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Supplemental Information

miR-199a-3p Modulates MTOR and PAK4 Pathways and Inhibits Tumor Growth in a Hepatocellular Carcinoma Transgenic Mouse Model

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SUPPLEMENTAL FIGURES

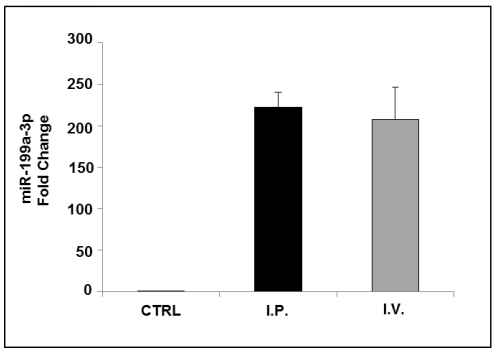

Figure S1. Comparison between intraperitoneal and intravenous systemic administration approaches for *in vivo* miRNA delivery. To find the most efficient and functional route for miRNA administration, intraperitoneal (I.P.) and intravenous (I.V.) injection approaches were compared. Mice received a single dose (2,5mg/kg) of miR-199a-3p by I.P. (n=3) or by I.V. (n=3). Three untreated mice were used as control (CTRL). After 24h, the animals were sacrificed and the levels of miR-199a-3p in livers measured by droplet digital polymerase chain reaction (ddPCR). Results (represented as mean + SD) show that efficacy of the two systemic approaches was comparable.

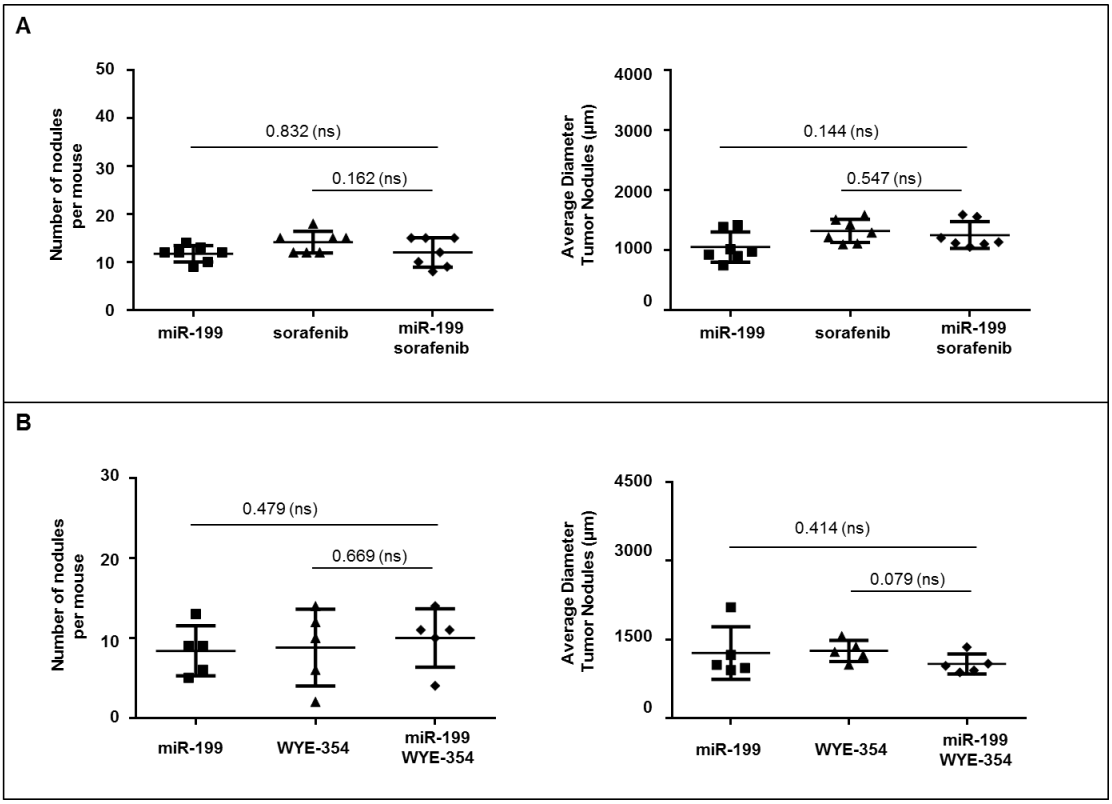

Figure S2. The combination of miR-199a-3p with sorafenib or WYY-354 does not elicit an additive effect. To verify a possible additive anti-tumor effect due to the combination of miR-199a-3p and the tested compounds, additional groups of mice were established. (A) In the context of the experiment described in Figure 1D, 7 mice received a combination of miR-199a-3p and sorafenib. No significant effect on the number and size of nodules was detected. (B) In the context of the experiment described in Figure 4, 5 mice received a combination of miR-199a-3p and WYE-354. No significant differences were observed between single agents and their combination.

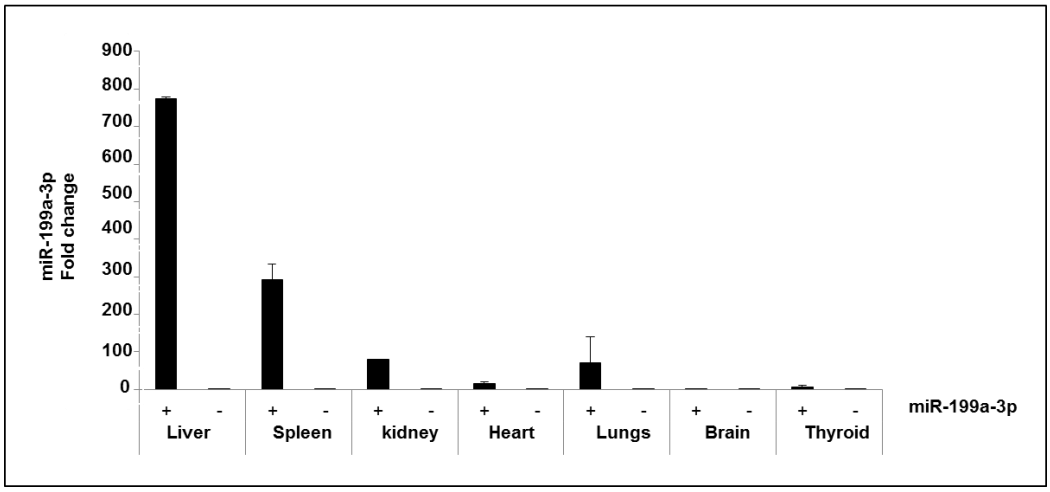

Figure S3. Distribution of miR-199a-3p in various mouse organs following its i.p. administration. To show the delivery of miR-199a-3p molecules *in vivo*, a droplet digital polymerase chain reaction (ddPCR) analysis was performed on several mouse organs. Mice injected with the miRNA (+) were compared with untreated animals (-). All the data represent an average from three animals of each condition (mean + SD)

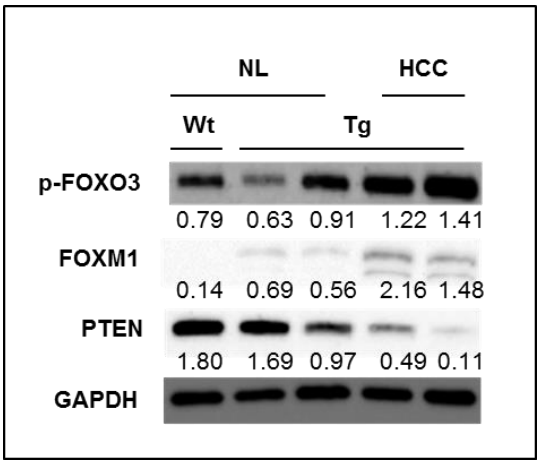

Figure S4. Differential expression of phospho-FOXO3, FOXM1 and PTEN in normal liver and cancer tissues in TG221 mice. Western blot analysis of liver proteins from normal liver of B6D2F2 wild type (wt), TG221 transgenic mice, and liver hepatocellular carcinoma (HCC) from TG221. The analyses showed that HCCs exhibited a higher phosphorylation of FOXO3, upregulation of FOXM1, and downregulation of PTEN compared to normal livers. A slight upregulation of FOXM1 and downregulation of PTEN were observed in normal liver from TG221 mice compared to wt mice. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal normalizer.

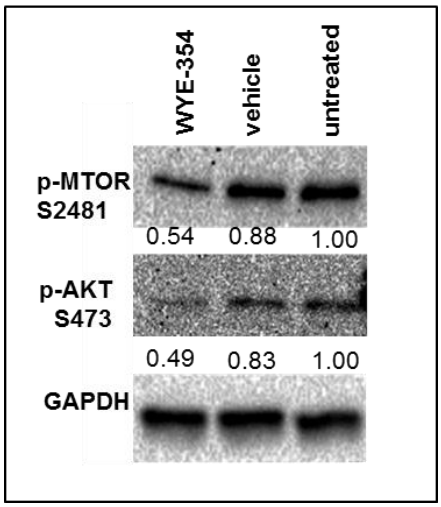

Figure S5. WYE-354 inhibited *in vivo* phosphorylation of AKT and MTOR. Western blot analysis of liver protein extracts from mice treated with WYE-354 and controls. The inhibition of phosphorylation of Akt (S-473) and MTOR (S-2481) was detected in mice treated with WYE-354 compared to control animals. GAPDH was used as an internal normalizer.

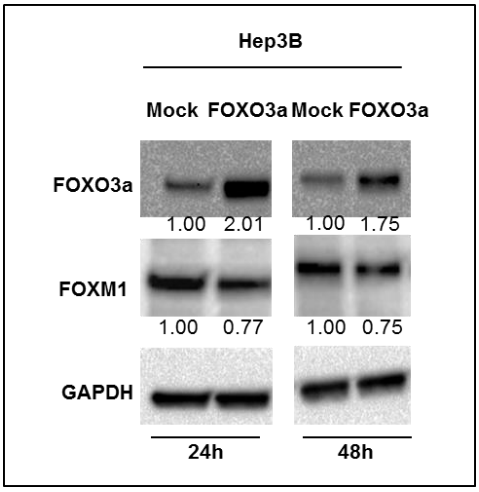

Figure S6. FOXO3A over-expression inhibited FOXM1 expression. Hep3B cells were transfected with a mammalian plasmid vector over-expressing FOXO3a. Cells were collected 24 and 48 h after transfection, and proteins were analyzed by western blot. The over-expression of FOXO3a induced a decrease in FOXM1 levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal normalizer.

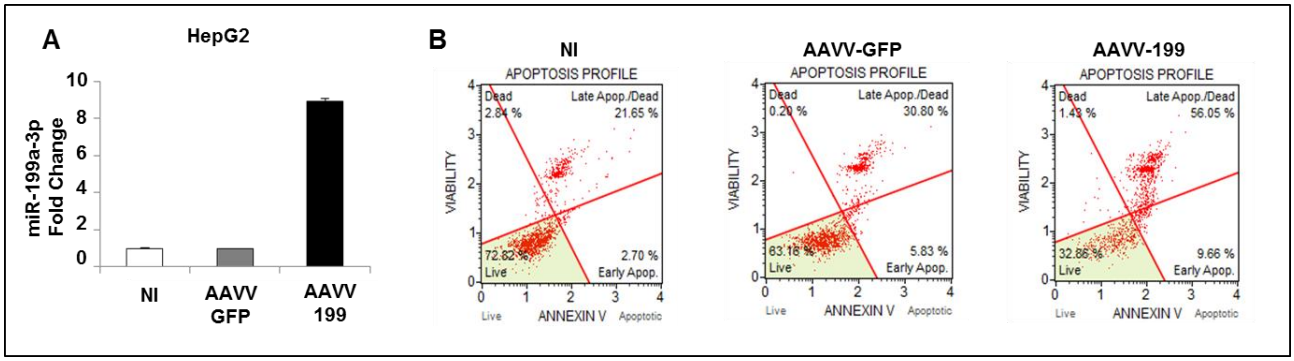

Figure S7. Increased expression of miR-199a-3p enhanced apoptosis levels in HepG2 cells. (A) HepG2 cells were infected with an Adeno Associated Virus (AAVV) expressing miR-199a-3p (AAVV-199). The increase in miR-199a-3p expression levels was detected in the infected cells by quantitative polymerase chain reaction (qPCR). All the data reported are an average of the experiment performed in triplicate. **(B)**. The increased expression of miR-199a-3p caused a statistically significant increase in the percentage of total apoptotic cells compared to the controls ($p < 0.0001$), while AAVV-green fluorescent protein (GFP) effect on cells viability was comparable to the uninfected control (NI)**.** Data are represented as mean + SD.

												Genotype	TG221	TG221	TG221	TG221	TG221	TG221	TG221	TG221	TG221	TG221	TG221	TG221	TG221	TG221	TG221
												Tumor	HCC	HCC	HCC	HCC	HCC	HCC	HCC	HCC	HCC	HCC	HCC	NL	NL	NL	NL	NL
												miR-199a-3p	no	no	no	no	no	no	no	yes	yes	yes	yes	no	no	no	no	no
Gene Symbol	**Agilent ProbeName**	**Foxm1 induced**	**GeneName**	**Organism**	**EntrezGene ID**	**Genbank Accession**	**Average HCC**	**Average HCC + miR-199**	**Average Normal Liver**	**p-value**	**q-value**																		
Acta2	A_52_P420504	no	actin, alpha 2, smooth muscle, aorta	Mus musculus	11475	NM_007392	4.33	0.95	1.00	4.97E-04	9.29E-04		1.95	1.87	3.10	5.07	5.01	10.13	3.20	1.03	1.34	0.76	0.67	1.09	1.49	1.10	0.90	0.39
Afp	A_51_P510891	no	alpha fetoprotein	Mus musculus	11576	NM_007423	245.05	355.63	1.03	8.74E-10	1.25E-08		216.47	182.68	170.58	191.27	259.32	307.34	387.67	780.58	186.88	223.34	231.71	3.29	0.58	0.47	0.35	0.44
Angpt2	A_51_P201982	no	angiopoietin 2	Mus musculus	11601	NM_007426	0.61	0.39	0.17	1.03E-03	1.70E-03		0.88	0.66	1.07	0.48	0.34	0.45	0.39	0.42	0.38	0.48	0.28	0.23	0.06	0.22	0.23	0.13
Birc5	A_55_P1983773	yes	baculoviral IAP repeat-containing 5	Mus musculus	11799	NM_001012273	19.32	7.55	1.37	4.91E-07	4.23E-06		15.67	12.09	13.45	28.30	18.65	34.21	12.86	5.70	8.50	6.00	10.00	0.90	0.62	3.00	1.36	0.99
Birc5	A_55_P1983768	yes	baculoviral IAP repeat-containing 5	Mus musculus	11799	NM_009689	1.66	0.51	0.10	1.15E-06	6.19E-06		1.36	1.00	1.18	2.56	1.55	3.00	0.95	0.55	0.61	0.39	0.50	0.07	0.03	0.26	0.08	0.07
Birc5	A_55_P1983769	yes	baculoviral IAP repeat-containing 5	Mus musculus	11799	NM_001012273	0.51	0.20	0.04	1.33E-05	4.08E-05		0.43	0.27	0.17	0.61	0.65	1.16	0.29	0.17	0.20	0.17	0.26	0.04	0.04	0.08	0.05	0.02
Ccnb1	A_55_P1952256	yes	cyclin B1	Mus musculus	268697	NM_172301	0.41	0.10	0.03	1.08E-06	6.19E-06		0.21	0.34	0.28	0.54	0.43	0.83	0.24	0.10	0.13	0.08	0.10	0.03	0.03	0.07	0.02	0.02
Ccnb1	A_55_P2128668	yes	cyclin B1	Mus musculus	268697	NM_172301	0.43	0.24	0.05	6.24E-06	2.98E-05		0.21	0.23	0.28	0.32	0.45	1.01	0.49	0.19	0.26	0.17	0.35	0.04	0.04	0.08	0.03	0.04
Ccnb1	A_55_P2065671	yes	cyclin B1	Mus musculus	268697	NM_172301	4.13	1.68	0.22	1.59E-07	1.71E-06		2.25	2.60	3.88	3.24	3.91	8.93	4.08	1.51	1.59	1.47	2.15	0.16	0.26	0.42	0.09	0.16
Ccnb2	A_51_P457528	yes	cyclin B2	Mus musculus	12442	NM_007630	9.33	3.14	0.74	1.05E-06	6.19E-06		6.05	6.61	4.52	13.94	8.08	17.88	8.27	3.00	3.99	2.22	3.33	0.53	0.47	1.50	0.45	0.77
Cdc20	A_55_P1996946	yes	cell division cycle 20 homolog (S. cerevisiae)	Mus musculus	107995	NM_023223	8.99	4.42	0.70	1.34E-05	4.08E-05		3.75	7.21	4.08	9.94	10.21	20.43	7.34	3.30	5.53	3.55	5.29	0.38	0.22	1.93	0.47	0.49
Cdc25b	A_52_P612382	yes	cell division cycle 25 homolog B (S. pombe)	Mus musculus	12531	NM_023117	0.83	0.24	0.14	3.79E-05	9.59E-05		0.37	0.79	0.59	0.69	0.76	2.00	0.61	0.27	0.32	0.17	0.18	0.12	0.08	0.19	0.15	0.17
Cdk1	A_55_P2048588	yes	cyclin-dependent kinase 1	Mus musculus	12534	NM_007659	0.68	0.27	0.06	1.42E-05	4.08E-05		0.73	0.44	0.69	0.49	0.86	1.25	0.31	0.25	0.23	0.29	0.31	0.03	0.03	0.19	0.04	0.02
Fgf1	A_55_P2047188	no	fibroblast growth factor 1	Mus musculus	14164	NM_010197	12.64	12.35	32.85	1.03E-05	3.68E-05		15.78	12.66	16.03	10.01	11.39	8.60	13.98	12.25	12.68	13.35	11.11	23.57	35.39	34.19	45.89	25.21
Fgf1	A_52_P538673	no	fibroblast growth factor 1	Mus musculus	14164	NM_010197	0.38	0.39	1.19	8.82E-06	3.61E-05		0.40	0.34	0.46	0.24	0.32	0.54	0.38	0.30	0.43	0.44	0.40	0.88	1.18	1.33	1.73	0.85
Foxm1	A_52_P28806		forkhead box M1	Mus musculus	14235	NM_008021	1.29	0.59	0.21	6.30E-05	1.50E-04		0.62	1.04	0.80	1.36	1.65	2.72	0.85	0.63	0.70	0.36	0.67	0.20	0.17	0.42	0.14	0.12
Gpc3	A_52_P23225	no	glypican 3	Mus musculus	14734	NM_016697	25.44	45.63	0.10	2.75E-10	5.92E-09		22.08	18.49	28.39	23.74	21.82	19.82	43.72	46.36	21.00	34.88	80.28	0.04	0.04	0.28	0.08	0.05
Hgf	A_51_P484998	no	hepatocyte growth factor	Mus musculus	15234	NM_010427	0.28	0.50	0.80	1.75E-02	2.15E-02		0.34	0.35	0.52	0.23	0.08	0.26	0.17	0.26	0.27	0.97	0.52	1.42	0.49	0.84	0.73	0.50
Hhip	A_55_P1969276	no	Hedgehog-interacting protein	Mus musculus	15245	NM_020259	0.08	0.12	0.80	3.64E-04	7.11E-04		0.34	0.06	0.02	0.02	0.01	0.05	0.07	0.12	0.03	0.04	0.28	0.79	0.68	0.80	0.99	0.77
Igf1	A_55_P2085979	no	insulin-like growth factor 1	Mus musculus	16000	NM_184052	2.56	3.70	10.38	2.80E-03	4.01E-03		2.76	1.86	2.45	2.08	2.23	3.92	2.59	2.84	6.99	2.47	2.52	13.50	4.47	14.14	16.30	3.50
Igf1	A_55_P2085974	no	insulin-like growth factor 1	Mus musculus	16000	NM_010512	4.05	4.94	24.32	3.31E-04	6.78E-04		10.85	2.69	3.10	1.08	4.12	4.35	2.15	2.62	9.78	5.50	1.87	23.50	13.90	30.35	32.16	21.71
Igf1	A_55_P2031636	no	insulin-like growth factor 1	Mus musculus	16000	NM_010512	0.80	1.48	6.22	7.67E-05	1.74E-04		1.58	0.40	0.57	0.18	0.87	1.34	0.68	0.87	2.77	1.45	0.83	7.09	4.68	6.36	7.70	5.25
Igf1	A_55_P2085984	no	insulin-like growth factor 1	Mus musculus	16000	NM_010512	29.40	28.88	121.85	3.10E-03	4.31E-03		74.28	14.06	22.65	4.36	27.08	43.27	20.10	21.91	62.90	21.88	8.84	152.64	130.41	103.22	99.98	122.99
Igf1	A_55_P2031631	no	insulin-like growth factor 1	Mus musculus	16000	NM_010512	38.71	40.60	164.58	3.21E-03	4.32E-03		95.71	18.11	28.94	5.74	34.78	59.49	28.21	30.03	88.05	31.40	12.92	214.55	183.57	132.87	126.17	165.72
Lpl	A_52_P257812	no	lipoprotein lipase	Mus musculus	16956	NM_008509	9.52	10.96	1.70	8.67E-05	1.86E-04		13.40	8.97	12.45	5.18	14.34	7.30	4.99	14.14	11.46	10.07	8.17	3.32	1.61	0.66	0.46	2.44
Lpl	A_51_P259296	no	lipoprotein lipase	Mus musculus	16956	NM_008509	67.82	69.95	7.67	9.23E-06	3.61E-05		117.19	78.17	96.15	48.12	66.73	36.53	31.82	71.07	70.00	91.13	47.62	14.34	6.04	3.46	2.82	11.71
Mst1	A_55_P1984098	no	macrophage stimulating 1 (hepatocyte growth factor-like)	Mus musculus	15235	NM_008243	108.66	100.01	79.97	4.43E-02	5.14E-02		125.37	100.99	85.13	84.00	113.43	132.32	119.40	131.02	104.71	80.82	83.48	87.63	90.70	66.89	68.60	86.03
Mst1	A_55_P2115392	no	macrophage stimulating 1 (hepatocyte growth factor-like)	Mus musculus	15235	NM_008243	99.08	91.21	71.96	3.65E-02	4.35E-02		109.24	90.69	77.19	77.47	105.64	121.65	111.69	117.58	96.26	75.00	75.99	82.33	81.17	58.72	59.84	77.73
Pdgfb	A_55_P2047310	yes	platelet derived growth factor, B polypeptide	Mus musculus	18591	NM_011057	1.58	1.22	0.58	7.56E-04	1.35E-03		0.76	1.75	2.41	1.74	1.53	1.23	1.60	0.93	1.65	1.36	0.96	0.54	0.40	0.99	0.53	0.45
Pdk1	A_51_P406429	no	pyruvate dehydrogenase kinase, isoenzyme 1	Mus musculus	228026	NM_172665	1.61	1.51	6.44	2.57E-03	3.82E-03		2.54	1.57	1.88	1.05	1.10	2.27	0.84	1.29	1.93	1.31	1.50	3.57	1.67	10.69	10.24	6.03
Pld1	A_55_P2104487	no	phospholipase D1	Mus musculus	18805	NM_001164056	5.06	5.63	1.45	1.70E-05	4.58E-05		4.91	4.62	5.69	6.61	4.25	4.70	4.64	5.33	4.04	7.40	5.74	1.75	1.98	0.91	0.60	1.99
Pten	A_52_P24843	no	phosphatase and tensin homolog	Mus musculus	19211	NM_008960	1.83	2.23	2.70	1.29E-02	1.64E-02		2.45	1.64	1.82	1.52	1.76	1.62	1.97	1.76	1.92	2.95	2.30	2.62	2.39	2.09	2.91	3.48
Pten	A_51_P275350	no	phosphatase and tensin homolog	Mus musculus	19211	NM_008960	19.58	24.09	27.57	8.68E-04	1.49E-03		20.10	16.96	20.50	20.68	18.37	18.43	22.05	22.98	21.45	28.38	23.56	28.72	28.50	22.39	25.33	32.94
Scd2	A_51_P129464	no	stearoyl-Coenzyme A desaturase 2	Mus musculus	20250	NM_009128	120.54	102.19	1.08	4.08E-12	1.76E-10		104.39	97.13	82.64	166.15	150.83	118.63	124.03	118.50	80.01	112.39	97.88	0.81	0.48	1.65	1.30	1.14
Tff3	A_51_P456208	no	trefoil factor 3, intestinal	Mus musculus	21786	NM_011575	609.00	1010.38	124.46	9.13E-03	1.19E-02		898.70	451.82	795.32	133.85	681.09	272.54	1029.69	1284.51	1287.18	697.91	771.92	226.44	329.86	0.97	0.51	64.50
Tgfb2	A_65_P10913	yes	transforming growth factor, beta 2	Mus musculus	21808	NM_009367	1.07	0.68	0.18	1.07E-03	1.70E-03		0.20	0.96	1.61	0.65	0.56	1.73	1.77	0.51	0.67	0.73	0.83	0.11	0.16	0.33	0.18	0.12
Tgfb2	A_51_P317640	yes	transforming growth factor, beta 2	Mus musculus	21808	NM_009367	0.35	0.23	0.07	2.12E-03	3.25E-03		0.10	0.39	0.58	0.22	0.22	0.43	0.52	0.14	0.22	0.33	0.25	0.03	0.06	0.15	0.11	0.03

Table S1. Expression data of genes from Figure 3