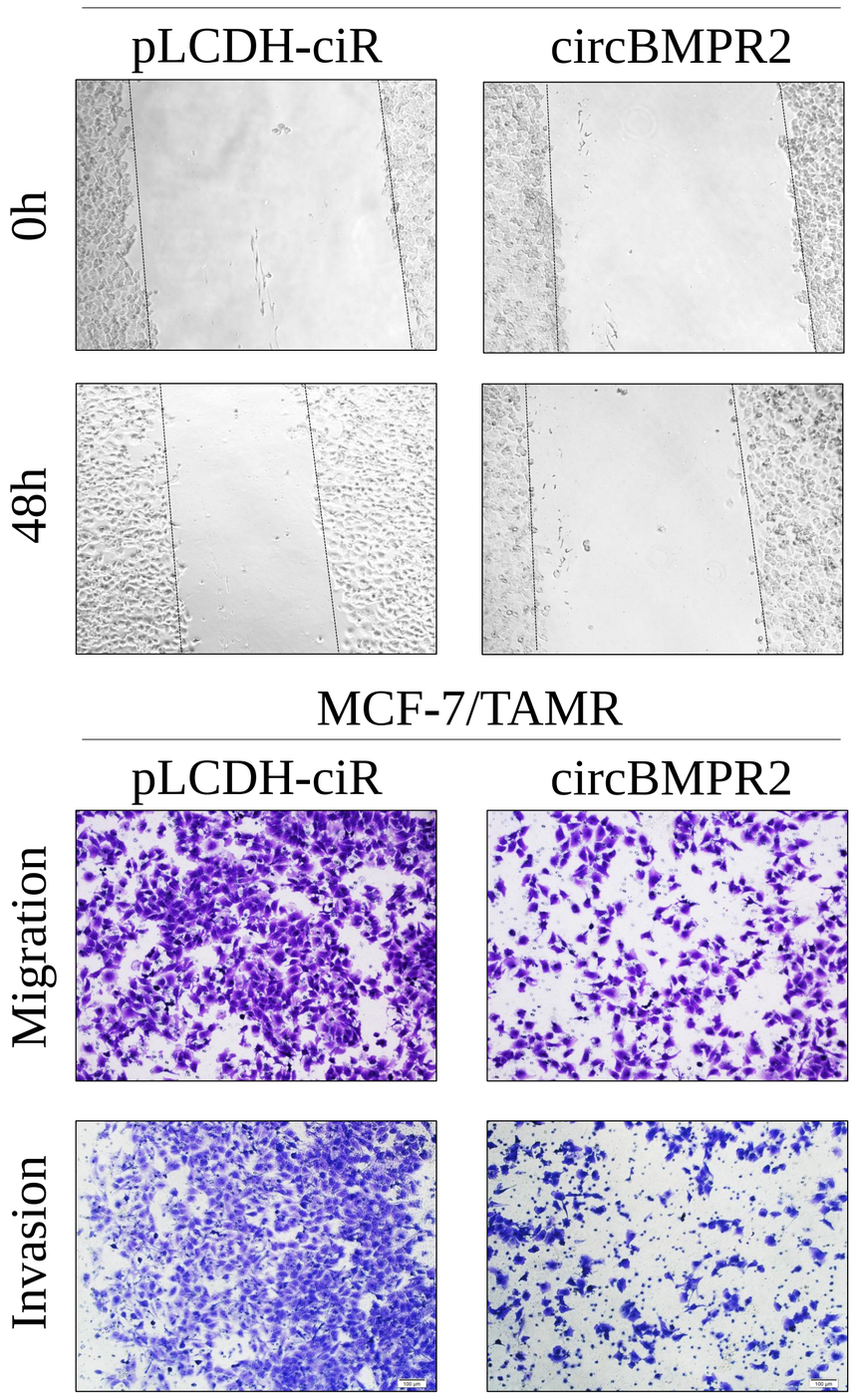

MCF-7/TAMR
Relative proliferation absorbance at 570nm
pLCDH-ciR
circBMPR2
***
***
1
2
3
4
5
Time （day）

F

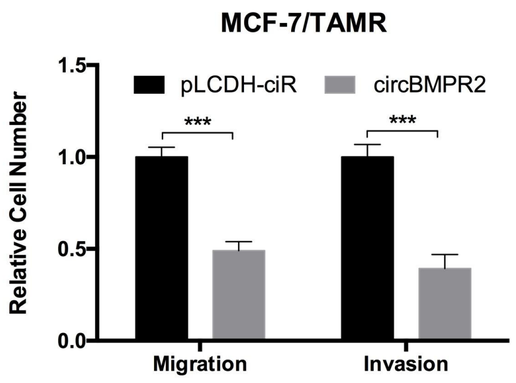

pLCDH-ciR
circBMPR2
ZEB1
ZEB2
Fibronectin
N-cadherin
E-cadherin
Vimentin
MMP2
MMP9
Snail
Slug
Actin
MCF-7/TAMR

D

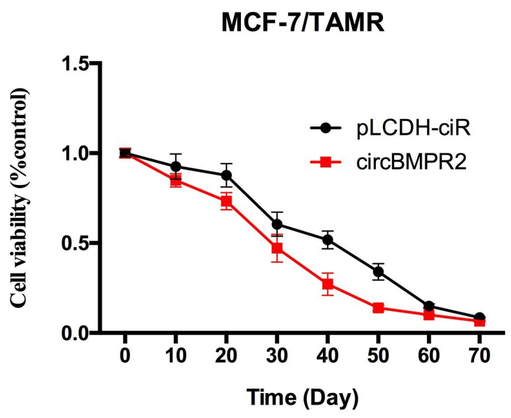

MCF-7/TAMR
Relative Distance
1.5
1.0
0.5
0.0
**
pLCDH-ciR
circBMPR2

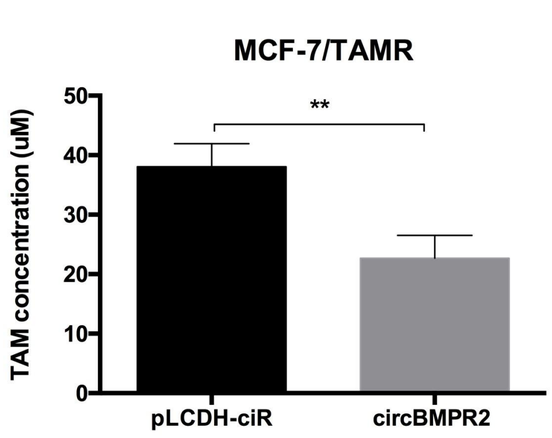

MCF-7/TAMR
pLCDH-ciR
circBMPR2
0h
48h
MCF-7/TAMR
pLCDH-ciR
circBMPR2
Migration
Invasion

E

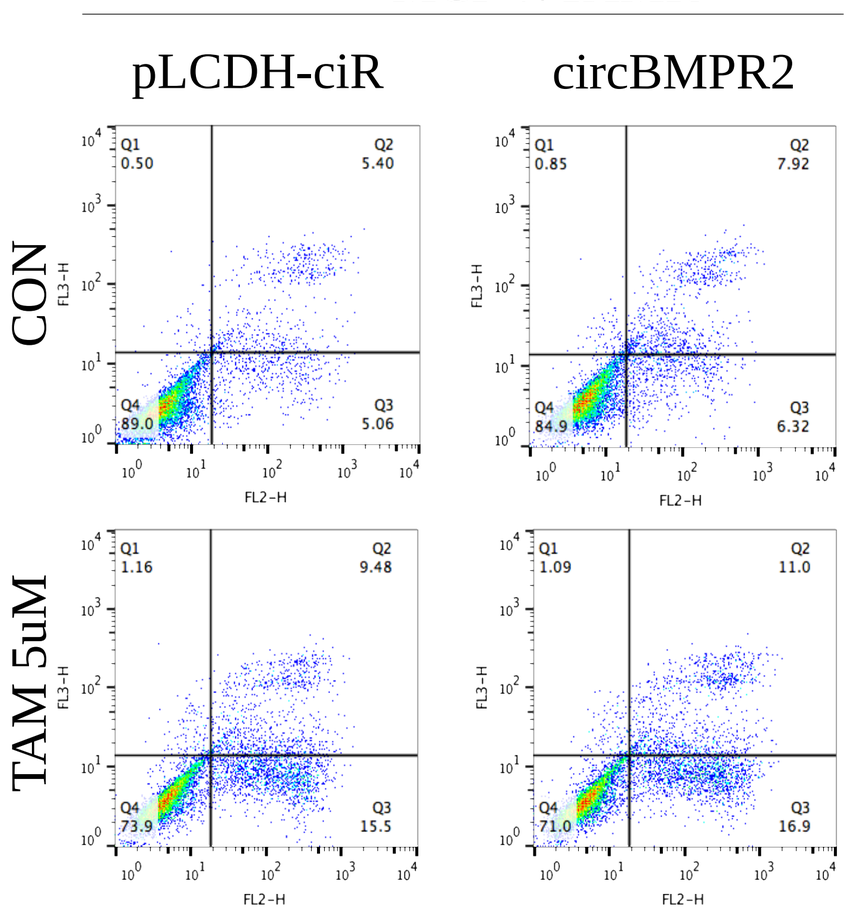

MCF-7/TAMR
pLCDH-ciR
circBMPR2
Relative Cell Number
1.5
1.0
0.5
0.0
***
***
Migration
Invasion

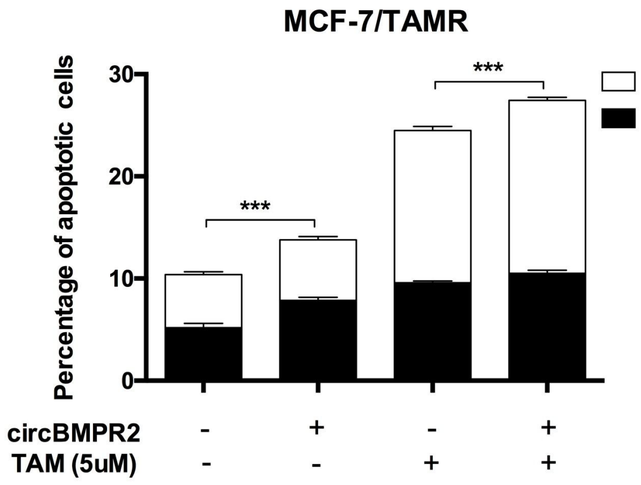
G


MCF-7/TAMR
Cell viability (%control)
1.5
1.0
0.5
0.0
pLCDH-ciR
circBMPR2
0
10
20
30
40
50
60
70
Time (Day)

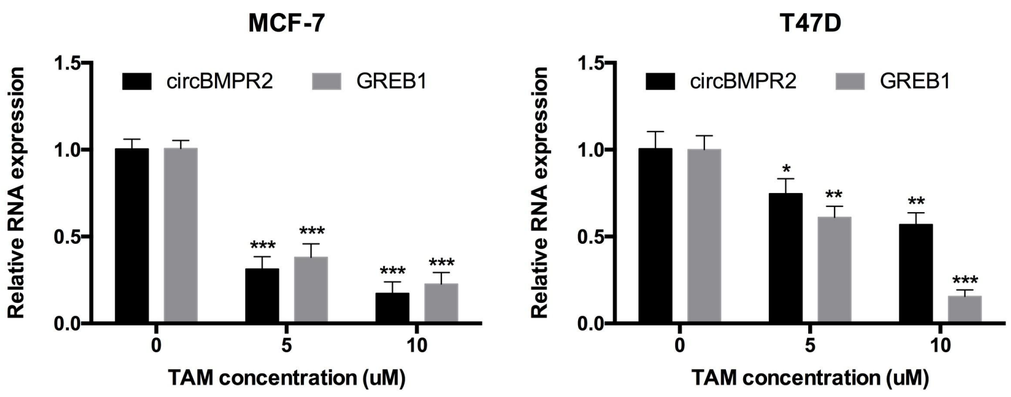

MCF-7/TAMR
TAM concentration (uM)
50
40
30
20
10
0
**
pLCDH-ciR
circBMPR2

H

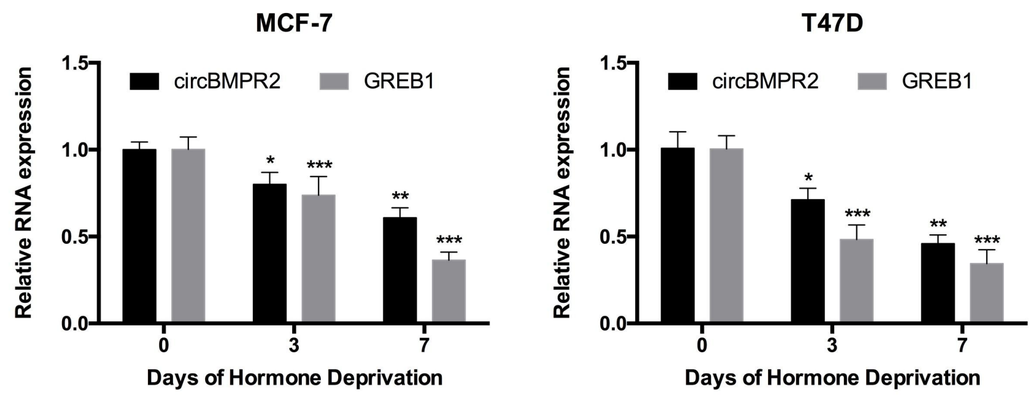

MCF-7/TAMR
pLCDH-ciR
circBMPR2
CON
TAM 5uM
Q1
0.50
Q2
5.40
Q4
89.0
Q3
5.06
Q1
0.85
Q2
7.92
Q4
84.9
Q3
6.32
Q1
1.16
Q2
9.48
Q4
73.9
Q3
15.5
Q1
1.09
Q2
11.0
Q4
71.0
Q3
16.9
FL2-H
FL3-H


MCF-7/TAMR
Percentage of apoptotic cells
30
20
10
0
***
***
Early apoptosis
Late apoptosis
circBMPR2
TAM (5uM)
-
+
-
+
-
-
+
+

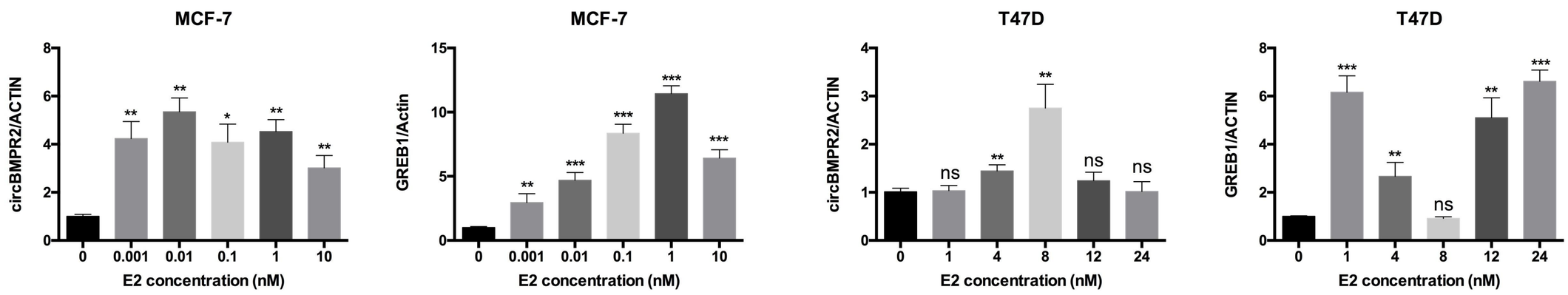
A


MCF-7
T47D
circBMPR2
GREB1
Relative RNA expression
TAM concentration (uM)
0
5
10

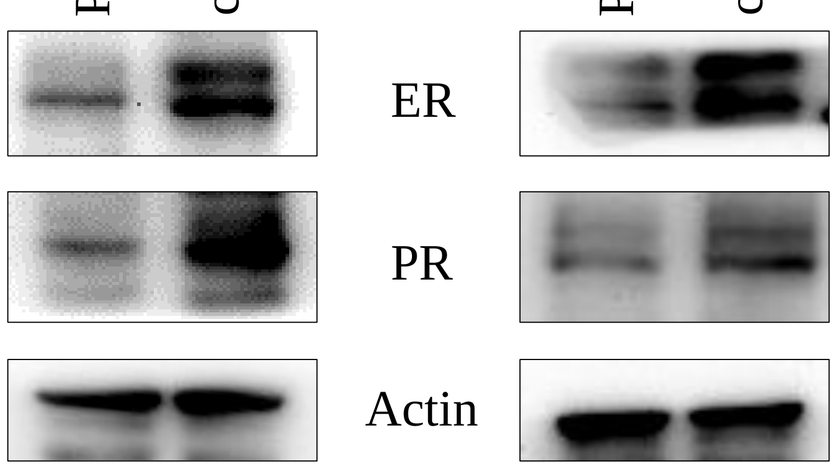
B


MCF-7
T47D
circBMPR2
GREB1
Relative RNA expression
Days of Hormone Deprivation
0
3
7

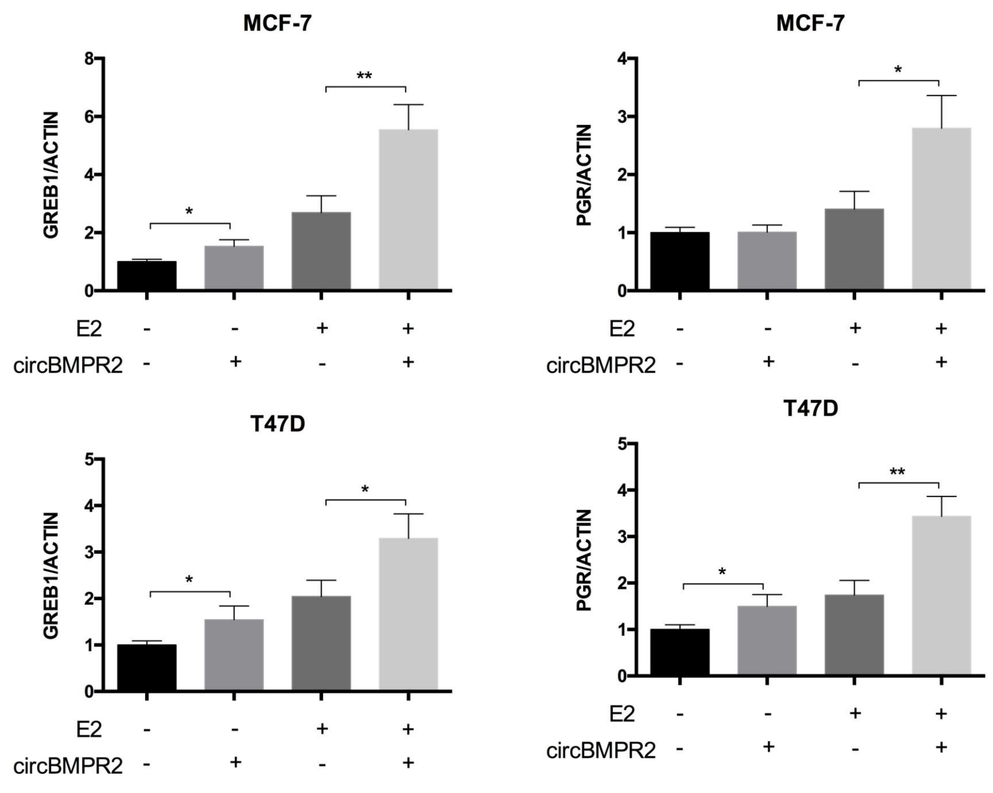
C


MCF-7
MCF-7
T47D
T47D
circBMPR2/ACTIN
GREB1/Actin
GREB1/ACTIN
E2 concentration (nM)
0
0.001
0.01
0.1
1
10
4
8
12
24
ns

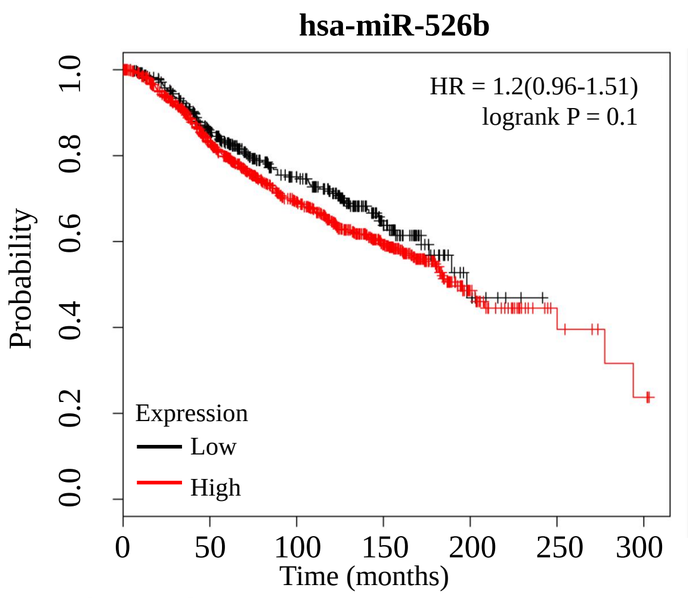
D


MCF-7
MCF-7/TAMR
pLCDH-ciR
circBMPR2
ER
PR
Actin

E

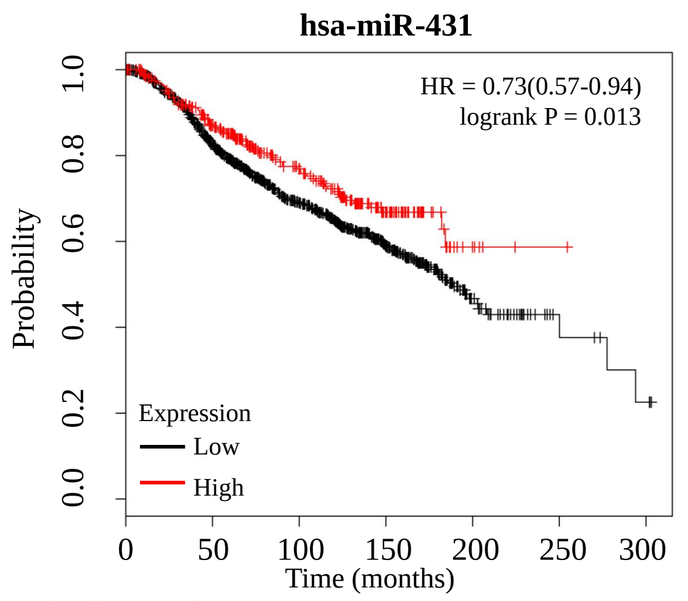

MCF-7
MCF-7
T47D
T47D
GREB1/ACTIN
PGR/ACTIN
E2
circBMPR2
-
+

A



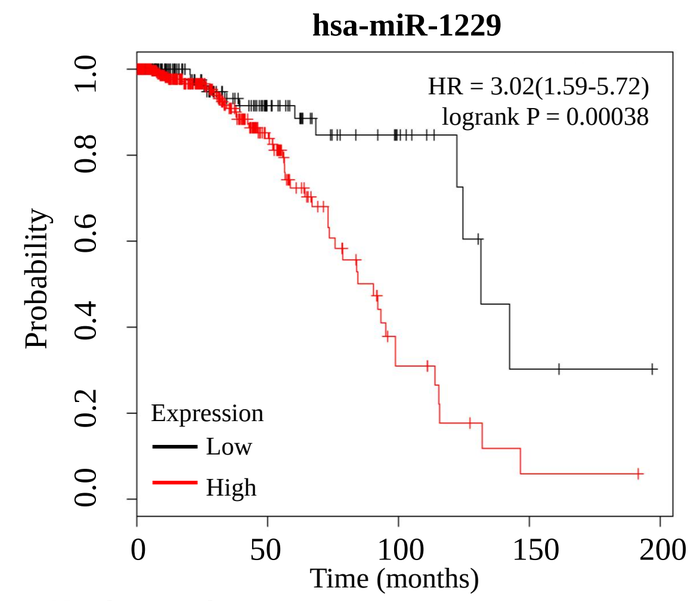
Number at risk

Low	397	288	166	60	8	0	0
High	865	628	373	197	39	9	3



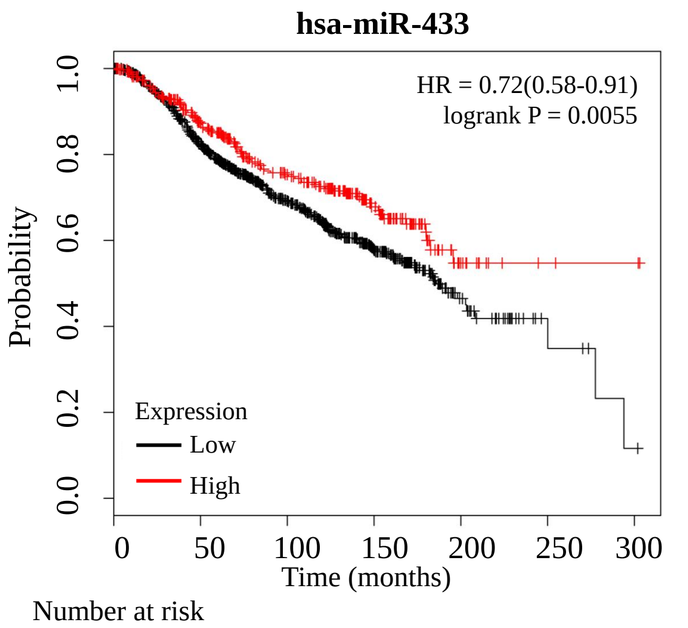
Number at risk

Low	940	678	400	201	42	8	3
High	322	238	139	56	5	1	0



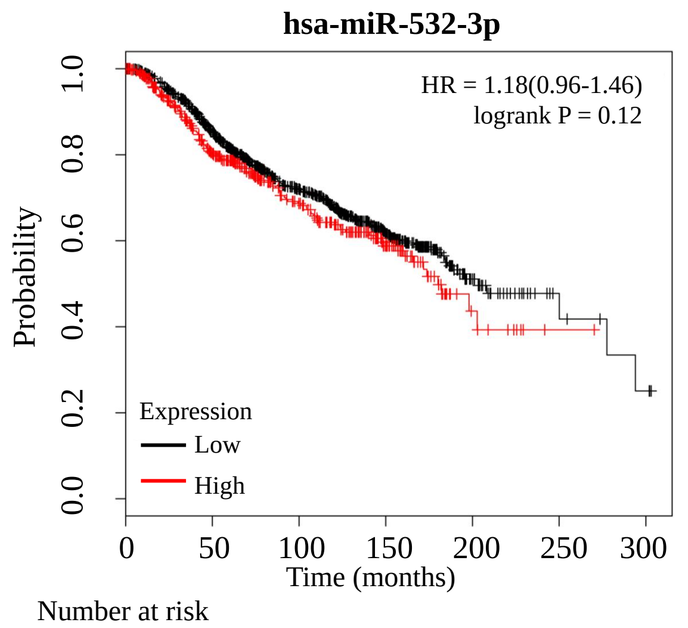
Number at risk

Low	165	39	12	2	0
High	414	64	9	1	0



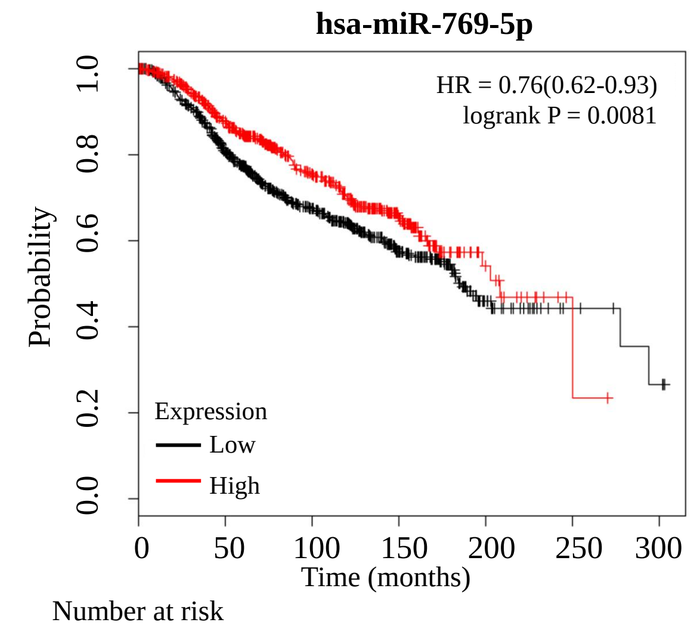
Number at risk

Low	886	638	368	178	34	6	1
High	376	278	171	79	13	3	2



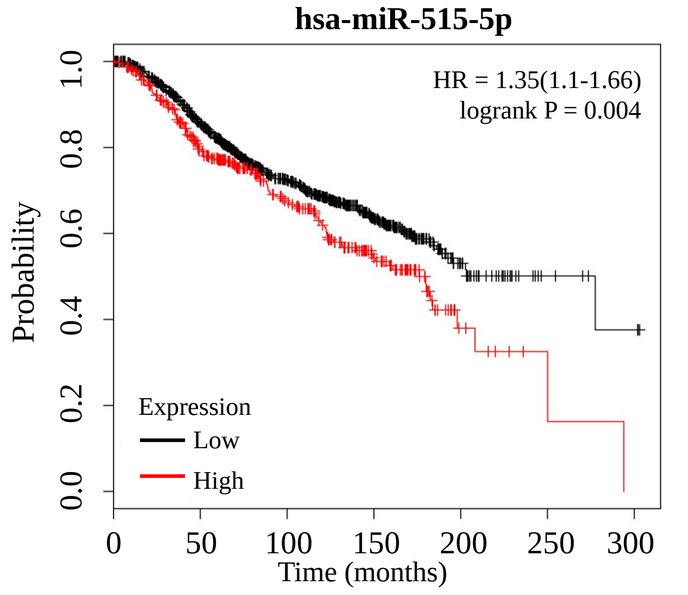
Number at risk

Low	861	653	393	193	37	8	3
High	401	263	146	64	10	1	0



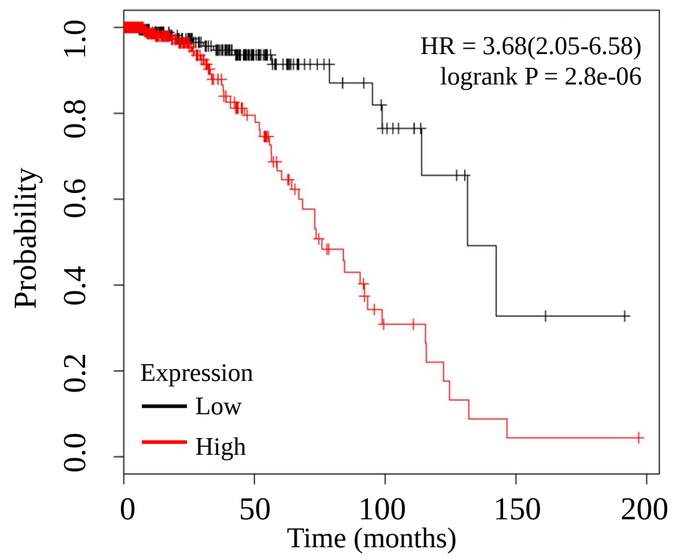
Number at risk

Low	702	493	296	154	31	7	3
High	560	423	243	103	16	2	0



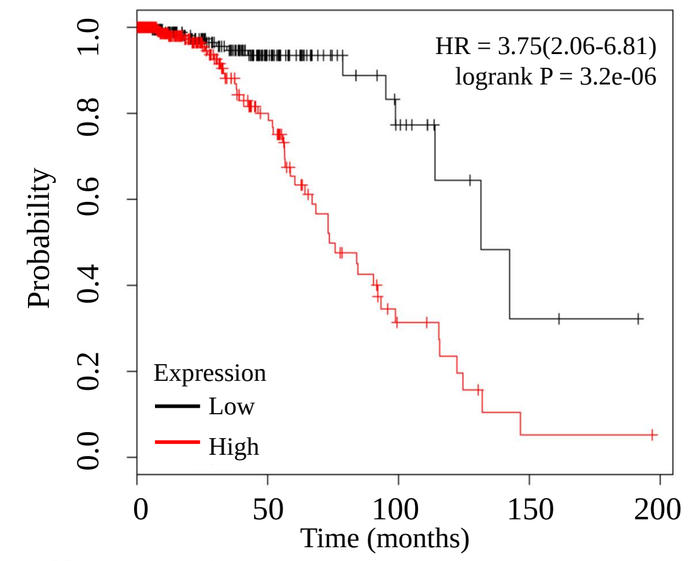
Number at risk

Low	881	659	396	193	39	7	3
High	381	257	143	64	8	2	0



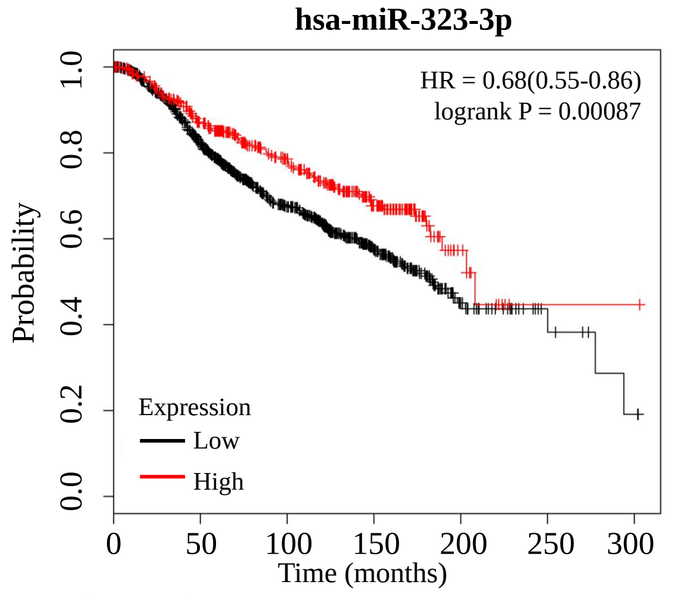
Number at risk

Low	222	55	13	2	0
High	357	48	8	1	0



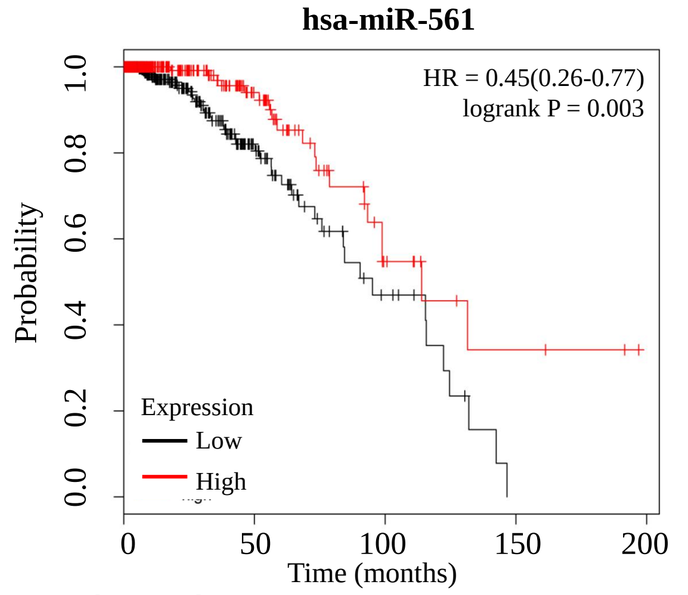
Number at risk

Low	220	54	12	2	0
High	359	49	9	1	0



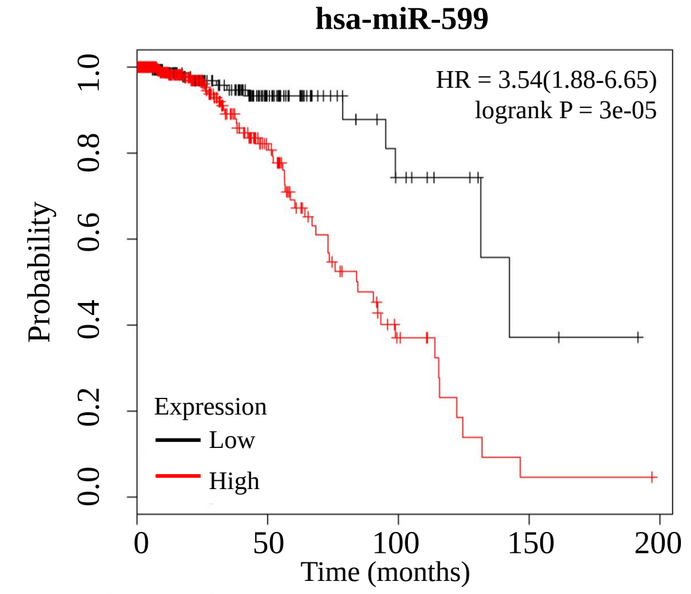
Number at risk

Low	864	614	346	165	35	8	2
High	398	302	193	92	12	1	1



Number at risk

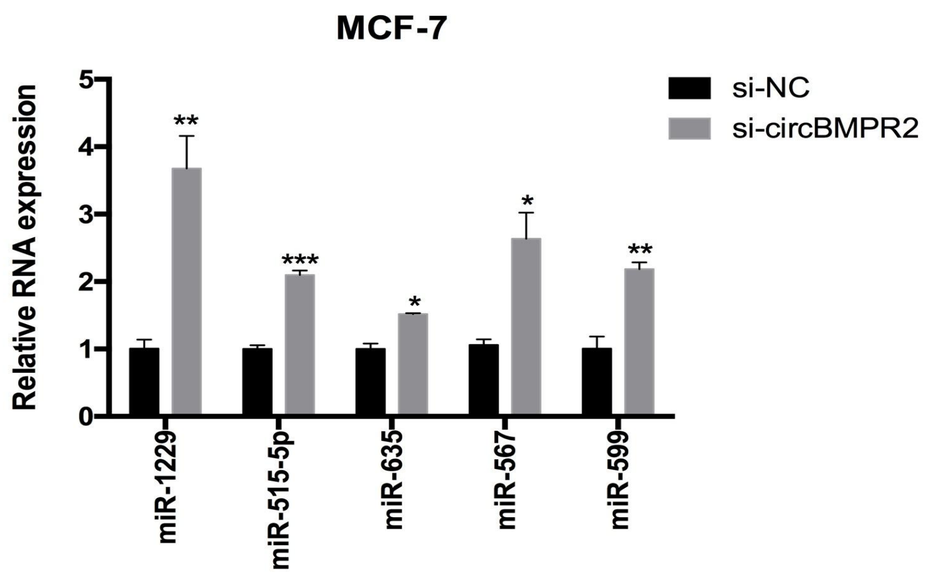
Low	326	51	11	0	0
High	253	52	10	3	0



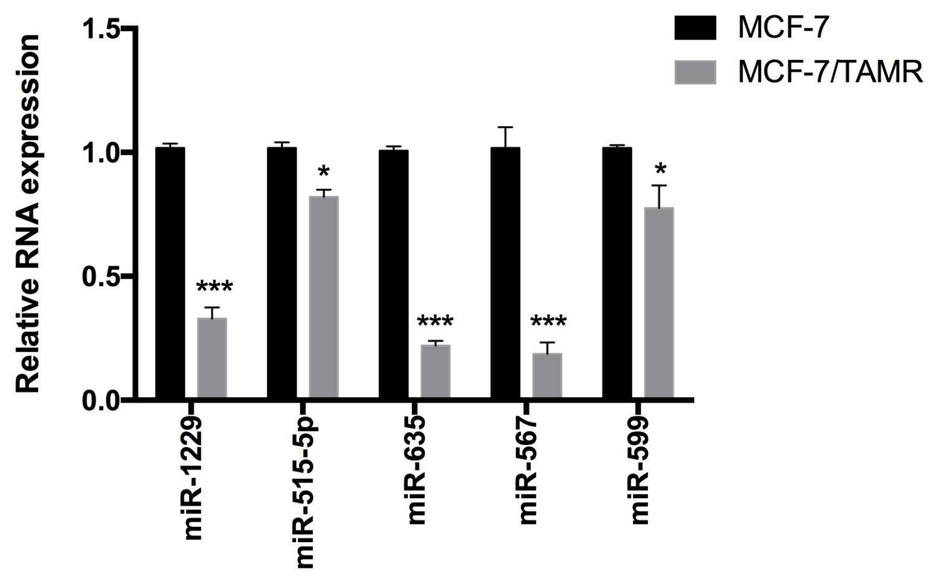
Number at risk

Low	186	47	10	2	0
High	933	56	11	1	0

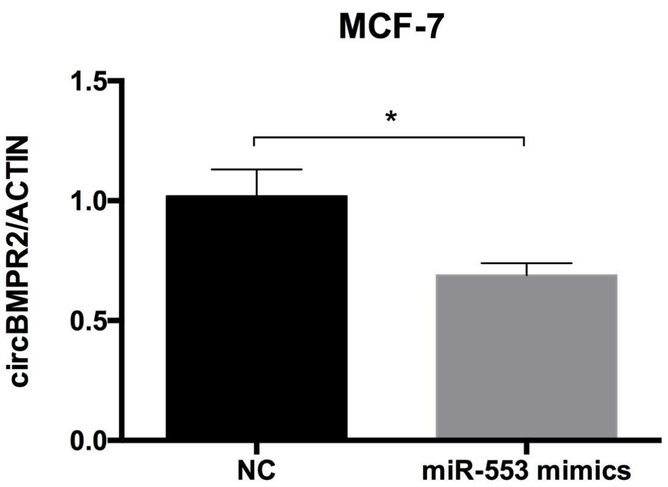
A

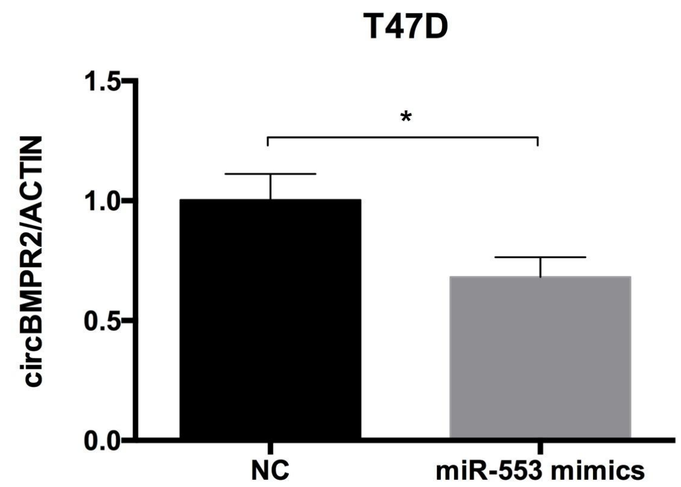

MCF-7
si-NC
si-circBMPR2
Relative RNA expression
0
1
2
3
4
5
**
***
*
*
**
miR-1229
miR-515-5p
miR-635
miR-567
miR-599

B

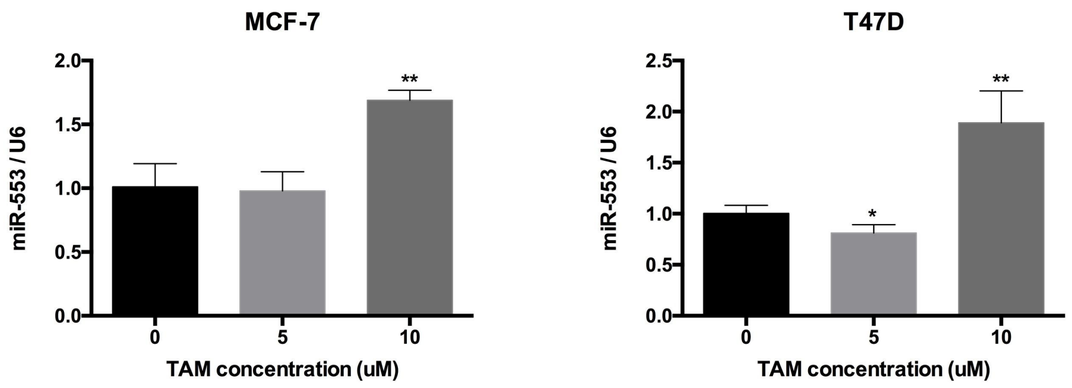

MCF-7
MCF-7/TAMR
Relative RNA expression
0.0
0.5
1.0
1.5
***
*
***
***
*
miR-1229
miR-515-5p
miR-635
miR-567
miR-599

C

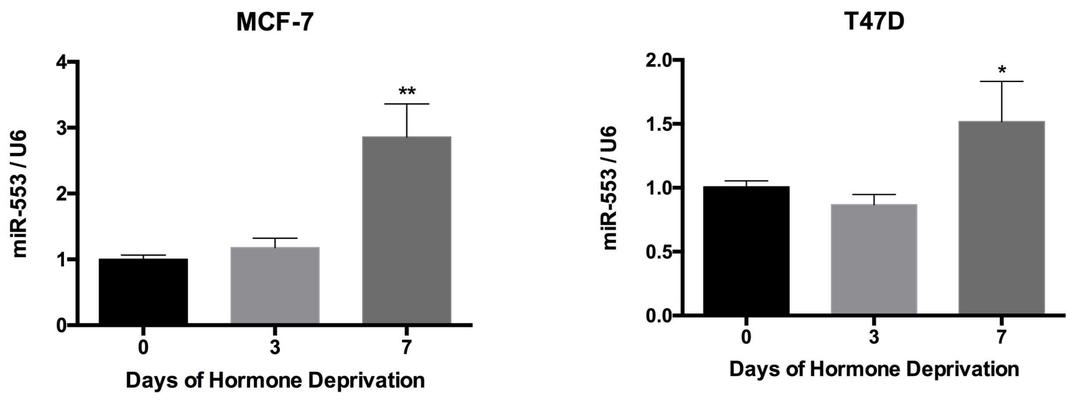

MCF-7
circBMPR2/ACTIN
0.0
0.5
1.0
1.5
*
NC
miR-553 mimics


T47D
circBMPR2/ACTIN
0.0
0.5
1.0
1.5
*
NC
miR-553 mimics

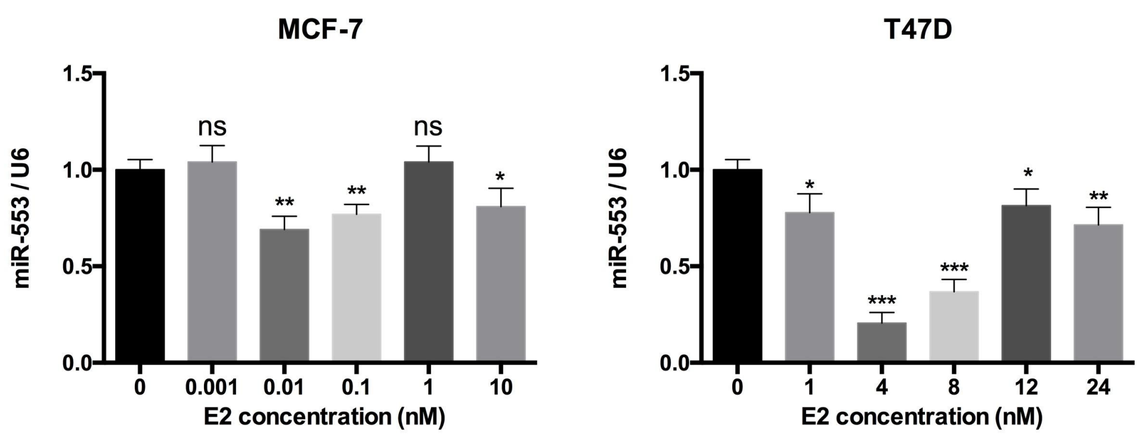
A


MCF-7
T47D
miR-553 / U6
2.0
1.5
1.0
0.5
0.0
2.5
**
*
0
5
10
TAM concentration (uM)

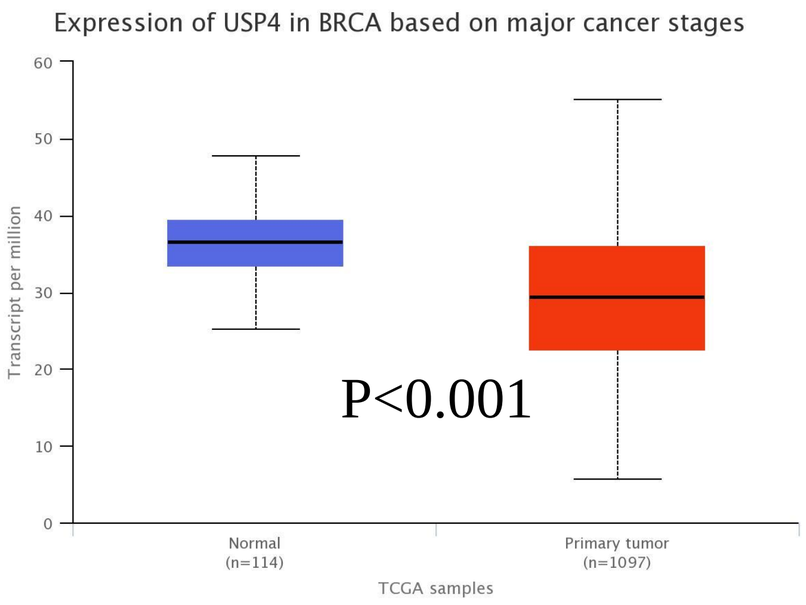
B

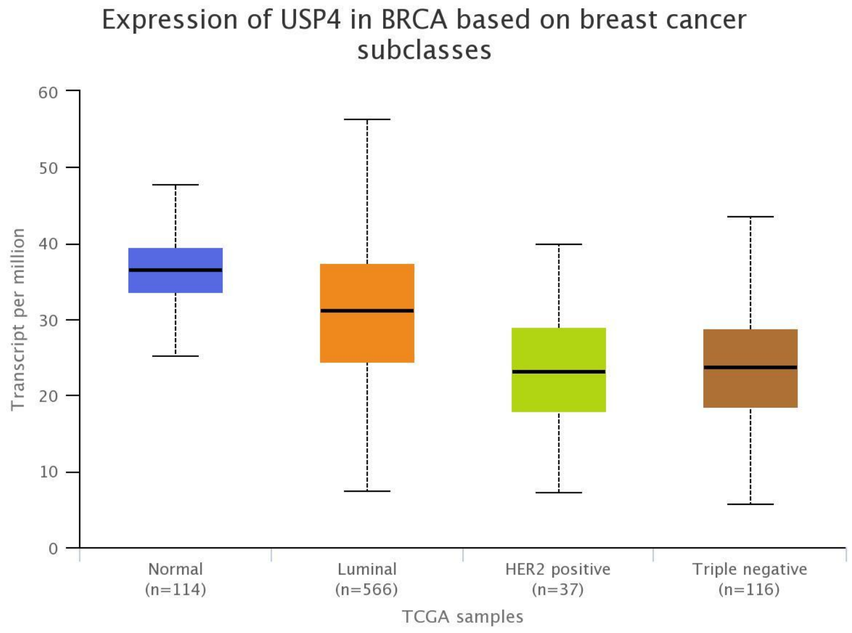

MCF-7
T47D
miR-553 / U6
4
3
2
1
0
**
*
0
3
7
Days of Hormone Deprivation

C

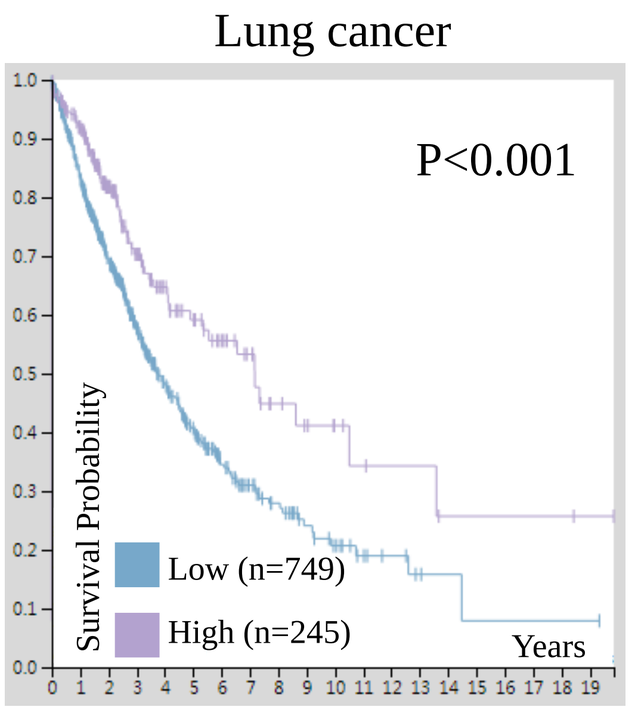

MCF-7
T47D
miR-553 / U6
1.5
1.0
0.5
0.0
ns
**
*
***
0
0.001
0.01
0.1
1
10
4
8
12
24
E2 concentration (nM)

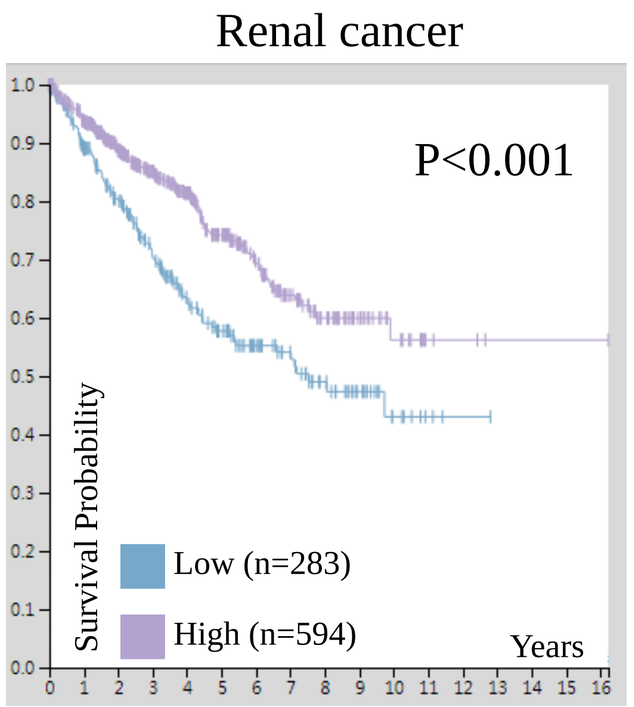
A


Expression of USP4 in BRCA based on major cancer stages
Transcript per million
P<0.001
Normal (n=114)
Primary tumor (n=1097)
TCGA samples

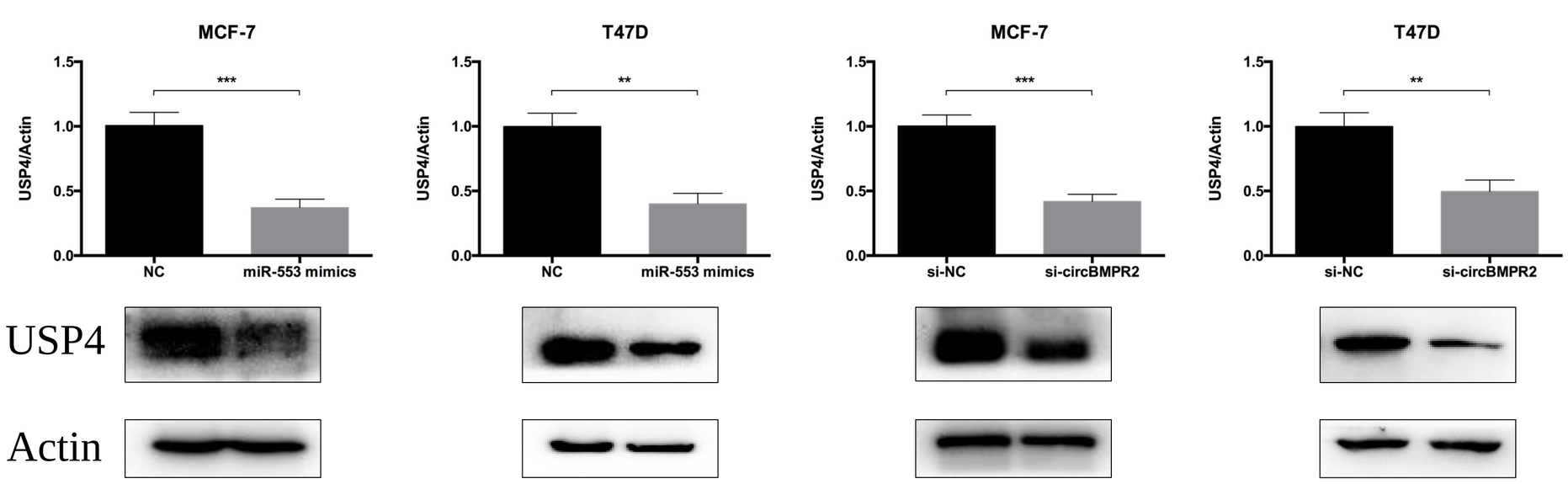

Expression of USP4 in BRCA based on breast cancer subclasses
Transcript per million
Normal (n=114)
Luminal (n=566)
HER2 positive (n=37)
Triple negative (n=116)
TCGA samples

B

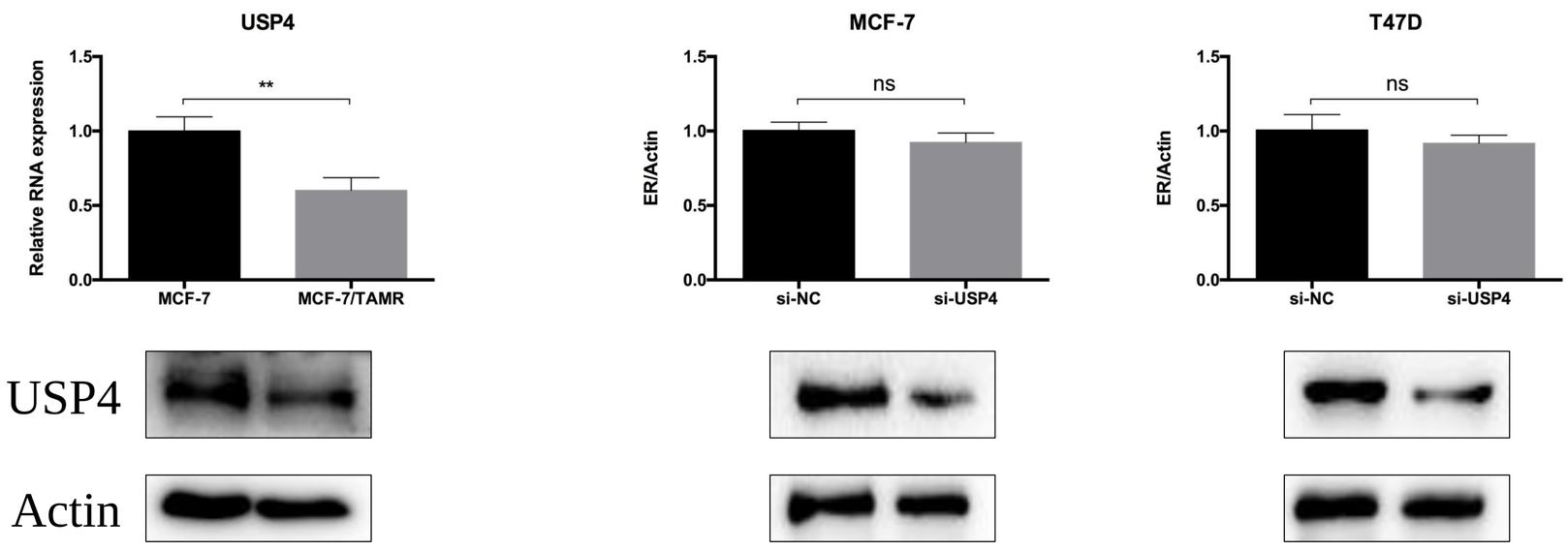

Lung cancer
P<0.001
Survival Probability
Low (n=749)
High (n=245)
Years


Renal cancer
P<0.001
Survival Probability
Low (n=283)
High (n=594)
Years

C


MCF-7
T47D
MCF-7
T47D
USP4/Actin
NC
miR-553 mimics
si-NC
si-circBMPR2
USP4
Actin

D


USP4
Relative RNA expression
MCF-7
MCF-7/TAMR
USP4
Actin

E

MCF-7
T47D
ER/Actin
ns
si-NC
si-USP4

Figure S1 CircBMPR2 inhibits proliferation, migration, and invasion of breast cancer cells. (A) The qRT-PCR assay was used to validate the knockdown efficiency of circBMPR2 in MDA-MB-231 and MDA-MB-468 cells. (B) MTT assay showed inhibited proliferation after circBMPR2 knockdown in MDA-MB-231 and MDA-MB-468 cells. (C) Knockdown of circBMPR2 promoted cell cycle progression in MDA-MB-231 and MDA-MB-468 cells. (D) Transwell migration assay was used to measure metastasis capacity in MDA-MB-231 and MDA-MB-468 cells transfected with si-circBMPR2 or si-NC. (E) CircBMPR2 knockdown led to increased expression of mesenchymal markers and decreased epithelial markers in MDA-MB-231 and MDA-MB-468 cells. (F) The qRT-PCR assay was used to examine the expression of circBMPR2 after transfection with pLCDH-circBMPR2 in MDA-MB-231 and MDA-MB-468 cells. (G-H) Overexpression of circBMPR2 promoted proliferation and migration of MDA-MB-231 and MDA-MB-468 cells. (*P < 0.05, **P < 0.01, and ***P < 0.001, Student's t-test)

Figure S2 CircBMPR2 overexpression attenuates tamoxifen resistance of breast cancer cells. (A) The expression of circBMPR2 was decreased in MCF-7/TAMR cells compared to their parental cells. (B) The qRT-PCR assay was used to examine the expression of circBMPR2 after transfection with pLCDH-circBMPR2 in MCF-7/TAMR cells. (C) Overexpression of circBMPR2 inhibited proliferation of MCF-7/TAMR cells as detected by MTT assay. (D) Wound healing assay revealed that circBMPR2 overexpression inhibited migration ability of MCF-7/TAMR cells. (E) Transwell assay was performed to detect the migration and invasion ability of MCF-7/TAMR cells with circBMPR2 overexpression. (F) Western blot was used to detect the effect of circBMPR2 overexpression on EMT-related proteins in MCF-7/TAMR cells. (G) Overexpression of circBMPR2 could reduce tamoxifen resistance of MCF-7/TAMR cells. (H) CircBMPR2 overexpression promoted tamoxifen-induced apoptosis in MCF-7/TAMR cells. (**P < 0.01, and ***P < 0.001, Student's t-test)

Figure S3 Tamoxifen treatment inhibits expression of circBMPR2 and circBMPR2 promotes expression of ER in breast cancer cells. (A) MCF-7 and T47D cells were treated with varying concentrations of tamoxifen for 7 days. The qRT-PCR assay indicated decreased circBMPR2 and GREB1 expression in a concentration-dependent manner. (B) Estrogen deprivation inhibited cirBMPR2 and GREB1 expression in a time-dependent manner. (C) MCF-7 and T47D cells were estrogen starved for 3days and treated with indicated concentrations of estrogen for 6h. The qRT-PCR assay was used to examine the expression of circBMPR2 and GREB1. (D) Overexpression of circBMPR2 was able to restore the expression of ER and PR. (E) Overexpression of circBMPR2 promoted the effect of estrogen on the expression of its target genes as detected by qRT-PCR assays. (*P < 0.05, **P < 0.01, and ***P < 0.001, Student's t-test)

Figure S4 The association between predicted miRNAs of circBMPR2 and overall survival of breast cancer patients. (A) Kaplan-Meier Plotter tool was used to detect the association between the expression of several putative miRNAs and the overall survival of breast cancer patients.

Figure S5 The association between circBMPR2 and predicted miRNAs. (A) The qRT-PCR assay was used to evaluate the effect of circBMPR2 on the expression of several putative miRNAs. (B) The expression of several putative miRNAs in MCT-7/TAMR and parental cells. (C) Overexpression of miR-553 led to decreased expression of circBMPR2 in MCF-7 and T47D cells. (*P < 0.05, **P < 0.01, and ***P < 0.001, Student's t-test)

Figure S6 The expression of miR-553 was promoted by tamoxifen treatment and inhibited by estrogen treatment. (A) The expression of miR-553 was increased after tamoxifen treatment for 7 days in MCF-7 and T47D cells. (B) Estrogen deprivation promoted miR-553 expression in a time-dependent manner. (C) The expression of miR-553 was inhibited after estrogen treatment. (*P < 0.05, **P < 0.01, and ***P < 0.001, Student's t-test)

Figure S7 The expression of USP4 was decreased in breast cancer tissues and associated with better prognosis of breast cancer patients. (A) The expression of USP4 was decreased in breast cancer tissues and negatively associated with clinical stages of breast cancer according to the TCGA database. (B) The expression of USP4 was associated with better prognosis of breast cancer patients according to the Protein Atlas database. (C) Overexpression of miR-553 or circBMPR2 knockdown led to decreased expression of USP4. (D)The qRT-PCR and western blot assays showed reduced expression of USP4 in MCF-7/TAMR cells compared to their parental cells. (E) USP4 knockdown led to decreased protein expression level of ER. (***P < 0.001, Student's t-test)

Table S1. The putative miRNAs for circBMPR2.

miRNA ID	Sequence (3'-5')	Site Type	Context + score	Context + score percentile
hsa-miR-1229	GACACCCUCCCGUCACCACUCUC	7mer-m8	-0.271	97
hsa-miR-323-3p	UCUCCAGCUGGCACAUUACAC	7mer-m8	-0.173	98
hsa-miR-431	ACGUACUGCCGGACGUUCUGU	7mer-m8	-0.219	95
hsa-miR-433	UGUGGCUCCUCGGGUAGUACUA	7mer-1a	-0.103	91
hsa-miR-515-5p	GUCUUUCACGAAAGAAAACCUCUU	7mer-m8	-0.135	89
hsa-miR-526b	UGUCUUUCACGAAGGGAGUUCUC	7mer-1a	too_close	NA
hsa-miR532-3p-	ACGUUCGGAACCCACACCCUCC	7mer-1a	-0.159	91
hsa-miR-553	UUUUGUUUUAGAGUGGCAAAA	7mer-m8	-0.248	93
hsa-miR-561	UGAAGUUCCUAGAAUUUGAAAC	7mer-1a	-0.006	90
hsa-miR-567	CAAGACAGGACCUUCUUGUAUGA	7mer-m8	-0.075	90
hsa-miR-599	CAAACUAUUUGACUGUGUUG	7mer-m8	-0.134	95
hsa-miR-635	CCUGUAACAAAGUCACGGGUUCA	7mer-1a	-0.157	84
hsa-miR-769-5p	UCGAGUCUUGGGUCUCCAGAGU	7mer-1a	-0.164	93

Table S2. Primers used for qRT-PCR.

Gene	Forward	Reverse
circBMPR2	CATACCGTTTCTGCTGTT	CCCTTTTGATTTCTCCC
BMPR2	GAGGAGGCTTTCTTGGTGG	CTTTCGCTTCGGTGCTTC
GREB1	GGTCTGCCTTGCATCCTGATC	CCTGCTCCAAGGCTGTTCTC
ERα	GCTTACTGACCAACCTGGCA	GGATCTCTAGCCAGGCACATTC
PGR	GTCGCCTTAGAAAGTGCTGTC	GCTTGGCTTTCATTTGGAACG
USP4	TAGACACGCTGGAACAGGTTGC	CTCCTCGTACAGCTTCACAGTC
ZEB1	GGCATACACCTACTCAACTACGG	TGGGCGGTGTAGAATCAGAGTC
ZEB2	TGCACAGAGTGTGGCAAGG	CTGCTGATGTGCGAACTGTAGG
Vimentin	GGCAAAGCAGGAGTCCACTG	CTGGCGTTCCAGGGACTCAT
E-cadherin	GCTCACATTTCCCAACTCCTC	CTCTGTCACCTTCAGCCATCC
Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
miR-553	AAAACGGTGAGATTTTGTTTT	-

Table S3 Antibodies used in the study

Antigen	Supplier	Catalog #
p53	Cell Signaling Technology	9282
Rb	Cell Signaling Technology	9309
PARP	Cell Signaling Technology	9532
caspase8	Cell Signaling Technology	4790
cleave caspase8	Cell Signaling Technology	9746
caspase9	Abcam	ab32539
Actin	Proteintech	60008-1-Ig
USP4	Santa Cruz	sc-376000
Fibronectin	Proteintech	15613-1-AP
N-cadherin	Proteintech	YT2988
E-cadherin	Proteintech	20874-1-AP
Vimentin	Cell Signaling Technology	5741
ZEB1	Proteintech	21544-1-AP
ZEB2	Proteintech	14026-1-AP
Snail	Cell Signaling Technology	3879
Slug	Cell Signaling Technology	9585
ER	Cell Signaling Technology	13258
PR	Cell Signaling Technology	3153
HRP-anti-mouse	Cell Signaling Technology	7076
HRP-anti-rabbit	Cell Signaling Technology	7074