OMTN, Volume *25*

Supplemental information

miR-497 defect contributes to gastric cancer tumorigenesis and progression via regulating CDC42/ITGB1/FAK/PXN/AKT signaling

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Construction of miRNA 497 knockout (KO) C57BL/6 mice by CRISPR/Cas9-mediated genome engineering

Summary:

The mouse mi-RNA 497 gene (mi-RNA Base: MI0004636; Ensembl: ENSMUSG00000105220) is located on mouse chromosome 11. 5P and 3P of mouse mi-RNA 497 were selected as target site. Cas9 mRNA and gRNA generated by *in vitro* transcription were then injected into fertilized eggs for KO mouse productions. The founders were genotyped by PCR followed by DNA sequencing analysis. The positive founders were breeding to the next generation which was genotyped by PCR and DNA sequencing analysis.

Results

Injected mRNA was named as Mouse miRNA-497-gRNA3(VB160318-1030dzc) and Mouse miRNA-497-gRNA4(VB160318-1031ady) (Table S1, 2). The generation of founder 0 and 1was showed in Table S3.

Table S1. Summary of VB160318-1030dzc.

Vector ID	VB160318-1030dzc
Vector Name (official)	pRP[CRISPR]-hCas9-U6>20nt_GCCTGCTAAACTACTTTTGC
Date Created (Pacific Time)	2016-03-17
Size	8507 bp
Vector Type	Regular plasmid CRISPR vector (single gRNA)
Inserted gRNA	20nt_GCCTGCTAAACTACTTTTGC
Inserted Nuclease	hCas9
Target Sequence	GCCTGCTAAACTACTTTTGC

Copy Number	High
Bacterial Resistance	Ampicillin
Cloning Host	Stbl3

Table S2. Summary of VB160318-1031ady.

Vector ID	VB160318-1031ady
Vector Name (official)	pRP[CRISPR]-hCas9-U6>20nt_GTTGTCTGATACCAGTTATC
Date Created (Pacific Time)	2016-03-17
Size	8507 bp
Vector Type	Regular plasmid CRISPR vector (single gRNA)
Inserted gRNA	20nt_GTTGTCTGATACCAGTTATC
Inserted Nuclease	hCas9
Target Sequence	GTTGTCTGATACCAGTTATC
Copy Number	High
Bacterial Resistance	Ampicillin
Cloning Host	Stbl3

Table S3. Founder generation.

Founder generation			
Name of Injected mRNA	Mouse Mir497-gRNA3(VB160318-1030dzc)/ Mouse Mir497-gRNA4(VB160318-1031ady)		
Mouse Strain	C57BL/6		
Date of Birth	2016-08-13		
Founders (F0) Generated	♂	2	Mouse-ID#17, Mouse-ID#19
Mouse Strain	C57BL/6(Mouse-ID#17) × C57BL/6(WT)		
Date of Birth	2016-10-27		
Founders (F1) Generated	♂	2	Mouse-ID#7
	♀	1	Mouse-ID#10, Mouse-ID#11
Mouse Strain	C57BL/6(Mouse-ID#19) × C57BL/6(WT)		
Date of Birth	2016-10-28		
Founders (F1) Generated	♂	2	Mouse-ID#19, Mouse-ID#23

	♀	1	Mouse-ID#25

1. **F0 founder screening: The PCR products were generated from PCR genotyping using the primers and conditions listed below (Fig. 1). The amplicons were then purified and sent for DNA sequencing analysis. DNA sequencing using the primer listed below revealed that Mouse-ID#17 was missing 409 bases in one strand; Mouse-ID#19 was missing 411 bases in one strand.**
    - Mouse Mir497-F: 5'-CACCACCTCTGGACTGCCAACTC-3'
    - Mouse Mir497-R: 5'-CGTTCCTGATAACCATGTGCCCT-3'
    - Product Size: WT: 979 bp; MT: ~580 bp, delete: ~400 bp
    - Annealing Temp: 58℃
    - DNA Sequencing Primer (Forward Sequencing): 5'-GCCCTGTGTCTTCCAGCATTTC-3'

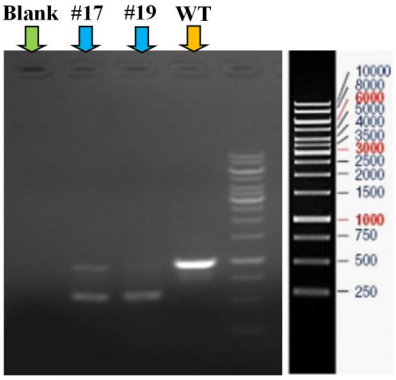


Fig.1. F0 founder PCR screening

2. **F1 founder screening: The PCR products were generated from PCR genotyping using the primers and conditions listed above (Fig.2). The amplicons were then purified and sent for DNA sequencing analysis. DNA sequencing revealed that Mouse-ID#7, #10, #11 were missing 409 bases in one strand; Mouse-ID#19, #23, #25 were missing 411 bases in one strand (See extended data and attachment).**

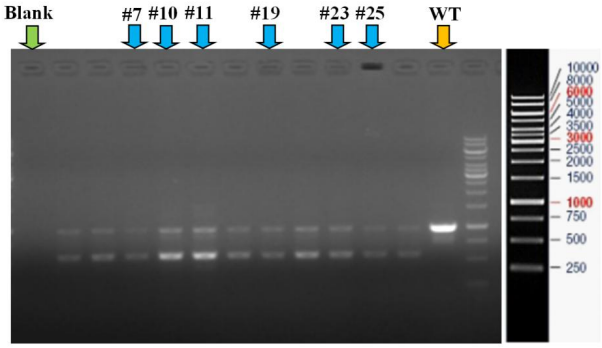


Fig.2. F1 founder PCR screening

Extended Data:

The deletion sequence is in black and the 5P and 3P of Mouse Mir497 are over-striking.

Mouse Mir497 sequence:

cctgcccccgccc**cagcagcacactgtggtttgta**cggcactgtggccacgtc**caaaccacactgtggtgttag**agcgagggta

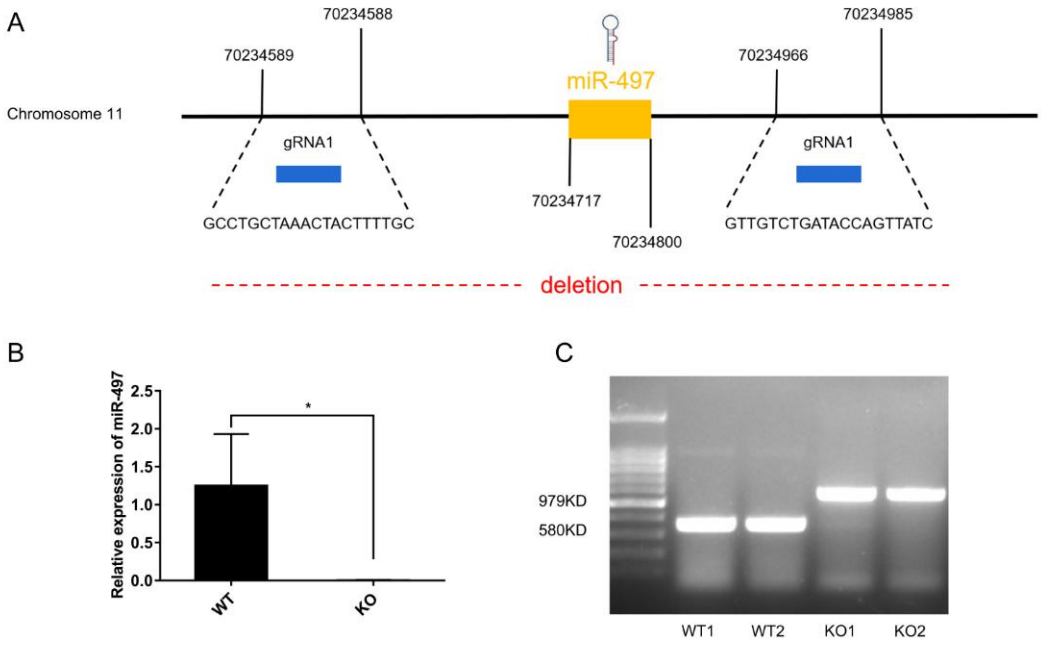
ID#17-F1-Mouse-ID#7, #10, #11:
ttttgctggttcctgattgttcccccccccccagctttttttgggggggccatgtttgccattcacacctctgtctcactctaggtgggggtcttcacggcactg
cctgtgctgtcttcctctcgacccaccccagtcctgcccccgccc**cagcagcacactgtggtttgta**cggcactgtggccacgtc**caaaccacac**
tgtggtgttagagcgagggtatgggaggcaccgatgagcctggccctgggaggccaccctggagaagcaacacacacacacacacacacaca
cacacacacacacacacaccgtctagggtattgtgatgaagttcttgcaaggtgggacaggagacactggaaagagcccctctgcaacccccag
gttgtctgataccagttatcaggaac

ID#19-F1-Mouse-ID#19, #23, #25:
tttgctggttcctgattgttcccccccccccagctttttttgggggggccatgtttgccattcacacctctgtctcactctaggtgggggtcttcacggcactg
cctgtgctgtcttcctctcgacccaccccagtcctgcccccgccc**cagcagcacactgtggtttgta**cggcactgtggccacgtc**caaaccacac**
tgtggtgttagagcgagggtatgggaggcaccgatgagcctggccctgggaggccaccctggagaagcaacacacacacacacacacacaca
cacacacacacacacacaccgtctagggtattgtgatgaagttcttgcaaggtgggacaggagacactggaaagagcccctctgcaacccccag
gttgtctgataccagttatcaggaaccct

Relevant Reagents	
Water	Sigma, Cat. No. W1503
EDTA	Sigma, Cat. No. E7889
Trizma Hydrochloride Solution	Sigma, Cat. No. T2663
Proteinase K	Merck, Cat. No. MK539480
Taq DNA Polymerase	Takara, Cat. No. R007
dNTP	Takara, Cat. No. 4030
Agarose	Biowest Agarose, Regular
DNA Marker	FermentasGeneRuler™ 100bp DNA Ladder #SM0241
0.5×TBE	Tris Bio Basic Inc, TBO194-500g EDTA Shanghai Sangon, 0105-500g Boric Acid, Shanghai Sangon, 0588-500g

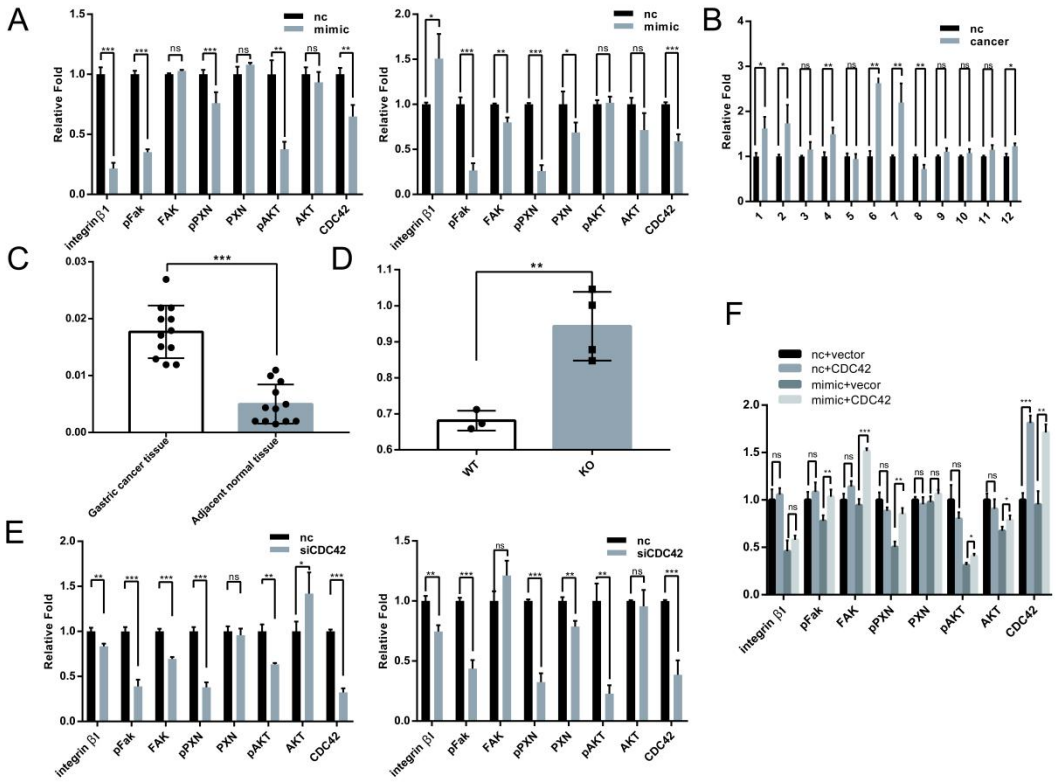
Table S4. Antibodies used in the present study.

Target	Company	Catalog number	Dilution
ITGB1	Cell Signaling Technology	#34971	1:1000 (WB)
FAK	Cell Signaling Technology	#3285	1:1000 (WB)
pY397-FAK	Cell Signaling Technology	#3281	1:1000 (WB)
CDC42	Abcam	#ab187643	1:5000 (WB)
Paxillin	Abcam	#ab32084	1:5000 (WB)
pY118-Paxillin	Abcam	#ab109547	1:1000 (WB)
AKT	Proteintech	#10176-2-AP	1:2000 (WB)
pS473-AKT	Proteintech	#66444-1-IG	1:2000 (WB)
GAPDH	Santa Cruz Biotechnology	#sc-47724	1:1000 (WB)
pY397-FAK	Abcam	#ab81298	1:1000 (IF)
pY118-Paxillin	Abcam	#ab109547	1:1000 (IF)
CDC42	Proteintech	10155-1-AP	1:50-500 (IHC)



Supplementary Figure 1. The generation of miR-497 knockout mice.

(A) The schematic representation of generation of miR-497 knockout mice. (B) qPCR was used to validate the efficiency of miR-497 knockout. (C) Southern blot of genomic DNA from mice of the indicated genotypes; WT band, 979 kb; disrupted band, 580 kb. (P<0.05).



Supplementary Figure 2. The quantification of the experimental results of Western blotting and IHC.

(A) Western blot was used to detect the effect of miR-497 transfection in AGS (left) and HGC (right) cells on CDC42/ITGB1/FAK/PXN/AKT pathway. (B) Quantitation of CDC42 expression in GC tissues / para-carcinoma tissues, which was detected by Western blot. (C) Quantitation of the IHC staining of CDC42 in GC tissues / para-carcinoma tissues, indicating that the expression of CDC42 was up-regulated in GC tissues. (D) Quantitation of CDC42 expression in the gastric tissues of miR-497$^{-/-}$ mice (n=4) and WT mice (n=3), which was detected by Western blot. (E) Western blotting was used to detect the change of CDC42/ITGB1/FAK/PXN/AKT pathway in AGS (left) and HGC (right) cells, after CDC42 was depleted. (F) Western blot was used to detect the effect of CDC42 and miR-497 on CDC42/ITGB1/FAK/PXN/AKT pathway (ns, no significance; *, P<0.05; **, P<0.01; ***, P<0.001).