OMTN, Volume *13*

Supplemental Information

Antisense Oligonucleotides against miR-21 Inhibit the Growth and Metastasis of Colorectal Carcinoma via the DUSP8 Pathway

Tao Ding, Panpan Cui, Ya Zhou, Chao Chen, Juanjuan Zhao, Hairong Wang, Mengmeng Guo, Zhixu He, and Lin Xu

Material and methods

Cell culture

Human colon carcinoma cell line HCT116 was obtained from the Shanghai Institutes for Biological Sciences Cell Resource Center (Catalog Number: TCHu99, the latest identification in 2018) and cultured in McCoy's 5A medium (Life Technologies Corporation, Grand Island, USA), containing 100IU/mL penicillin, 100 μg/mL streptomycin, 20mM glutamine and 10% fetal bovine serum (Gibco, Grand Island, USA), incubated in a humidified incubator at 37°C with 5%CO_2.

Xenografts model antitumor assay in nude mice

For *in vivo* study, Athymic nude mice (BALB/C, 4-6 weeks old, female) were purchased from the Shanghai Laboratory Animal Research Center (Shanghai, China). Animals were in line with the *Guidelines for the Care and Use of Laboratory Animals* (Ministry of Health, People's Republic of China, 1998), and all the experimental procedures were approved by the ethical guidelines of Shanghai Medical Laboratory Animal Care and Use Committee (permit number: 2013018). For the preparation of subcutaneous xenograft model, cells were subcutaneously implanted into the right flanks of nude mice at 4.5×10^6 HCT116 cells density (n=12). Then seven days after tumor cells inoculation with confirmation of successful maturation of tumors, 12 mice were divided randomly into two groups (6 mice per group). The 100μg plasmid of p-miR-21-ASOs or p-Cont was given locally by direct injection into the tumor tissues of nude mice at four times every three days. Meanwhile, tumor volume was calculated by the formula: volume (mm^3)= length×width^2/2. After 18 days of treatment, all mice were sacrificed. Tumor tissues were stored at -80°C for further experiments.

RNA and miRNA isolation and qRT-PCR

Total RNA was isolated from cell lines or tumor tissues with RNAiso Plus reagents (Takara, Ohtsu, Japan) according to the manufacturer's instructions. Taq-Man MicroRNA Reverse Transcription Kit (ThermoFisher Scientific, USA) and PrimeScrip™RT Reagent Kit (Takara, Kusatsu, Japan) were used for reverse transcription of miRNA-21 and indicated genes respectively. qRT-PCR was performed to quantify mature miRNA-21 expression and mRNA expression using the miR-21 probe of Taq-Man (Life Technologies Corporation, Grand Island, USA) and SYBR® Premix Ex Taq™ II (Takara, Kusatsu, Japan). Data collection was accomplished on the CFX96™ Real-Time System (Bio-Rad, Hercules, USA). Relative expression level was normalized and calculated using the relative quantification ($2^{-\Delta\Delta Ct}$) method. The indicated genes and their primer sequences were shown in Tab. S1.

Immunofluorescence

Ki-67 (Proliferating Cell Nuclear Antigen) was used to assess the proliferation of tumor cells in tumor tissues after locally injected the plasmid of p-miR-21-ASOs or p-Cont. All frozen sections of tumor tissues were cut into 5μm thick slides. After washed with PBS for thrice and blocked with 10% normal goat serum for 30 minutes at room temperature and were incubated with rabbit-monoclonal anti-human anti-Ki-67 antibody (1:100, Santa Cruz Biotechnology, CA) at 4°C

for overnight; then, the sections were washed in PBS and incubated with a secondary antibody of Alexa Fluor 594 conjugated goat-anti-rabbit IgG (1:250, Invitrogen, Grand Island, USA) for 1 hour in the dark at room temperature. After washed in PBS for thrice, the slides were counterstained, mounted with SlowFade Gold Antifade Reagent with DAPI (40, 6-diamidino-2-phenylindole) (ThermoFisher Scientific, USA), and left for ten mins in the dark at room temperature before examination by fluorescence microscopy (Zeiss Axioplan 2, Gottingen, Germany).

TUNEL assay was performed according to the manufacturer's protocol. In brief, after antigen retrieval, the slides were washed in PBS thrice and incubated with 50ul of TUNEL reaction mixture (In situ Cell Death Detection Kit, Roche, Mannheim, Germany) for 2 hours at 37°C. Finally, the slides were also counterstained by DAPI after washed in PBS and evaluated by fluorescence microscopy. Image J software was used for analyzing the fluorescence intensity of images, and the mean optical density values represented by the relative number of apoptotic cells.

Gene expression microarray and analysis

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies). After quantile normalization of the raw data, genes that at least 1 out of 2 samples had flags in Detected ("All Targets Value") were chosen for further data analysis. Differentially expressed genes with statistical significance between the two groups were identified through Volcano Plot filtering. Differentially expressed genes between the two samples were identified through Fold Change filtering. Hierarchical Clustering was performed using the R scripts. GO analysis was performed in the standard enrichment computation method.

Statistical data analysis

All data were presented as means ± standard deviation (SD) from at least three independent experiments. Unpaired Student's *t*-test (two-tailed) was performed to evaluate the statistical significance by using GraphPad Prism 6 software. Differences were considered as statically significant when probability *P* values less than 0.05 ($P<0.05$).

Fig. S1

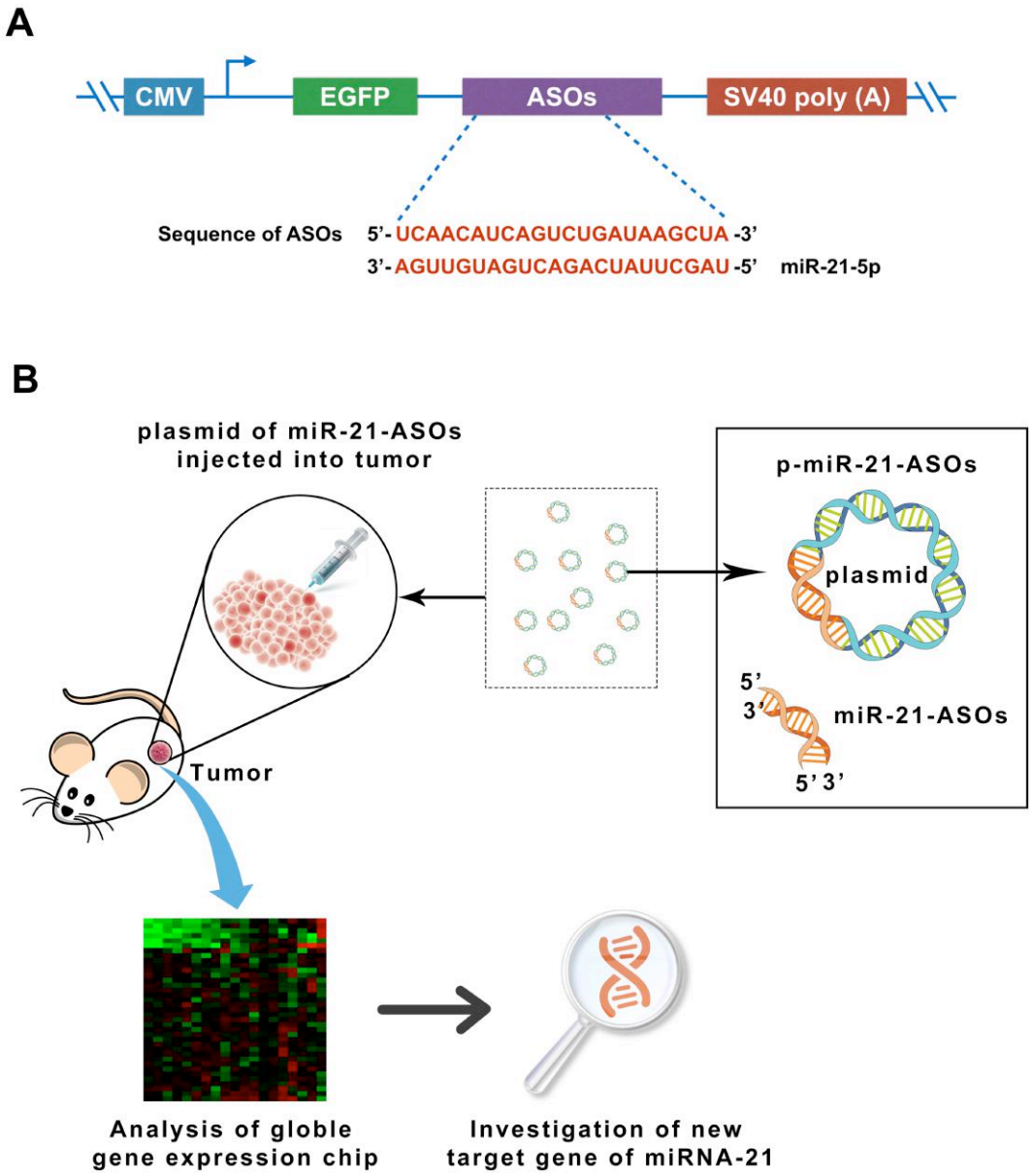

Fig. S1. The design of the current study.

(A) The sequence of ASOs against miR-21. **(B)** The sketch map for the current research study. A xenografts model of CRC in nude mice was established. After local treatment of p-miR-21 ASO plasmids, the tumor tissue was obtained, and then global gene array analysis was performed. Next, the possible target molecule mechanism was investigated.

Fig. S2

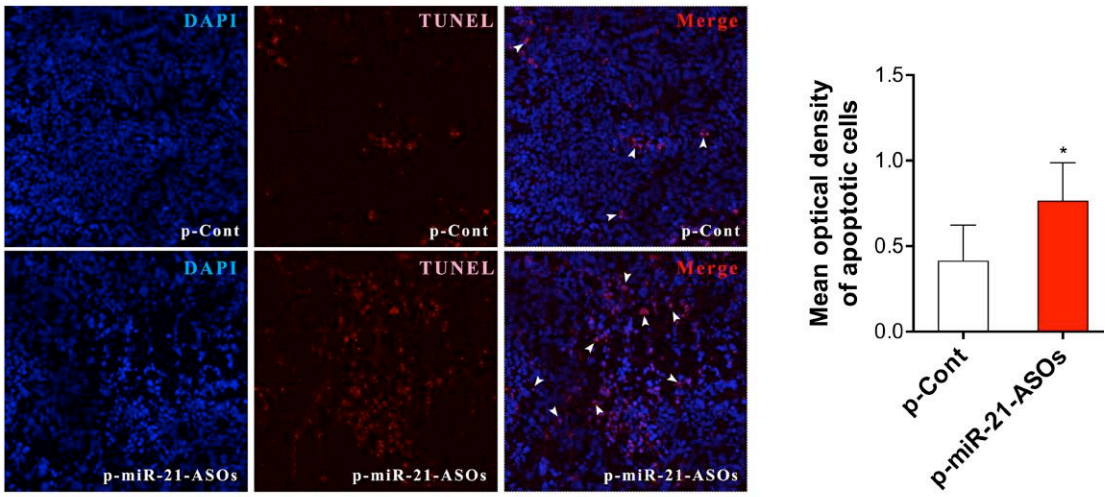

Fig. S2. The cell apoptosis in CRC tumor tissue.

The Human CRC SW620 cells were injected subcutaneously into the right flank of BALB/c nude mice (n =10 per group). Seven days later, the plasmid of p-miR-21-ASOs (100μg) or p-Cont (100μg) was locally given by subcutaneous injection into the tumor tissues of nude mice four times every three days. Six days after the last injection, tumor tissues were collected. The apoptosis in tumor tissue was analyzed by TUNEL assay, and the numbers of relative apoptotic cells were calculated with mean optical density by Image J software. **P*<0.05.

Fig. S3

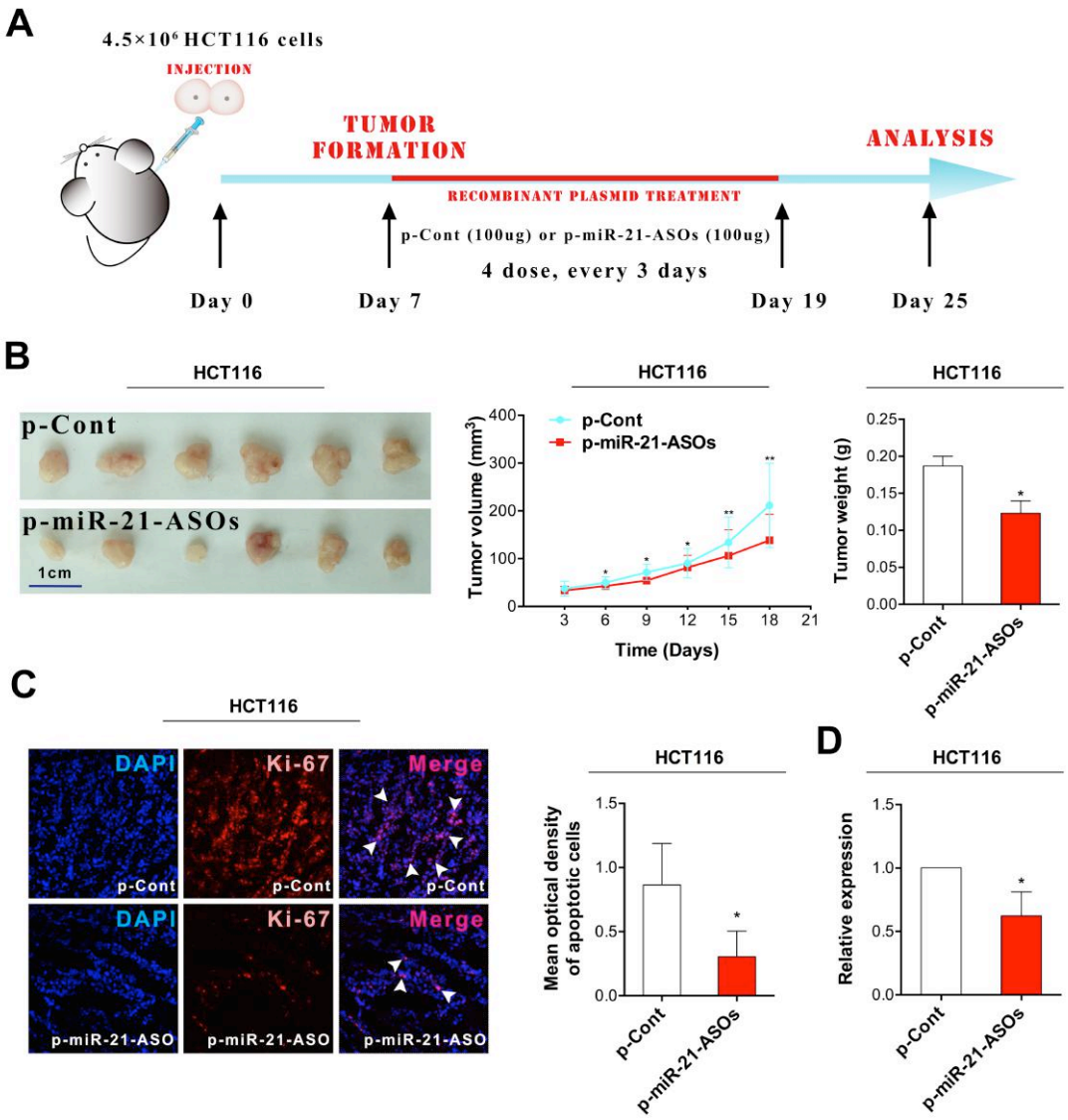

Fig. S3. ASOs against miR-21 inhibited the growth and metastasis of HCT116 human colorectal carcinoma cells *in vivo*.

The Human CRC HCT116 cells were injected subcutaneously into the right flank of BALB/c nude mice (n =6 per group). Seven days later, the plasmid of p-miR-21-ASOs (100μg) or p-Cont (100μg) was locally given by subcutaneous injection into the tumor tissues of nude mice four times every three days. Six days after the last injection, tumor tissues were collected. (**A**) The schematic diagram of the *in vivo* experiment. (**B**) The representative image, growth curve and weight of tumors were shown. (**C**) The expression of Ki-67 in tumor tissues was analyzed by immunofluorescence assay, and the mean optical density of Ki-67 was calculated with Image J software. (**D**) Real-time PCR detected the expression of miR-21 in tumor tissues. * P<0.05.

Fig. S4

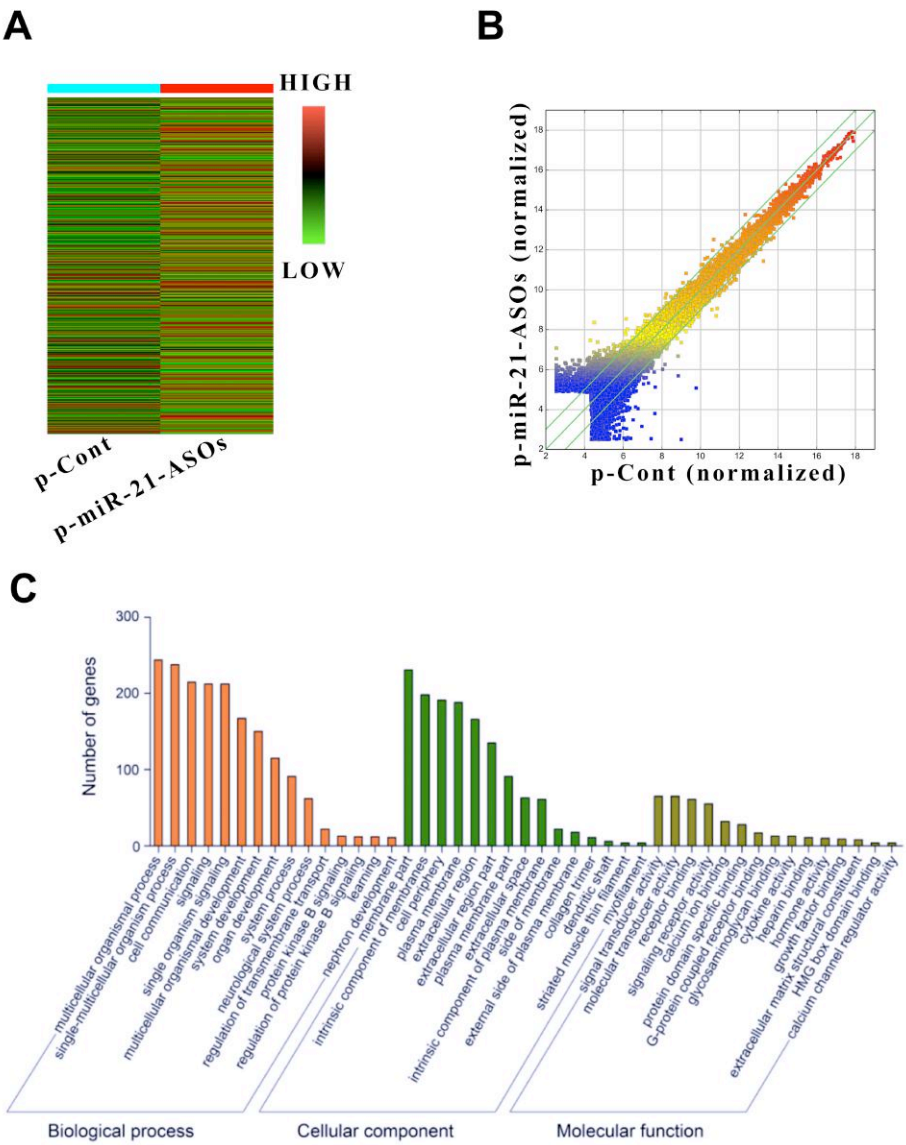

Fig. S4. The analysis of global gene expression in CRC tumor tissue.

The Human CRC SW620 cells were injected subcutaneously into the right flank of BALB/c nude mice (n =10 per group). Seven days later, the plasmid of p-miR-21-ASOs (100μg) or p-Cont (100μg) was locally given by subcutaneous injection into the tumor tissues of nude mice four times every three days. Six days after the last injection, tumor tissues were collected. The global gene expression was analyzed and matched by cDNA Microarray chip. (**A**) The heatmap and (**B**) scatterplot of global gene expression. (**C**) The GO terms of upregulated genes.

Tab. S1. The primer sequence used for Real-time quantitative PCR

Gene	Primer sequence (5'-3')
CDK2	F: TTCTGCCATTCTCATCGG
	R: ATGGGTGTAAGTACGAACAGG
CDK3	F: GTTTCTGCCACTCACATCGG
	R: ACCACAGTGTCACCACCTCAT
CDK4	F: TTCGTGAGGTGGCTTTACTG
	R: GATATGTCCTTAGGTCCTGGTCT
CDK6	F: CTCCGAGGTCTGGACTTTCT
	R: TGCTCTGTACCACAGCGTGA
MMP-2	F: TATGGCTTCTGCCCTGAGAC
	R: CACACCACATCTTTCCGTCA
MMP-9	F: CGCAGACATCGTCATCCAGT
	R: GAAATGGGCGTCTCCCTGAA
E-Cadherin	F: GCCGAGAGCTACACGTTCAC
	R: CACACCATCTGTGCCCACTT
CXCR4	F: ATCAGTCTGGACCGCTACCT
	R: ATCTGCCTCACTGACGTTGG
DUSP8	F: TCCCGAGGAAGGTGATGGAT
	R: AGCTTGGAGCAGCAGATGTT
STAG2	F: TCCTTCTGGTCCAAACCGAAT
	R: ACCGACTGCATAGCACTCTTG
PDZD2	F: TCTGTACTGTGTACCTCACCAA
	R: CCCTGCGCTTTTCACCATAG
CCL-1	F: CTCATTTGCGGAGCAAGAGAT
	R: GCCTCTGAACCCATCCAACTG
B3GAT2	F: TTGTCATCATCATGCTCGACG
	R: CACCGCGTAGGGAGAGAAGTA
GAPDH	F: GCACCGTCAAGGCTGAGAAC
	R: TGGTGAAGACGCCAGTGGA

F: Forward primer, R: Reverse primer

Table 2. Over 4-fold upregulation genes (195) in p-miR-21-ASOs injection group

Target Gene	Fold Change	Target Gene	Fold Change	Target Gene	Fold Change
lnc-ABCF2-1	4.0019413	SLC13A3	4.5040221	SHANK2	5.2867591
FNDC1	4.0044525	VEPH1	4.5318044	PLA2G2F	5.2883952
MAFA	4.005127	MPZ	4.5958694	CTSZ	5.2955003
WFIKKN1	4.0144908	XLOC_l2_011874	4.6325043	PAX3	5.2991057
B9D1	4.0258092	SNAP91	4.6575066	CFAP57	5.3390694
PFKFB1	4.0531942	ZNF853	4.6700345	DUPD1	5.3955058
KRT28	4.0651444	TOM1L2	4.6727699	PRSS27	5.4060592
FAM71E2	4.0651655	HAUS8	4.6778831	CCDC180	5.4716187
IZUMO3	4.0751589	ARID1B	4.7406921	ARHGAP23	5.4862433
XLOC_l2_013192	4.0766169	AGER	4.7446346	CLIC3	5.4940186
CRIP2	4.0865744	DOK7	4.7594	ZBTB21	5.5444394
TCTE3	4.0965991	CEMP1	4.7822996	SLC22A16	5.6031663
EFHD1	4.0978126	IGFALS	4.7905072	LOC653712	5.6173601
LZTS1	4.1484566	lnc-DTYMK-3	4.7949099	CTSL3P	5.6233257
LMX1B	4.1511308	ANKRD63	4.7980314	BCAS1	5.6241743
PNMA6A	4.186282	TSPYL5	4.833729	GLTSCR1	5.6293081
lnc-LANCL2-1	4.1891252	FABP3	4.847703	HRCT1	5.6563194
TCEB3C	4.2038424	MROH7	4.8489689	SCN2A	5.6782446
NRG2	4.2299699	ADARB2	4.8505488	CHIT1	5.7129183
ADAMTSL1	4.2448101	TNRC6C-AS1	4.8854302	KIF18B	5.7133915
CHD5	4.2492647	TPH1	4.8917182	THY1	5.7146003
OR2G6	4.2791786	ATP5L2	4.9048622	DNM1	5.7399895
GDPD2	4.2808367	OR10G3	4.9055102	OR1K1	5.7498416
DNAI2	4.2945051	LOC645427	4.9265739	PSCA	5.7807845
CTLA4	4.2999044	CRTAC1	4.9383969	TRIM17	5.7890347
ALOX12P2	4.3114379	CNTLN	4.9489138	MUC13	5.8172338
BCAM	4.3444348	NPPB	4.9567284	LOC101929469	5.8240482
HCCAT5	4.3514487	TMEM164	4.9885039	DKK3	5.8456684
KIF18B	4.3767895	SLC27A6	5.05487	TLR4	5.8486749
TNNI3	4.383735	AIDA	5.1122228	ABHD11-AS1	5.9031635
NUTM1	4.4087204	OR4B1	5.1124205	LINC00544	5.9538072
LOC100132111	4.4106271	CCDC183	5.1307371	COL25A1	5.9563263
MUC22	4.4112472	LOC100131910	5.131792	LOC102723640	5.9698712
LINC00856	4.4162211	UROC1	5.1570016	ELL	5.9992539
P4HA2	4.4187276	LINC00589	5.1700108	DOC2B	6.0063698
MYEOV2	4.423228	CXorf49B	5.1925097	PRO1082	6.0244277
PHKG1	4.4482371	DLG2	5.2214627	PPAN-P2RY11	6.0281303
SPATA21	4.4531821	RBMY2EP	5.2477631	SYNGAP1	6.0409326
SPRR2D	4.4644025	C17orf74	5.266649	OR9Q2	6.0735196
FAM25C	4.4675352	FAM83G	5.2693427	CCDC134	6.0805149

Continued

Target Gene	Fold Change	Target Gene	Fold Change	Target Gene	Fold Change
JPH4	6.0977851	HNF4A	6.6072778	AVP	7.6798264
LOC100129581	6.1124404	TMEM200C	6.6725139	MFSD6L	7.7449155
FAM231A	6.1261437	TMEM8B	6.6888644	TMEM120B	7.7930766
WISP2	6.1461509	MXRA8	6.6904238	MAPK12	7.9343025
PRLH	6.1702259	LOC100126784	6.7854006	C11orf88	7.9495285
AHNAK2	6.2040431	**DUSP8**	6.790195	FBXO24	8.5436426
PYGM	6.2043527	CYP4F22	6.8349842	IL17F	8.6711095
LTBP3	6.2258383	C2orf74	6.8761108	TMCC2	8.7511723
QRFP	6.2635106	OR10AD1	6.9664758	CYP27C1	8.9903719
TTC28-AS1	6.2923691	CFAP46	6.9927111	MARK4	9.2951052
PRSS54	6.3822862	FAM71D	7.0038991	LENG1	9.35898
DTNA	6.3890924	RFPL4B	7.0701793	NHLRC4	9.398378
ANTXRL	6.3984582	lnc-FNBP1L-1	7.1551966	ZNF316	9.4462473
MOBP	6.4207067	MGAT3	7.2282159	ACOXL	9.5724837
MIR7-3HG	6.4209444	PAX6	7.3033091	SRCAP	9.671568
CD34	6.4370961	LOC645752	7.3050594	KCNK3	10.0640194
TGIF1	6.4470144	lnc-LBH-1	7.3052381	DCLK1	10.2690463
MPDU1	6.4530907	ZNF81	7.3150909	TAS1R3	10.668181
RBMY1B	6.510512	SEC16B	7.3177392	LOC100996890	10.7557752
SPACA5	6.5355937	IL17B	7.3226625	TTTY1	11.8119832
GALNT9	6.55416	OR2T34	7.3514473	CFAP69	11.8831277
TET3	6.5613105	YJEFN3	7.3739064	FANK1	12.9244197
TMEM262	6.5700999	NRDE2	7.4863526	TBCB	12.9618766
SLC24A4	6.5793771	SCN1B	7.5941163	GPR101	14.6200567
AGAP2	6.5845338	ADAM19	7.633617	SPATA19	16.0544986