

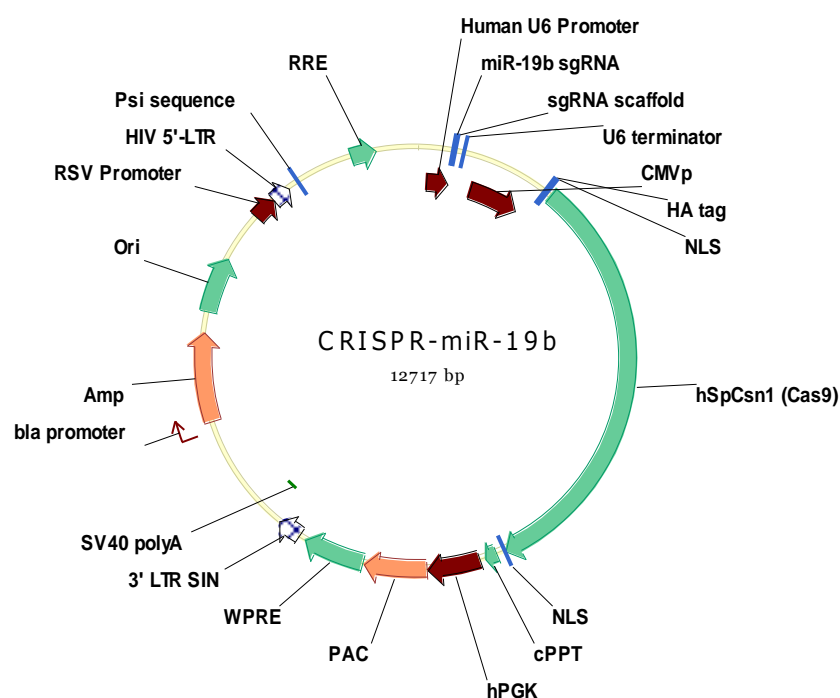
Product Description

MicroRNAs (miRNAs), also known as “mature miRNA” are small endogenous RNAs (approximately 20-24 nucleotides in length), non-coding RNA molecules that can inhibit protein expressions of target mRNAs, by interacting mainly to its 3’ untranslated regions (3’UTR) and thus degrade mRNAs or inhibit translation. MicroRNAs have been implicated in critical processes including differentiation, apoptosis, proliferation, and the maintenance of cell and tissue identity; furthermore, their misexpression has been linked to cancer and other disease. Currently, three general methods are used for miRNA loss-of-function studies: genetic knockouts, antisense oligonucleotide inhibitors ([Meister et al. 2004](#); [Krützfeldt et al. 2005](#); [Ørom et al. 2006](#)) and sponges ([Ebert et al. 2007](#)). Here, we established a system for construction of all-in-one expression vectors containing a guide RNA expression and Cas9 expression cassette. CRISPR/Cas9 systems provide a platform for high efficiency genome modulation and editing. Using a specifically designed gRNA, Cas9 can be directed to the target sequence, making double-strand breaks (DSBs) that silence a gene of interest.

Result

A. Establishment of an all-in-one construction system for CRISPR/Cas9 mediated knockout of miRNAs

*See RNAi core sgRNA service (Customized C6-15)



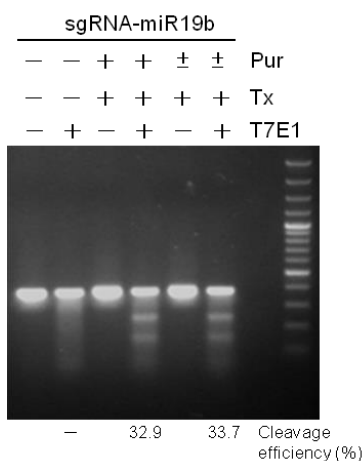
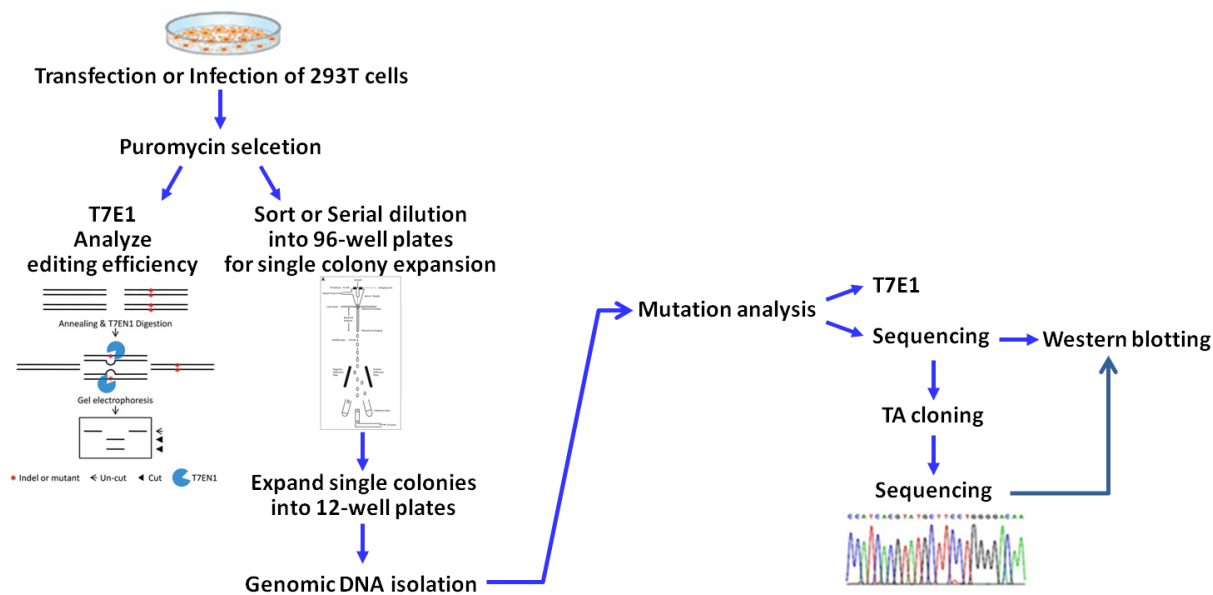
has-TP53 3'UTR	5' -UGUGAAAUGCUGGCAUUUGCACC...- 3'
has-miR19b	3' -GUGAUGGUGUCAGUCAAAACGUACC <u>UAAACGUGU</u> -5'
has-miR19a	3' -CGUCCGGUGGUAGUCAAAACGUAUC <u>UAAACGUGU</u> -5'
has-miR19b gDNA	3' -GTGATGGTGTCTAGTCAAACGT <u>ACCTAAACGTGT</u> -5'
CRISPR miR19b gRNA	3' -CATGGTGTCTAGTCAAACGT-5'

Fig. 1: Knockout strategy for making Cas9 all-in-one lenti-CRISPR vector targeting miR19b. (A) Schematic diagram of the all-in-one CRISPR/Cas9 vector construction. (B) Gene structure of TP53 and has-miR19a or has-miR19b

targeting sequence within 3'UTR. The seed region of miR19a or miR19b were marked in green; underlined sequences were the mature miRNA and the PAM sequence of miR19b was in cyan. The predicted cleavage site was indicated with a red triangle. The sgRNA was designed for miR19b by using CRISPR DESIGN (<http://crispr.mit.edu/>).

B. Workflow of CRISPR/Cas9 mediated gene engineering platform

To test the efficiency of our system, 293T cells were transfected with all-in-one CRISPR/Cas9-miR19b-KO expression plasmids. Check to make sure the cells should be 60-80% confluent prior to transfection. The transfection efficiency varies according to cell type and the transfection reagent used. The optimal conditions must be determined empirically. At 24-48 hr post-transfection, the medium was replaced with fresh medium containing puromycin. After enrichment by puromycin, sort or dilute cells into 96-well plates for single clone isolation. In parallel, the pool populations were assayed with T7E1 to detect the target cleavage effect. miR19b-sgRNA showed efficient cleavages in inducing indels by T7E1 assay (32.9%, Fig.2B) in the targeted loci and was therefore chosen for subsequent studies. Expand single colonies into 24- or 12-well plates until the cells were dense enough. Different clonal lines were further culture and harvest for gDNA isolation (T7E1 and sequencing, Fig.3A;3B) and protein extraction (western blot. Fig.3C).



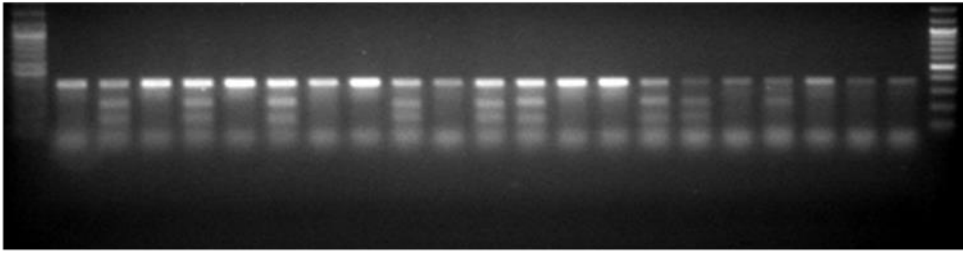
Gene	Sequence	PAM	PCR products	Expected cut products
miR19b	G <u>T</u> ACCACAGTCAGTTTGGCA	TGG	382 bp	146 bp 236 bp

Fig. 2: Flow chart of the cell line development strategy. (A) A procedure of selection and identification of knockout clones. (B) Detection of indels in miR19b knockout 293T cells by T7E1 (T7 endonuclease I) assay.

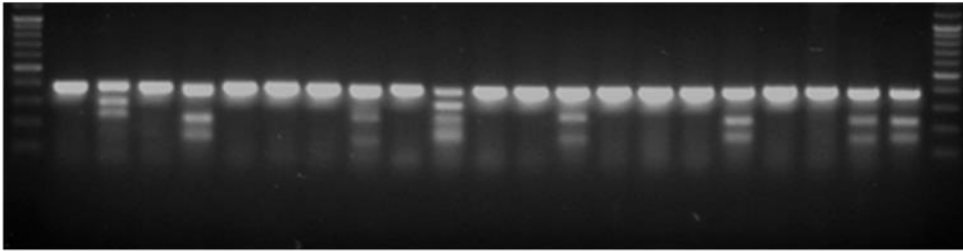
C. Results

Screening of the clones by using T7E1 assay and sequencing confirmation. The miR19b target genes, PTEN, TP53 or TP53INP1 increased in the 12 edited clonal lines, validating functional gene knockout.

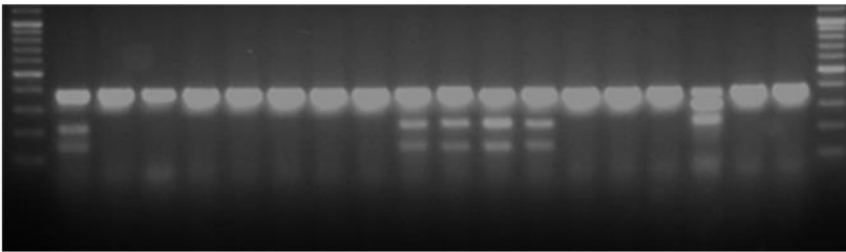
Clone # 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21



Clone # 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42



Clone # 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60



miR19b-5p *miR19b-3p

miR19b-wt CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TGC AAAACT GACT GTGG TAGTG

Allele 1	<p>KO#N-1 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TGC AAAACT GACT GTGG TAGTG (+1bp)</p> <p>(N: 1~60)</p> <p>KO#1-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TGC AAAACT GACT GTGG TAGTG (+1bp)</p> <p>KO#2-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATC -ATGC-----TGACTGTGGTAGTG (-10bp, +4bp)</p> <p>KO#4-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TG--AAACTGACTGTGGTAGTG (-2bp)</p> <p>KO#6-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TG-----TGGTAGTG (-12bp)</p> <p>KO#9-2* CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TG-----AAAAGT GACT GTGG TAGTG (-9bp)</p> <p>KO#11-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG-----AAACTGACTGTGGTAGTG (-14bp)</p> <p>KO#12-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TGC-----ACTGTGGTAGTG (-7bp)</p> <p>KO#15-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TGC A-----TGTGGTAGTG (-9bp, +1bp)</p> <p>KO#16-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TGC AAAACT GACT GTGG TAGTG (+57bp)</p> <p style="text-align: center;">ATTAGGCGTTTTCGCGTGTCTCGCATGTACGGGCCAGAAATTCATGAAGAATCTG</p>
Allele 2	<p>KO#18-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATC-----ACA (-63bp)</p> <p>KO#22-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TGC-AAACTGACTGTGGTAGTG (-1bp)</p> <p>KO#23-2** CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TGC-----ACTGACTGTGGTAGTG (-3bp)</p> <p>KO#25-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT-----CTGACTGTGGTAGTG (-23bp)</p> <p>KO#29-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG-----AAAGT (-30bp)</p> <p>KO#31-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG-----AAAAGT GACT GTGG TAGTG (-11bp)</p> <p>KO#34-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TGC A-----TAGTG (-14bp, +1bp)</p> <p>KO#38-2* CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TGC-----AAAAGT GACT GTGG TAGTG (-9bp)</p> <p>KO#41-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGT-----AAACTGACTGTGGTAGTG (-15bp)</p> <p>KO#42-2* CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TGC-----AAACTGACTGTGGTAGTG (-9bp)</p> <p>KO#43-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT-----AAACTGACTGTGGTAGTG (-23bp)</p> <p>KO#51-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG-----AAACTGACTGTGGTAGTG (-11bp)</p> <p>KO#52-2** CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TGC-----ACTGACTGTGGTAGTG (-3bp)</p> <p>KO#53-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA-----CTGACTGTGGTAGTG (-7bp)</p> <p>KO#54-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAAT-----AAAAGT GACT GTGG TAGTG (-6bp)</p> <p>KO#58-2 CAC TGT TCT ATG GTT AG TTT TGC AGG TTG CAT CCAG CTGT GTG ATATT CTG CTGTG CAAATCCA TG-AAAAGT GACT GTGG TAGTG (-1bp, +2bp)</p> <p style="text-align: center;">TA</p>

Genotype	Clone #	Allele 1		Allele 2	
		Insertion	Deletion	Insertion	Deletion
Homozygous	1	1	—	1	—
	3	1	—	1	—
	5	1	—	1	—
	7	1	—	1	—
	8	1	—	1	—
	10	1	—	1	—
	13	1	—	1	—
	14	1	—	1	—
	17	1	—	1	—
	19	1	—	1	—
	20	1	—	1	—
	21	1	—	1	—
	24	1	—	1	—
	26	1	—	1	—
	27	1	—	1	—
	28	1	—	1	—
	30	1	—	1	—
	32	1	—	1	—
	33	1	—	1	—
	35	1	—	1	—
	36	1	—	1	—
	37	1	—	1	—
	39	1	—	1	—
	40	1	—	1	—
	44	1	—	1	—
	45	1	—	1	—
	46	1	—	1	—
	47	1	—	1	—
	48	1	—	1	—
	49	1	—	1	—
	50	1	—	1	—
	55	1	—	1	—
	56	1	—	1	—
	57	1	—	1	—
	59	1	—	1	—
	60	1	—	1	—

Genotype	Clone #	Allele 1		Allele 2		
		Insertion	Deletion	Insertion	Deletion	
Heterozygous	2	1	—	4	10	
	4	1	—	—	2	
	6	1	—	—	12	
	9	1	—	—	9	
	11	1	—	—	14	
	12	1	—	—	7	
	15	1	—	1	9	
	16	1	—	57	—	
	18	1	—	—	63	
	22	1	—	—	1	
	23	1	—	—	3	
	25	1	—	—	23	
	29	1	—	—	30	
	31	1	—	—	11	
	34	1	—	1	14	
	38	1	—	—	9	
	41	1	—	—	15	
	42	1	—	—	9	
	43	1	—	—	23	
	51	1	—	—	11	
	52	1	—	—	3	
	53	1	—	—	7	
	54	1	—	—	6	
	58	1	—	—	2	1

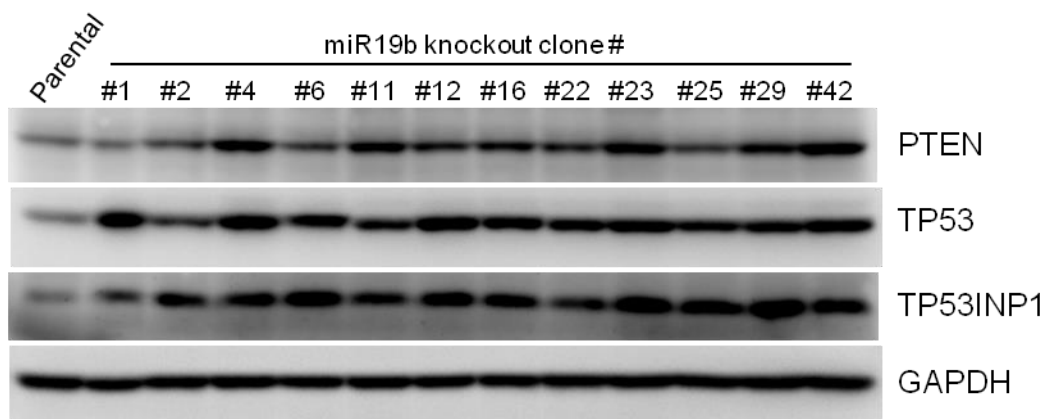


Fig. 3: Detection and identification of indels generated by CRISPR/Cas9 editing. (A) Detection of DNA cleavage by T7E1 assay. (B) Confirm the deletions and insertions generated by CRISPR/Cas9 in selected 60 knockout clones by DNA sequence identification. sgRNA sequence was highlighted by cyan and the deletion or insertion sequences were highlighted by red. (C) miR19b knockout increase the miR19b target genes PTEN, TP53, or TP53INP1, as detected by western blot.

D. Analysis of off-target effects by CRISPR/Cas9-miR19b knockout

We searched for potential off-target sites in the human genome that might be recognized by sgRNAs for miR19b genes (Fig.4A). The off-target sequences were predicted by CRISPR DESIGN (<http://crispr.mit.edu/>), those contain entire 20 bp sequence most similar to the target sequence and differ with 3-4 base mismatches. If the target genomic DNA fragment contains SNPs or allelic mutations, this may cause false positive results when using T7E1 to digest negative controls (off-2, Fig.4B). You may search for SNP information of a gene at NCBI website. No indels were detected at these sites (off-1,3,4,5,6) by T7E1 assay (Fig.4C) and sequence confirmation (data not shown). Two members of the cluster, mir19a and miR19b, belong to the same miRNA family (miR19), have only 1 different nucleotide in their mature sequences (Fig.1B). Analysis by sequence confirmation shows no off-target effects on miR19a in those 60 miR19b-knockout clones (data not shown), demonstrating that CRISPR/cas9 is specific and controllable crossing off-target effects among the miRNA at the same family or highly conserved miRNA family.

sgRNA		Sequence	PAM	Score	Mismatches	UCSC gene	Locus	Products size (bp)	Predicted cleavage size (bp)
miR19b	On	GTACCACAGTCAGTTTGGCA	TGG					382	146/236
	Off-1	AAACCACAGCCAGTTTGGCA	AAG	2.5	3 [1:2:10]		chr2:+25335265	356	108/248
	Off-2	CAGCCACITGTCAGTTTGGCA	GAG	2.5	3 [2:3:8]		chr15:-90607429	342	116/226
	Off-3	TTAICACAATCAGTTTGGCA	TGG	1.6	3 [1:4:9]	NR_029491	chrX:+133303702	391	142/249
	Off-4	AAACCACAGTQAGTTTGGCA	AAG	1.6	3 [1:2:11]		chr6:-155347594	349	123/226
	Off-5	TTACCACAICAGTTTGGCA	GAG	1.5	3 [1:9:10]		chr11:-131764311	406	100/306
	Off-6	GTCIGACAGTCAGTTTGGCA	AAG	1.3	4 [1:3:4:5]	NM_032119	chr5:+90151562	362	90/272

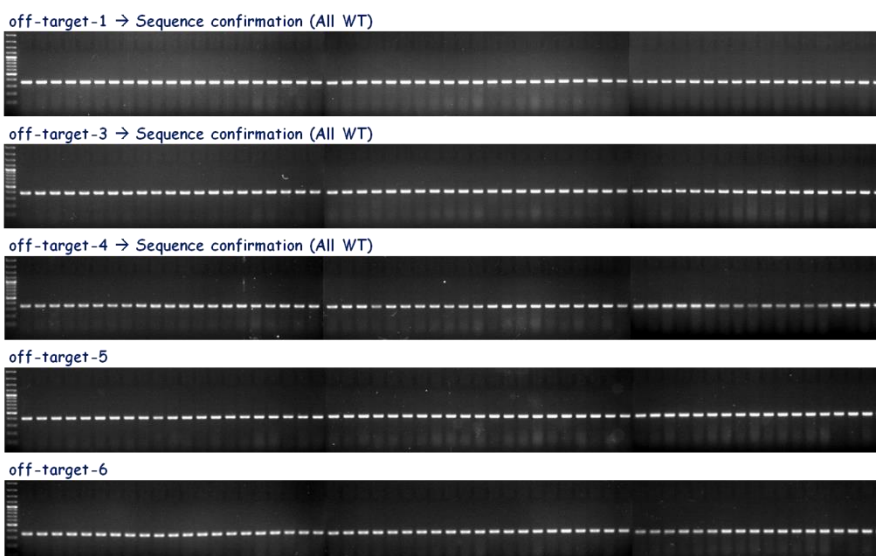
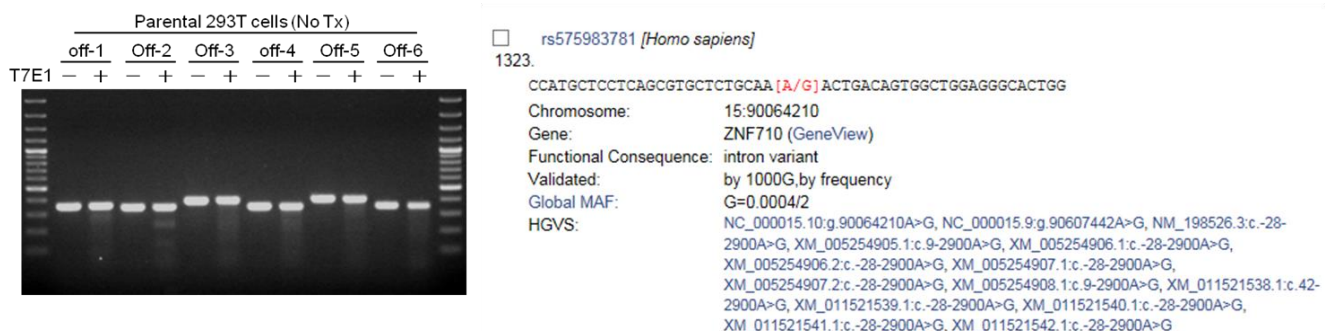
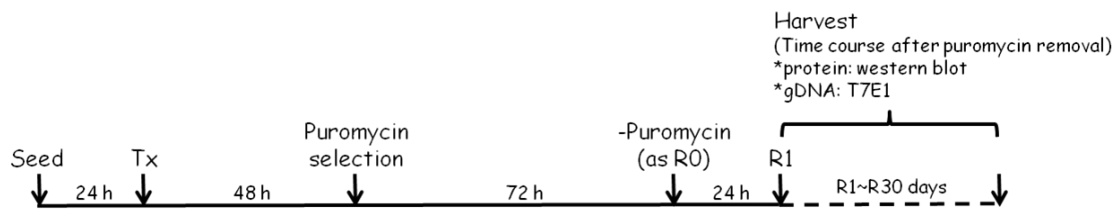


Fig. 4: Analysis of off-target effects. (A) Table of off-target sequences predicted by CRISPR DESIGN (<http://crispr.mit.edu/>). (B) Detection of potential SNP sites in non-transfected 293T cells. (C) No indels were detected at these sites (off-1,3,4,5,6) by T7E1 assay and sequence confirmation (data not shown).

E. Time course of miRNA expression by Western analysis

To test how long of the gene silence by CRISPR/Cas9-miRNA knockout can be expressed, we transiently transfected all-in-one CRISPR/Ca9 plasmids targeting miR20a or miR221 to 293T cells. At 72 hr post-puromycin selection, change media to standard growth medium 1-30 days prior to cell harvested. miR20a or miR221 target region was PCR amplified from the genomic DNA isolated from transfected 293T cells. Analysis of DNA cleavage by T7E1 at 1 Day post-puromycin selection to detect the target cleavage effect. Monitored the expression levels of CRISPR/Cas9 knockout by measured on Day 1 to Day 30 with western blot. The time-course results shown here indicate that although CRISPR/Cas9 can mediate inhibition of miRNA expression (T7E1, Fig.5B), the expression of miR20a or miR221 target gene, E2F1 or p27, gradually reduced and finally reached the same level as that of the non-transfected or negative control (Western, Fig.5C).



Gene	Sequence	PAM	PCR products	Expected cut products
miR20a	GACTAAAGTGCTTATAGTGC	AGG	376 bp	131 bp 245 bp
miR221	GGAAATCTACATTGTATGCC	AGG	366 bp	166 bp 200 bp

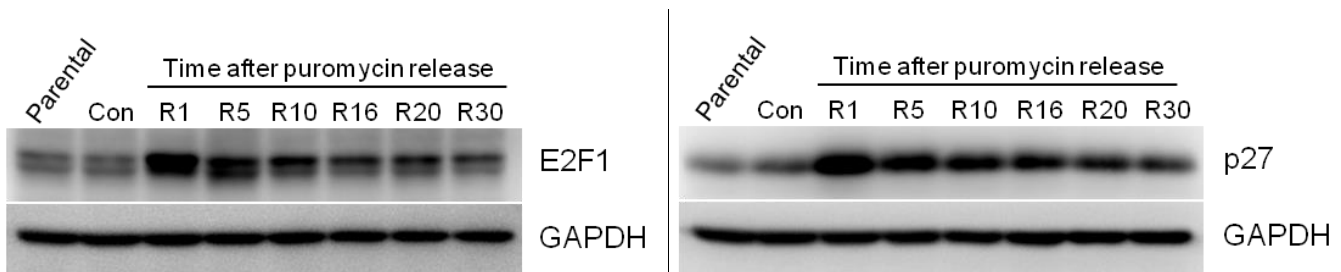
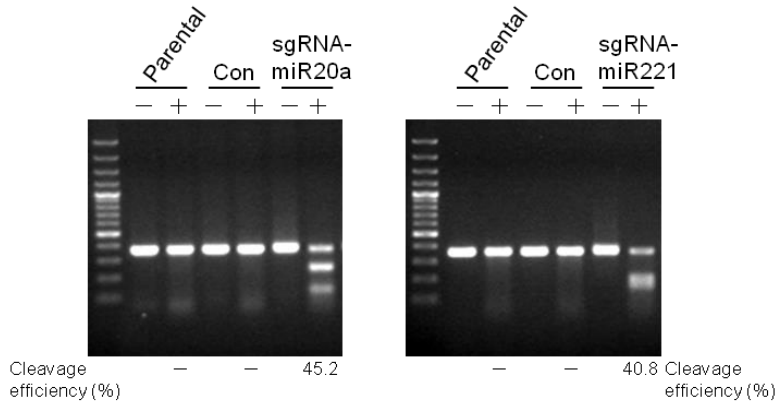


Fig. 5: Time course of sgRNAs targeted to miR20a or miR221 in 293T cells by transient transfection followed by 3-day puromycin enrichment. (A) Flow chart of the cell line development strategy. (B) DNA cleavage by T7E1 assay at Day 1 after puromycin removal. (C) Knockout of miR20a or miR221 by CRISPR/Cas9 can not be maintained stable or long-term expression by transient transfection of CRISPR/Cas9. The expression of miR20a or miR221 target gene, E2F1 or p27, is reduced after puromycin removal.