Supporting Information

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Materials and Methods

Reagents. Primers were purchased from either Sigma-Genosis or IDT. Antibodies were from the following vendors: c-Myc and eIF4E (Epitomics), Akt, pAkt, PUMA, and p21 (Cell Signaling Technology), p53 (EMD Chemicals), CDK4 (Applied Biological Materials), and β-actin (Sigma-Aldrich). Ly294002 and c-Myc-siRNA were purchased from Cell Signaling Technology. Tissue microarrays of colon cancer specimens were purchased from US Biomax.

Cell Culture. All cell lines were purchased from the American Tissue Culture Collection (ATCC), except for the p53 +/+ and p53 -/- HCT-116 cells, which were a generous gift from Dr. Bert Vogelstein (Johns Hopkins University). They were grown in either RPMI 1640 (Cambrex) or Dulbecco's modified Eagle's medium (DMEM) (Cambrex) supplemented with 10% FBS (Sigma-Aldrich) or 10% calf serum (for NIH/3T3 cells). All media contained 2 mM glutamine, 100 units of penicillin/ml, and 100 μg of streptomycin/ml. Cells were incubated at 37 °C and supplemented with 5% CO_2 in the humidified chamber.

Plasmids. To construct a plasmid expressing *miR-145*, we first amplified a DNA fragment carrying pre-*miR-145*, using genomic DNA from a healthy blood donor as a template. PCR reactions were performed using the high fidelity Phusion enzyme (New England Biolabs), and specific primers miR-145–5.1 and miR-145-NotI-3.1 (Table 2). The amplified fragment was first cloned into a PCR cloning vector and subsequently cloned into pCDH-CMV-MCS-EF1-copGFP (System Biosciences) at the EcoR1 and NotI sites. Expression of the mature *miR-145* was verified by TaqMan real-time RT-PCR. To generate a mutant *miR-145* expression vector, we adopted a two-step PCR procedure in which mutated seed sequences were introduced into primers (miR-145-mut-5.1 and miR-145-mut-3.1).

The luciferase-UTR reporter plasmid was constructed by introducing the c-Myc 3′-UTR region carrying a putative *miR-145* binding site into pGL3 control vector (Promega, Madison, WI). We first amplified the c-Myc 3′-UTR sequence by PCR using primers c-Myc-UTR-5.1 and c-Myc-UTR-NotI-3.1 (s-Table 2), and MCF10A cDNA as a template. The PCR product was also first cloned into a PCR cloning vector and then subcloned into a modified pGL3 control vector. To delete the putative *miR-145* binding site in the c-Myc 3′-UTR, we also adopted the two-step PCR method where two sets of overlapped primers, c-Myc-UTR-5.1, c-Myc-UTR-3.2, and c-Myc-UTR-5.2 and c-Myc-UTR-NotI-3.1 (s-Table 2), were used to amplify two fragments. The amplified two PCR fragments were then used as a template for the second PCR using primers c-Myc-UTR-5.1 and c-Myc-UTR-NotI-3.1

To clone the putative *miR-145* promoter, we amplified a DNA fragment (1.4 kb) from the genomic DNA of the healthy blood donor. PCR primers were miR-145p-KpnI-5.1 and miR-145p-XhoI-3.2 (s-Table 2) using the same cloning strategy as above. The PCR product was then subcloned into pGL3 basic (Promega) at KpnI and XhoI sites. All PCR products were verified by DNA sequencing. The similar strategy was used to make deletion constructs.

Cell Proliferation Assay. Cell growth assays were carried out by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays according to standard methods. In brief, cells were seeded in 96-well plates and incubated for various days before adding MTT. In addition, viable cells after transfection of *miR-145* were counted by trypan blue exclusion method in *Vi*-Cell XR analyzer (Beckman Coulter).

Soft Agar Assay. Soft agar assay was performed to assess the influence of *miR-145* on anchorage independent growth of the cells. A layer of base agar was first made in a 12-well plate with the final concentration of 0.4% (wt/vol) in culture medium. After it was solidified 5000 transfected cells per well were mixed with agar in growth media and 1 ml of the mixture was poured over the base agar in triplicates at a final concentration of 0.3% (wt/vol) of agar as a top layer. The cells were then incubated at 37 °C for 2 weeks. Colonies were photographed after staining with 0.05% (wt/vol) of crystal violet, and then counted.

Immunofluoresence Microscopy. Immunofluoresence staining was used to determine c-Myc expression in *miR-145* transfected cells as previously described (1). In brief, HCT-116 were transfected with vector control or *miR-145* expressing vector and were fixed with 3% paraformaldehyde Primary antibody against c-Myc was used to detect the c-Myc signal, followed by a secondary antibody conjugated with Alexa Fluor 560.

Immunohistochemistry (IHC). Paraffin-embedded tissue was pretreated at 65 °C for 2 h, followed by deparaffinization using standard procedures. Antigen retrieval was carried out in antigen retrieval solution before applying the primary pAkt antibody (Epitomics). Thereafter, slides were incubated for 2 h at room temperature followed by extensive washes with PBST and further incubated for 1 h at room temperature with the secondary antibody conjugated with horse radish peroxidase (HRP). HRP activity was detected using Histostain Plus kit (Invitrogen) according to the manufacturer's instruction. Finally, sections were counterstained with hematoxylin and mounted.

Cell Cycle Analysis. Cell cycle analysis was performed using the standard propidium iodide method. In brief, HCT-116 cells were first transiently transfected with either vector control or *miR-145* expression vector. One day later, cells were then split and grown for 24 h before harvesting for cell cycle analysis. After fixing at 70% ethanol, the cells were stained with propidium iodide along with RNase A. Finally, the cells were analyzed by FACS Vantage flow cytometer (Becton-Dickinson).

Patient Specimens. Freshly frozen specimens of matched breast and colon tumors from the same patients were obtained from Cooperative Human Tissue Network (CHTN) Midwestern Division. The use of these specimens in this study was approved by the Institutional Review Board of Southern Illinois University School of Medicine. Total RNA was isolated using TRIzol reagent (Invitrogen) for detection of miRNA expression.

Bioinformatics Analysis of Putative *miR-145* Targets. We used commonly cited miRNA prediction programs such as TargetScan4 (2), miRBase Target5 (http://microrna.sanger.ac.uk/targets), PicTar (3), and miRanda (4) (http://microrna.org). The *miR-145* binding site in c-Myc is not well conserved and the alignment of seed sequencing base pairing between *miR-145* and c-Myc at 3′-UTR is based on an early version of miRBase target (version 4). Clearly there is a base-pairing wobble (Fig. 4*C*). Despite the low conservation, our results strongly indicate that such an interaction plays an important role in silencing c-Myc expression. Thus, these results highlight the complexity of interactions

between miRNA and target gene, and the importance of experimental validation.

Chromatin Immunoprecipitation (ChIP) Assay. ChIP assay was based on the method of Nguyen *et al.* (5) with small modifications. In brief, MCF-7 cells were infected with Ad-p53 and Ad-GFP and incubated for 20 h. Alternatively, p53 +/+ and p53 -/- HCT-116 cells were treated with Doxo (1.0 μg/ml) for 16 h. Nuclear proteins bound to the genomic DNA were cross-linked by adding formaldehyde to a final concentration of 1% directly into the cell culture medium at room temperature on a rocking platform. The precleared chromatin solutions were incubated with anti-p53 antibody overnight at 4 °C with rotation.

Tumor Growth in Nude Mice. Athymic nude (nu/nu) mice (4–5 weeks old) were purchased from Harlan Sprague–Dawley and were maintained in the SIU School of Medicine's accredited animal facility. All animal studies were conducted in accordance with NIH animal use guidelines and a protocol approved by the SIU Animal Care Committee. Exponentially growing HCT-116 cells were first infected with vector control or *miR-145*. One day after infection, the cells were split and grown in a fresh medium for two more days before harvest for inoculation. A total of 1.5 million cells in 50% matrigel were s.c. injected into the flanks of each mouse.

1. Wu F, Chiocca S, Beck WT, Mo YY (2007) Gam1-associated alterations of drug responsiveness through activation of apoptosis. *Mol Cancer Ther* 6:1823–1830.
2. Grimson A, *et al.* (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell* 27:91–105.
3. Krek A, *et al.* (2005) Combinatorial microRNA target predictions. *Nat Genet* 37:495–500.
4. John B, *et al.* (2004) Human MicroRNA targets. *PLoS Biol* 2:e363.
5. Nguyen TT, Cho K, Stratton SA, Barton MC (2005) Transcription factor interactions and chromatin modifications associated with p53-mediated, developmental repression of the alpha-fetoprotein gene. *Mol Cell Biol* 25:2147–57.

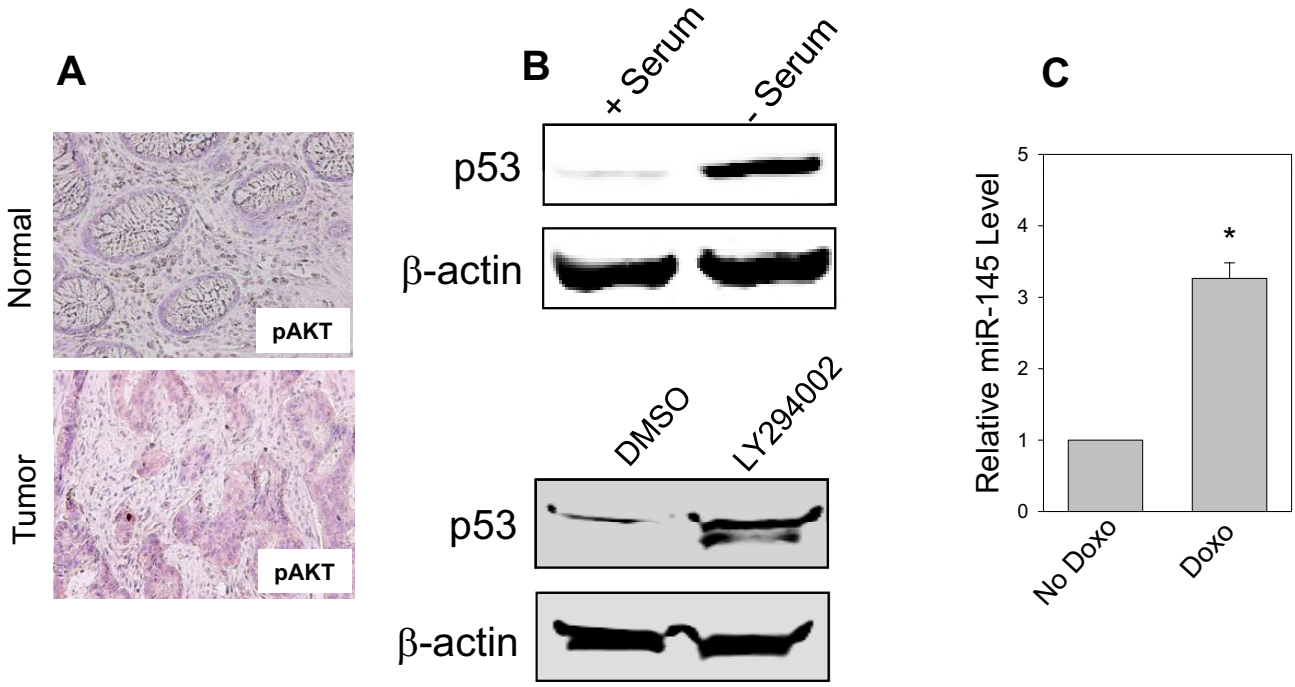

Fig. S1. Expression of miR-145 through Akt/p53 pathways. (*A*) Detection of activation of pAkt in colon cancer tissue specimens by immunohistochemistry. Shown here is a representative pair of matched samples. (*B*) Induction of p53 by serum starvation and the PI-3K inhibitor LY294002. HCT-116 cells were grown in serum-free medium or supplemented with 10%FBS for 24 h. Alternatively, HCT-116 cells were treated with 50 μM LY294002 for 16 h. C, Induction of miR-145 by Doxo. HCT-116 cells were treated with 1 μg/ml of Doxo for 16 h.

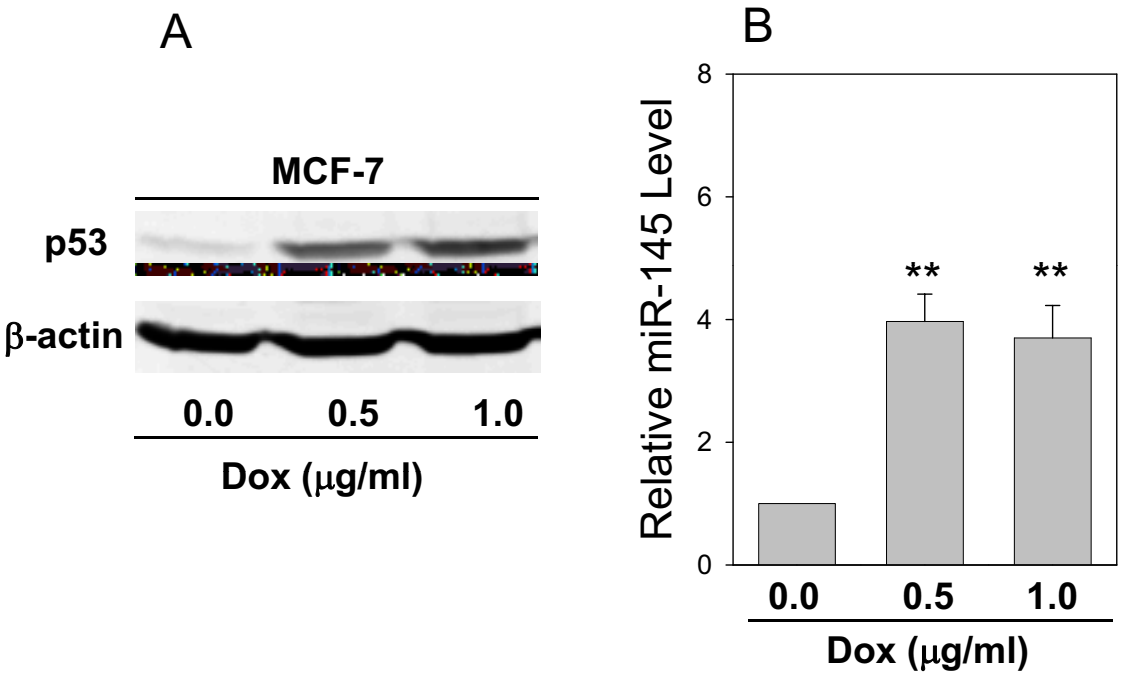

Fig. S2. p53 enhances the *miR-145* level. To determine induction of *miR-145* by the endogenous p53, MCF-7 cells were treated with doxorubicin (Dox) at 0.5 or 1.0 μg/ml for 24 h. The cells were separately harvested for protein (A) or RNA extraction (B). Expression of the endogenous *miR-145* was detected by TaqMan real-time RT-PCR. Values in B are means $\pm$ SE of three separate experiments and normalized to no Dox as 1. **, $P < 0.01$

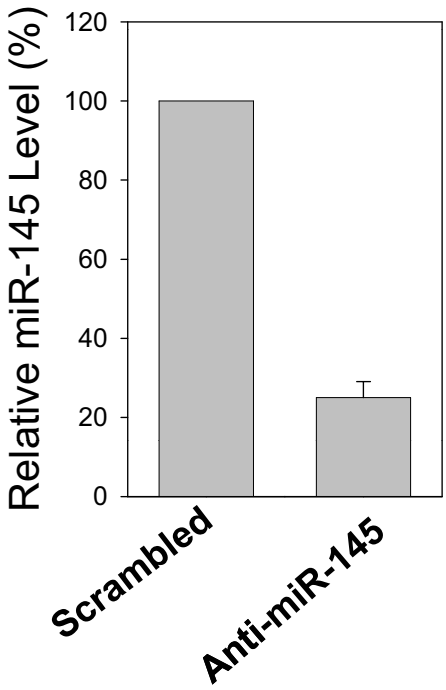

Fig. S3. Blockage of *miR-145* by anti-*miR-145*, as detected by TaqMan real-time PCR

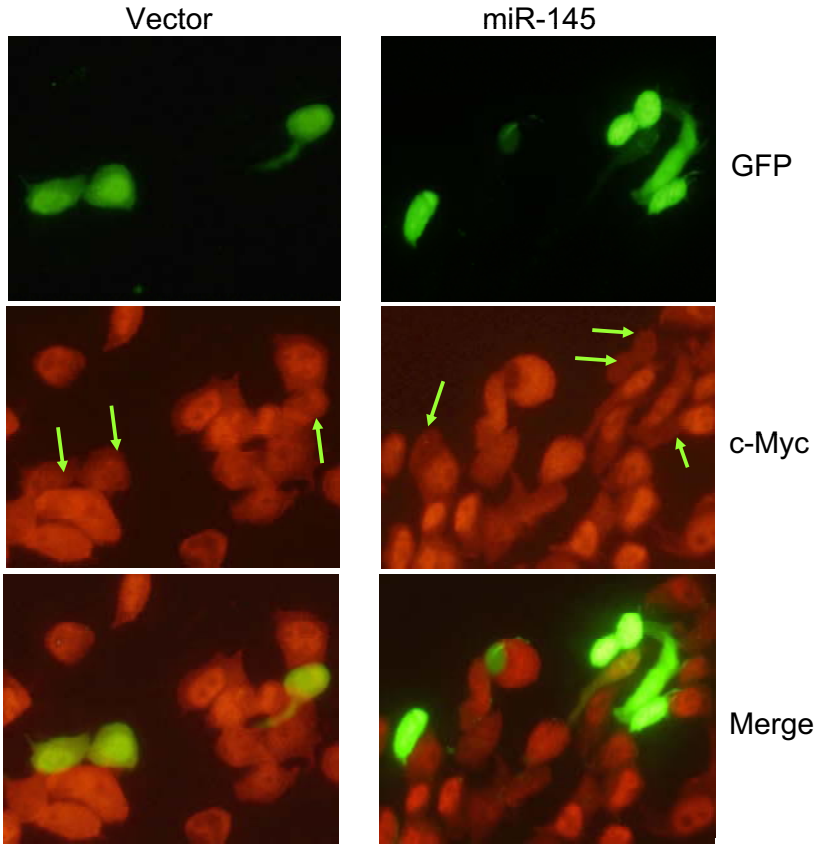

Fig. S4. Immunofluorescence staining reveals decreased levels of c-Myc in *miR-145* cells (*Right*) compared to vector control cells (*Left)*. Note the green cells in the merged picture for *miR-145* due to decreased expression of c-Myc (red). In contrast, the GFP positive cells became yellow in the merged picture because the c-Myc expression was not affected by the vector control.

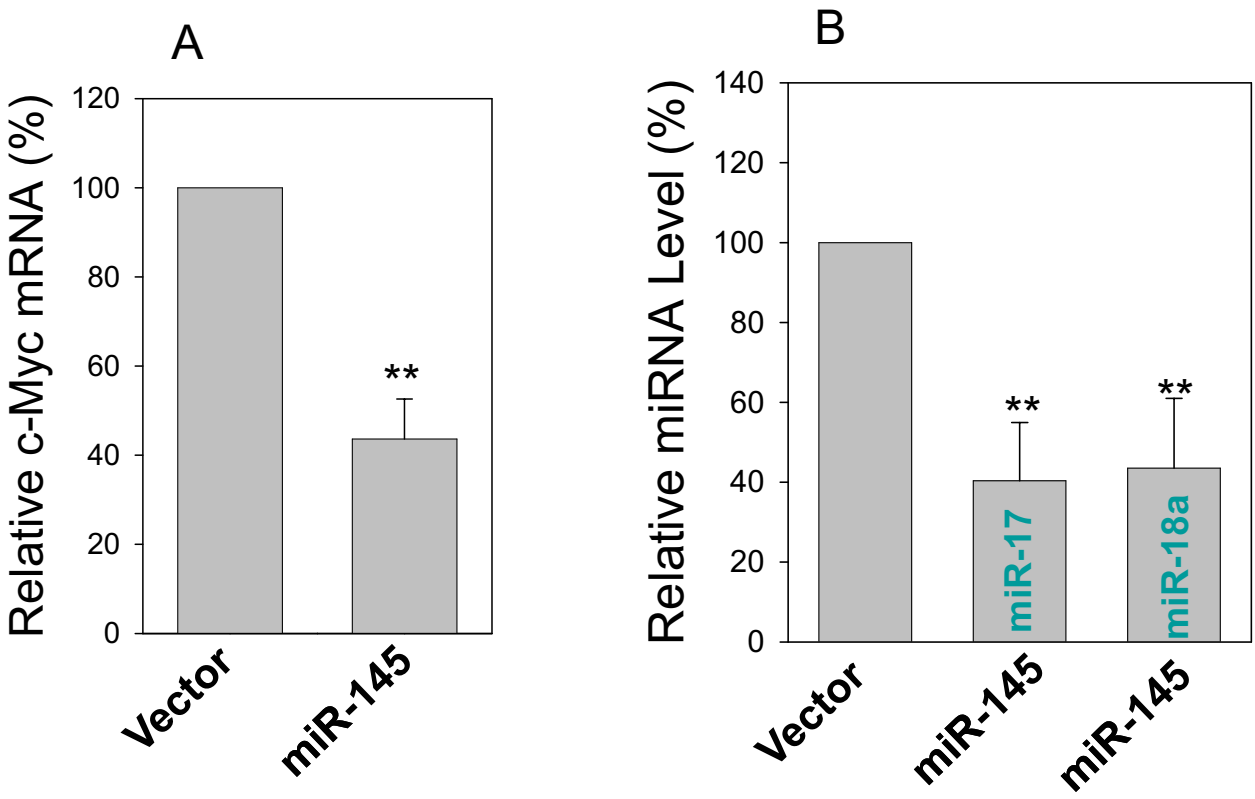

Fig. S5. Suppression of c-Myc mRNA (*A*) and *miR-17* and *miR-18a* (*B*) by *miR-145*. Cells were first transfected with vector alone or *miR-145* and then total RNA was extracted for real time PCR assays. Values are means ± SE of three separate Experiments and normalized to vector as 100%. **, $P < 0.01$.

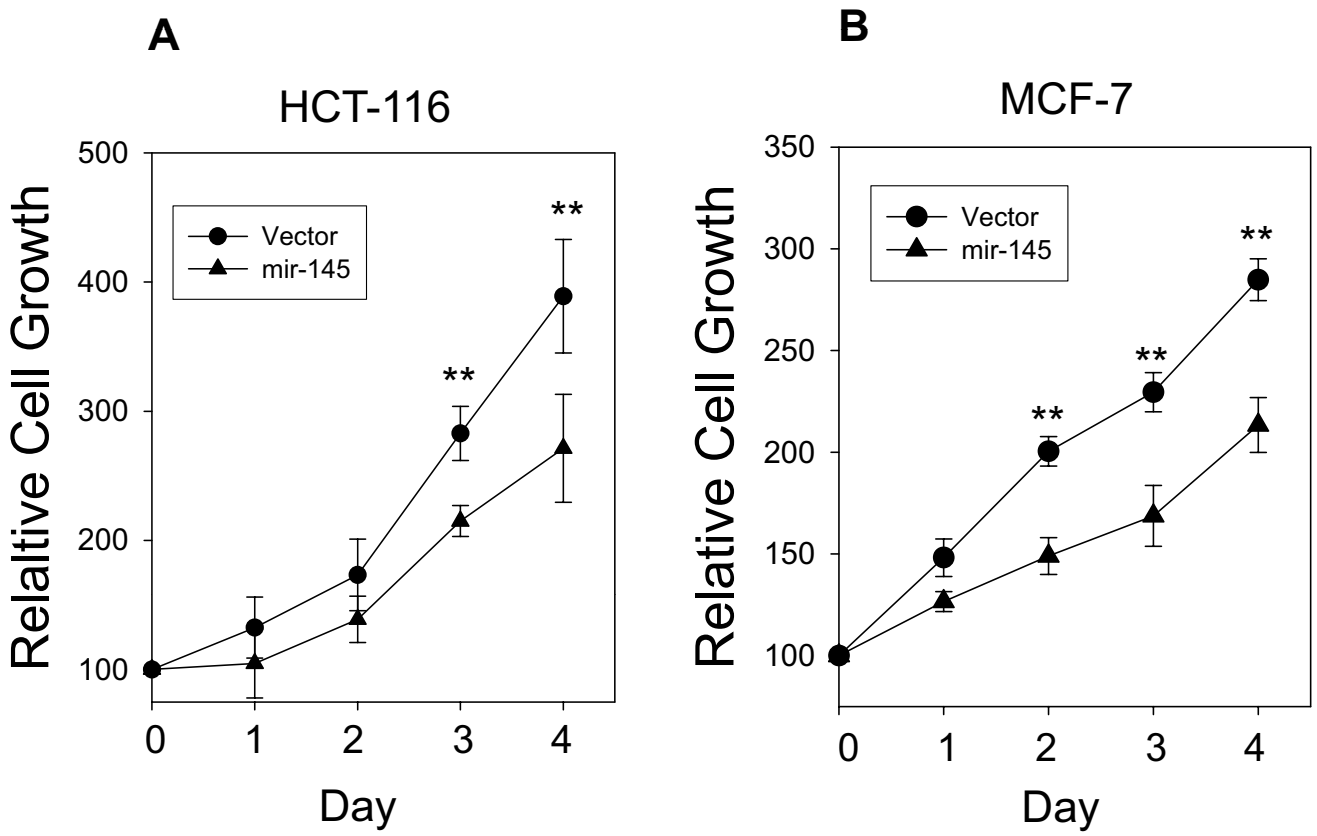

Fig. S6. *miR-145*-mediated cell growth inhibition. Cells were first transfected with vector control or *miR-145*. The transfected cells were then seeded in 96-well plates and incubated for indicated time points before MTT assays. (*A*) HCT-116 cells and (*B*) MCF-7 cells. Values are means ± SE of three separate experiments and normalized to day 0 as 100%. **, $P < 0.01$.

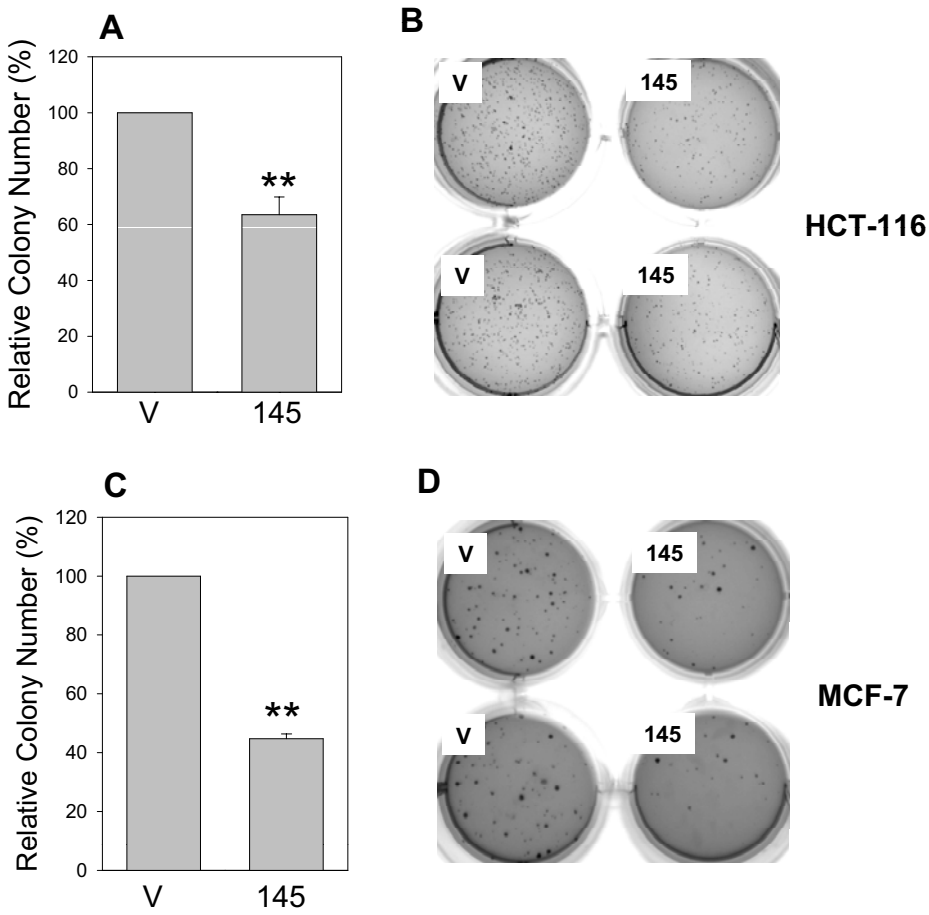

Fig. S7. Suppression of anchorage independent growth by *miR-145*. Cells were first transfected with vector control (V) or *miR-145* (145) expression vector and then grown in soft agar medium as described in *Materials and Methods*. Colonies were counted 2 weeks after seeding. Values in *A* and *C* are means ± SE of three separate experiments and normalized to vector as 100%. **, $P < 0.01$. B and D, Representative wells of colony formation.

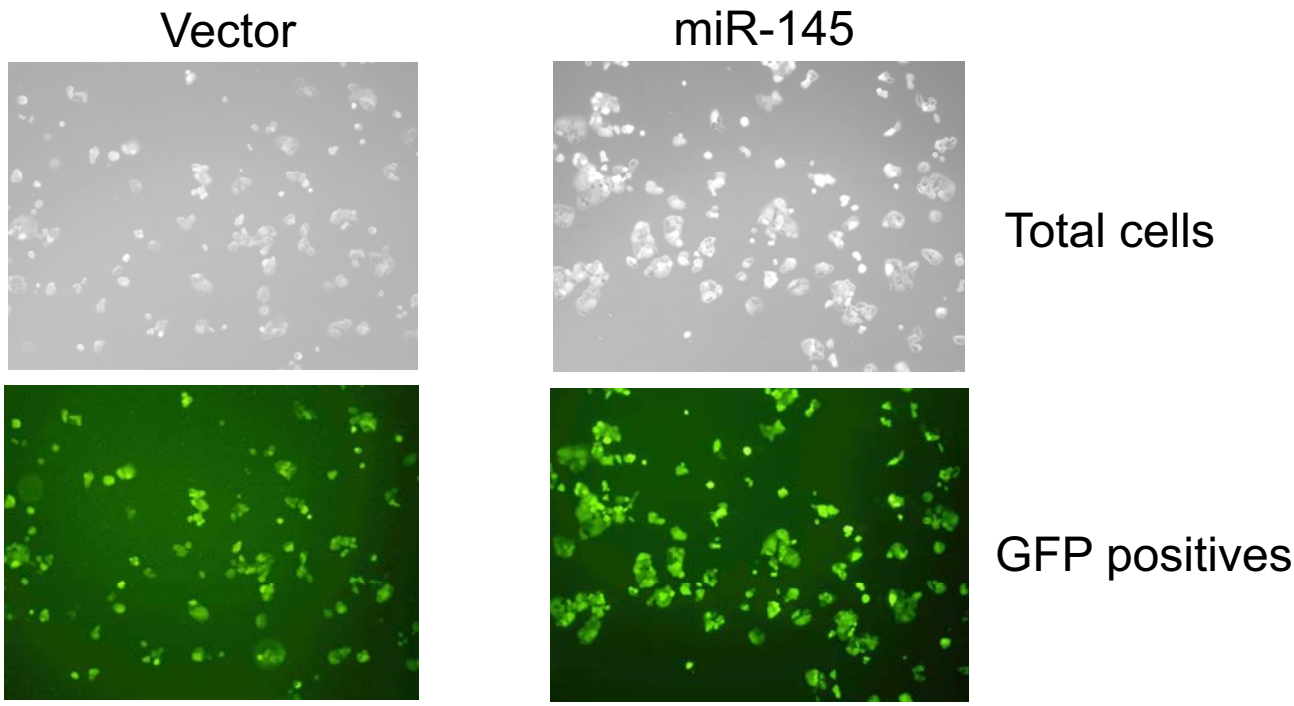

Fig. S8. HCT-116 cells used to inject into nude mice after infection of vector alone or *miR-145*. The picture reveals that cells for both vector and *miR-145* were almost 100% infected as indicated by GFP.

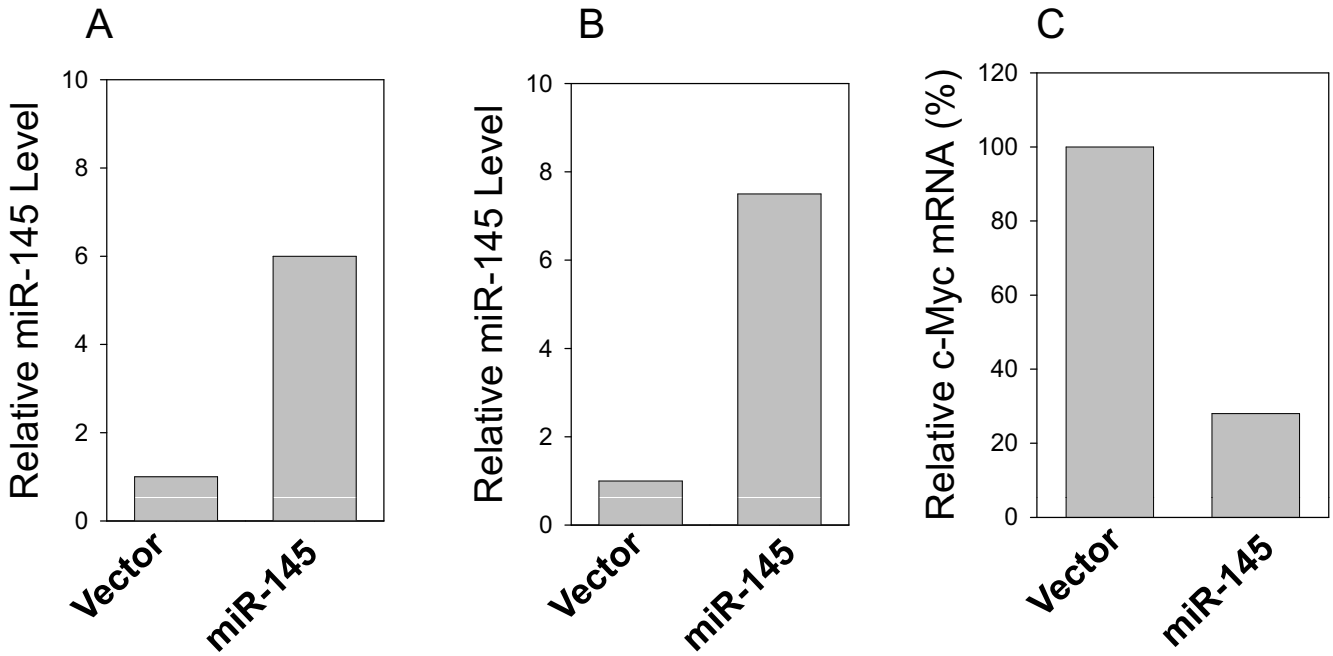

Fig. S9. Detection of *miR-145* levels in infected HCT-116 cells before injection (*A*) and in tumor harvested from mice (*B*) as well as c-Myc mRNA in tumor (*C*). The values are average of 2 measurements in each case.

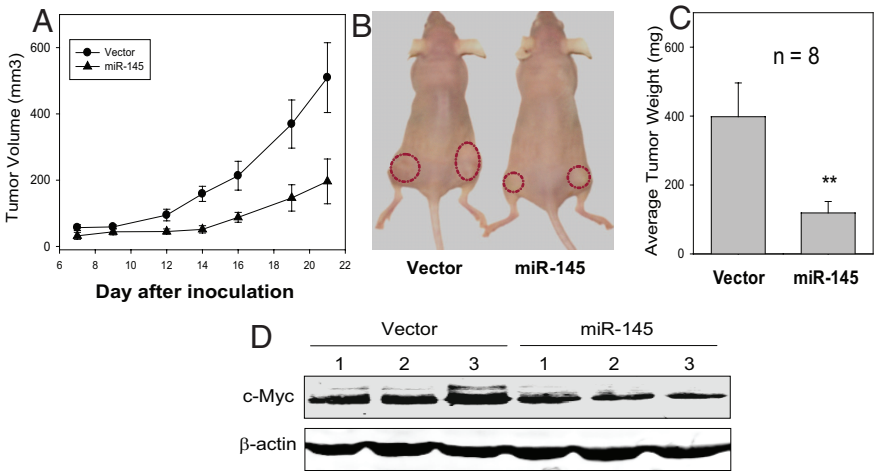

Fig. S10. Inhibition of tumor growth in vivo by *miR-145.* HCT-116 cells were infected with vector control or *miR-145* expression vector and then injected into nude mice as described in Materials and Methods. After injection, tumors were harvested at week 3 and weighed. (*A*) Tumor growth curve. (*B*) Representative tumor-bearing mice. (*C*) Average tumor weight. **, $P < 0.01$. (*D*) Suppression of c-Myc by *miR-145* in the xenograft tumors (three tumors for each of vector control group and *miR-145* group, respectively).

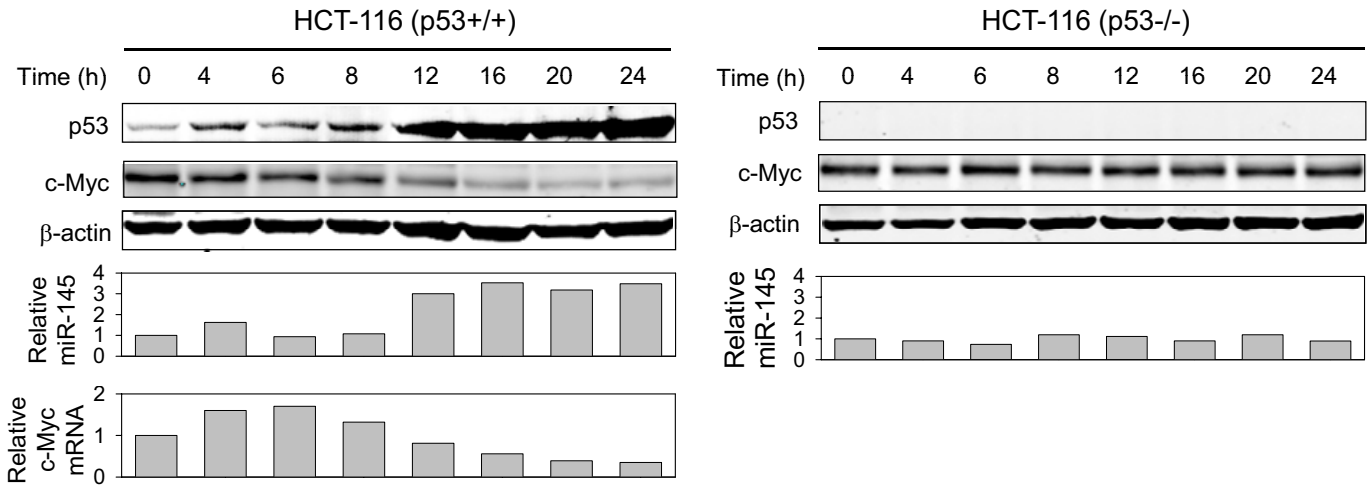

Fig. S11. The time-course effect of p53/doxo on miR-145, c-Myc protein and c-Myc mRNA. Cells were treated with doxo at 0.5 μg/ml for indicated times. Total RNA or protein was extracted for real-time RT-PCR or Western blot.

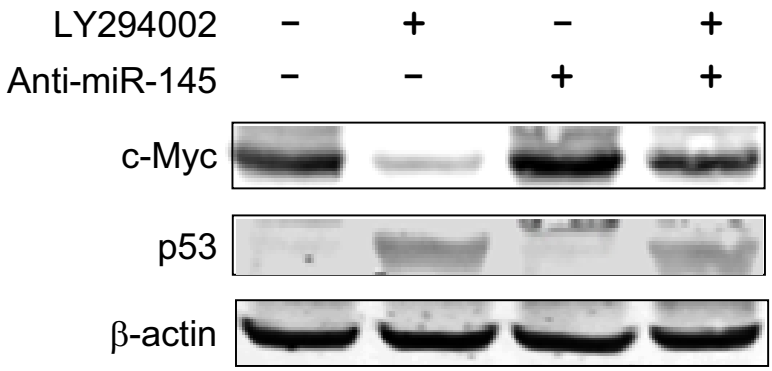

Fig. S12. Anti-miR-145 blocks LY294002/p53-mediated c-Myc repression. HCT-116 cells transfected with or without anti-*miR-145* were treated with 50 μM LY294002 for 16 h.

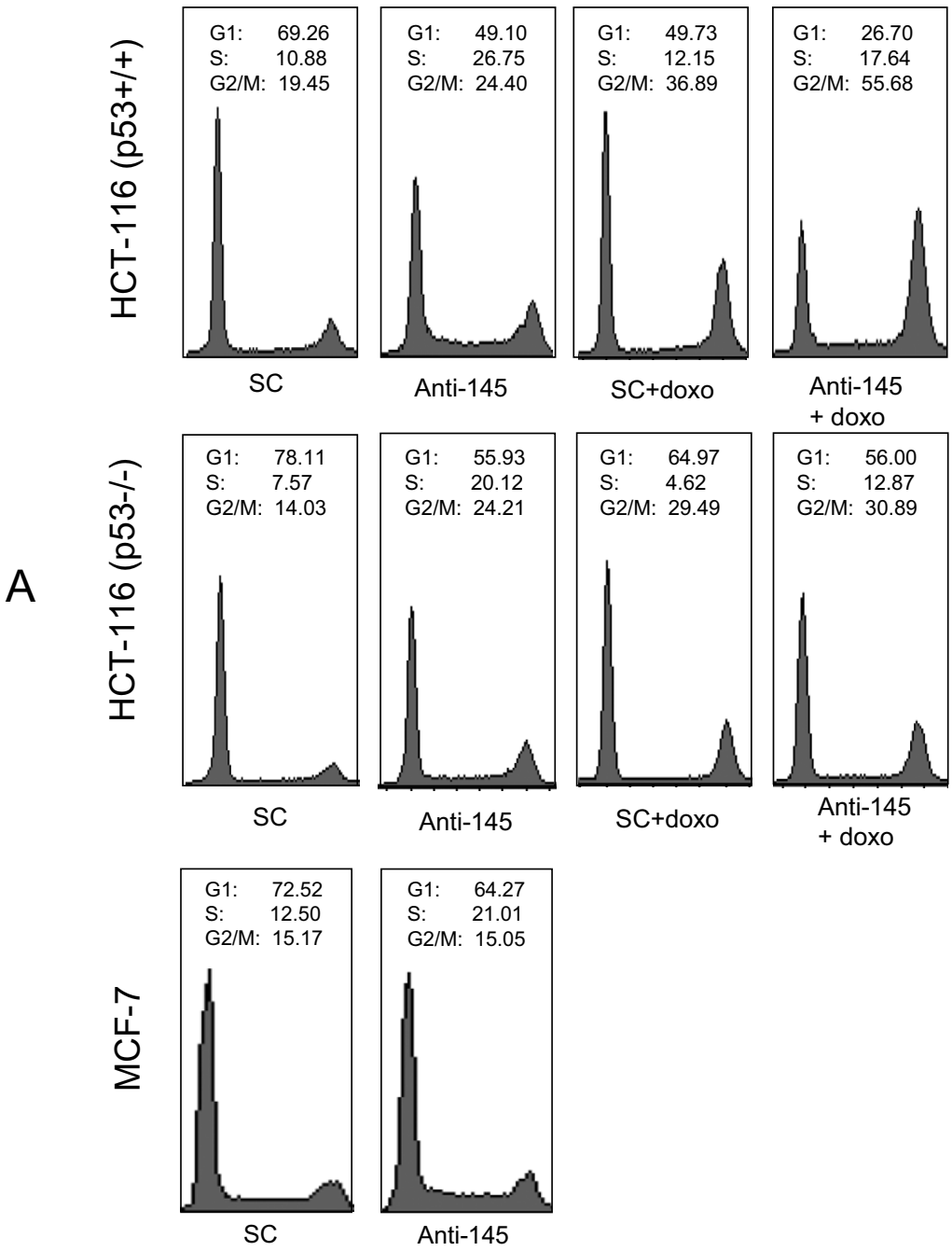

Fig. S13. Effect of anti-miR-145 and c-Myc-siRNA on cell cycle and apoptosis. (*A*) Cells were first transfected with scrambled oligo (SC) or anti-miR-145 (anti-145). For Doxo treatment, the cells were treated with Doxo (0.5 μg/ml) for 16 h before harvesting for cell cycle analysis. (*B*) HCT-116 cells were first transfected with p53 and then with anti-miR-145 and/or c-Myc-siRNA. The cells were harvested for cell cycle analysis 24 h later. C, Apoptosis analysis. Cell death ELISA plus kit (Roche) was used to measure the apoptotic activity per the manufacturer's recommendations. HCT-116 cells were first transfected with anti-miR-145 and then treated with Doxo (0.5 mg/ml) for 16 h before harvesting for the assay.

Table S1. Activation of pAkt in colon cancer specimens

Tissue	pAkt signal				Total positive*	
	−	+	++	+++		
Normal	16	5	2	0	23	30%
Tumor (G1)†	8	13	5	1	27	70%
Tumor (G2)	3	14	9	5	27	89%
Tumor (G3)	1	8	12	7	28	96%
Tumor (G4)	0	0	2	1	3	100%

*Positives (+, ++, and +++) among total number in the same group.
†Disease grade.

Table S2. Primers used in this study

Name	Sense	Sequence	Use
Mir-145 gene primers:			
miR-145–5.1	S	5′TAAGCAGCTCACCTAAAGTC	Cloning
miR-145-Not1–3.1	A	5′GCGGCCGCGTCATGTGGATGCAATACCG	Cloning
miR-145-mut-5.1	S TTAGATGCTAAGATGGGG	5′GGCTTCAGGTTCCCAGGAATCCC	Cloning
miR-145-mut-3.1	A GAGGACAAGGTGGGAGC	5′GATTCCTGGGAACCTGAAGCCGT	Cloning
Mir-145 promoter primers:			
Mir-145p-Kpn1–5.1	S	5′GGTACCAGCACTGTAGCTCAGGAAGAG	Cloning
Mir-145p-Kpn1–5.2	S	5′GGTACCTGTACCCCAGTGTGTCCATCTT	Cloning
Mir-145p-Kpn1–5.3	S	5′CATTCTTGAAGTGCTCAGATGC	Cloning
Mir-145p-Kpn1–5.6	S	5′GGTACCCAAATGTCTGACTTCTCCCAG	Cloning
Mir-145p-Xho1–3.2	A		