Supporting Information

Text

Construction of a lentiviral vector-based human miRNA library

We have modified the expression vector pSIF-H1-copGFP (Systems Biosciences Inc.) to provide two genetic markers, neomycin and GFP. An Internal Ribosomal Entry site (IRES) sequence from pIRES was inserted between the neomycin and GFP gene via an Xba I site, allowing both genes to be expressed with the same CMV promoter. A Multiple Cloning Site (MCS) was inserted in the expression cassette via the Bam HI and Eco RI sites, facilitating cloning of various miRNA minigenes downstream of the H1 RNA promoter. The resulting vector (pSIF-NEO-IRES-GFP, abbreviated as pSIF; Fig. S1A) is functional as designed.

Given that most mammalian miRNA genes are transcribed as pri-miRNAs usually over several kilobases (kb) or even hundreds of kb length (Cai et al., 2004; Cullen, 2004; Rodriguez et al., 2004), it is unrealistic to construct the lentiviral expression vector including the full-length miRNA gene or the host gene (the packaging limit for the lentiviral system is ~8.5kb). Two recent reports (Zeng & Cullen, 2005; Han et al., 2006) have investigated the molecular determinant of pri-miRNA processing by the Drosha-DGCR8 complex. A typical metazoan pri-miRNA consists of a stem of approximately 33 bp, with a terminal loop and flanking segments. The terminal loop is not essential, whereas the flanking ssRNA segments are critical for processing. The cleavage site is determined mainly by the distance (approximately 11 bp) from the stem-ssRNA junction. Drosha function also requires single-stranded RNA extensions located outside the pri-miRNA hairpin. The sequence (20 nts needed) of these RNA extensions was largely unimportant *in vitro*, but native sequences have been preferred in *in vivo* studies (Zeng & Cullen, 2005).

Based on the experimentally determined criteria above, we have decided to express minigenes (~140-150 bp; SI DataSet) of miRNAs consisting of precursor miRNA (~65 nts) and its adjacent native sequences (40-50 nts) at both the 5'- and 3'-ends. All precursor miRNA and its flanking 100bp sequences are retrieved from NCBI Human Genome Assembly 36 according to the miRNA information from miRBase 9.2 (Griffiths-Jones et al., 2006) using BioPerl (Stajich et al., 2002). As reported in the miRBase Release 9.2, May 2007 (Griffiths-Jones et al., 2006), there are 474 human miRNA precursors that include a total of 539 miRNAs (some precursors can produce 2 miRNAs by Dicer, Fig. S1B). For minigenes encoding the same miRNA, only one is constructed (e.g., hsa-let-7a-1 but not hsa-let-7a-2 or hsa-let-7a-3) to streamline the redundancy. Overall, we have chosen 420 human minigenes containing all 539 miRNAs (miRBase 9.2) as our targets for cloning. We employ a PCR-based total synthetic method to synthesize all the minigenes (Hoover & Lubkowski, 2002) with "TTTTT" downstream as a terminator for RNA polymerase III at their 3'-ends. The DNA fragments are also supplemented with two restriction sites at both ends. To minimize the impact of constitutive "U" stopping the RNA polymerase III, one "U" in four or more constitutive "U" nucleotides is modified with the consideration that the mature sequence and the base-pairing of the precursor stem are not jeopardized (see Notes in SI DataSet). The majority of the constructs are designed with restriction sites *Bam*HI and *Mfe*I, while ~10% of them (those containing either *Bam*HI or *Mfe*I in the minigenes) are supplemented with *Bgl*II and *Eco*RI sites. All PCR products are cloned into pSIF after restriction digestion and the resulting plasmid are confirmed by DNA sequencing.

We have been able to clone 367 out of 420 minigenes (SI DataSet). These 367 constructs can be used to overexpress 472 out of 539 human miRNAs. We have chosen constructs that contain minigenes for miR-508, miR-9, miR-21, miR-30d, miR-25, miR-200a, let-7b, and miR-301 to check transient transfection and expression

(Fig. S1D) and transduction with the miR-21 construct and the packaging plasmids. Forty-eight hrs after transient transfection into human embryonic kidney 293T cells, all miRNA are overexpressed compared with host cells transfected with the Vector control. We also transfect the miR-21 construct into other cell lines to achieve overexpression. The packaged miR-21 pseudoviral particles are able to transduce human lung carcinoma A549 cells (Fig. S1E). Pseudoviral particles can infect target cells but cannot replicate within them since the viral structural genes are absent, and the LTRs are designed to be self-inactivating upon transduction. The integration of the miR-21 minigene into the chromosomes of A549 cells is stable over >30 passages. miRNA overexpression is shown for miR-9, a low abundance miRNA in 293T (Fig. S1D), and miR-21, a high abundance miRNA in A549 on agarose gel electrophoresis (Fig. S1E).

Compared to the one constructed by the Agami laboratory (Voorhoeve et al., 2006), this library has three advantages (1) it is more comprehensive (367 constructs *versus* 197); (2) it has improved delivery options (lentivirus vs. retrovirus, and infectivity of non-mitotic cells); and (3) our constructs contain a *GFP* gene under control of the *NEO* gene promoter, allowing live-cell imaging fluorescence microscopy. We are cognizant that a new version of miRBase has been released (11.0; April, 2008) and we have undertaken efforts to update it. Our library is distributed by GeneCopoeia, Inc (Germantown, MD) under a worldwide nonexclusive license.

Cell culture

293T, PANC-1, Mia-PaCa-2, and Hs-766T cells (from ATCC) were cultured in DMEM with 10% fetal calf serum (FCS). BxPC-3 and AsPC-1 (from ATCC) were cultured in RPMI-1640 with 10% FCS. All medium contained 1x Antibiotic-Antimycotic (Invitrogen). All cell lines were cultured at 37°C, in a humidified atmosphere of 5% CO_2. Transient transfection was performed using Lipofectamine LTX (Invitrogen). Transfection efficiency for 293T and pancreatic cancer cell lines was >90% and 40-50%, respectively. For miRNA construct, we used the parental vector pSIF as a control (Supplemental Text 1). siRNAs were purchased from Sigma (St. Louis, MO) with the following clone IDs: NFKB1, NM_003998.2-2853s1c1; COX2, NM_000963.1-1227s1c1; KRAS, NM_004985.x-1160s1c1. For NKRF overexpression, the parental vector pCMV-SPORT6 (OpenBiosystems, Inc.) was used as a control. We used a modified antisense RNA to miR-301a (Anti-miR™-301a) purchased from Ambion (Austin, TX) to inactivate miR-301a; we used Anti-miR™ Negative Control #1 as a negative contro, which is a random-sequence RNA molecule that has been extensively tested in many human cell lines and tissues and does not produce any identifiable effects on known miRNA functions. At 48 h post-transfection, total RNAs were extracted with Trizol (Invitrogen) and quantitative real-time PCR (qPCR) was performed to measure the miRNA or mRNA expression levels with U6 snRNA (for miRNAs) or β-actin mRNA (for all mRNAs) as a reference (Chen et al, 2005). Dual luciferase-activity assays were performed 48 h after transfection according to the manufacturer's protocols (Promega). Total RNAs from a pool of 10 normal pancreases of healthy donors were obtained from Ambion (Austin, TX). The relative luminescence unit (RLU) of each well was either *luc* normalized to that of *Rluc*, or vice versa, before being compared with controls, which were arbitrarily set at 1.0. Values are represented as means with standard deviation (SD) from at least three independent experiments.

Cell line 293NF-κB and the reporter assays

We first packaged the PathNet® transcriptional reporter lentivector pTRF2-NF-κB –luc (System Biosciences) into pseudolentiviral particles with the packaging plasmids pFIV-34N and pVSV-G. The κB site upstream of a mCMV promoter and a firefly luciferase (*luc)* gene is 4 consecutive GGGACTTTCC repeats recognized by NF-κB (p50-RelA), because the p50-RelA heterodimer is the most abundant form of NF-κB in unstimulated cells and likely dominantes activation of the internal luciferase gene expression. After transducing 293T, we obtained a cell line carrying the TRE(NF-κB)-mCMV-luc construct, 293NF-κB. The 293NF-κB cells

demonstrated basal luciferase activity and >10-fold induced activity (i.e., stimulation with TNF-α, 10 ng/ml) compared with the unstimulated control. The integration of TRE(NF-κB)-mCMV–luc into the chromosomes of 293 cells was further confirmed by PCR amplification using their genomic DNAs as templates and DNA sequencing. Two additional constructs were used for the screening assay: pRL-TK (Promega) that carries a Renilla luciferase gene (*Rluc*) which is constitutively expressed and pGL3-promoter (Promega) with a constitutively expressed *luc*. In the first assay to find miRNAs that modulate NF-κB signaling, cells integrated with NF-κB–luc (293NF-κB) are co-transfected with two plasmids: a miRNA construct and pRL-TK (Fig. 1A) . For the second assay, 293T cells are co-transfected with three plasmids: a miRNA construct, pRL-NKRF, in which an *Rluc* gene is placed upstream of the 3′-UTR of *NKRF*, and pGL3-promoter. The readout *luc*/*Rluc* of the first assay and *Rluc*/*luc* of the second is used to grossly assess whether a miRNA modulates NF-κB activation and whether it targets the 3′-UTR of *NKRF* or other genes.

Western blotting

Total protein was isolated from 293T or pancreatic cancer cell lines (PANC-1, Mia-PaCa-2, AsPC-1, Hs-766T, and BxPC-3) from 6-well plates using M-PER mammalian protein extraction reagent (PIERCE, Rockford, IL). Protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Aliquots containing 50 μg of protein were separated on 12% Bis-Tris polyacrylamide gels (Sigma-Aldrich, St. Louis, MO) and then transferred to PVDF membranes (Invitrogen). Protein membranes were incubated in blocking agent [1× Tris-buffered saline (pH 7.5), 5% nonfat dried milk, 0.05% Tween 20] overnight at 4°C, then with anti-Nkrf antibody (Atlas Antibodies AB, Sweden), anti-Cox-2, or anti-β-actin antibody (Sigma-Aldrich) for 1 h at room temperature. The membranes were washed with 1× Tris-buffered saline containing 0.05% Tween 20, incubated with horseradish peroxidase-linked Goat anti-mouse Ig (Santa Cruz) or Goat anti-Rabbit Ig (PIERCE) for 1 h at room temperature, washed, and visualized with the SuperSignal West Pico Chemiluminescent Substrate kit (PIERCE).

Cell cycle analysis

Cells were resuspended in 1 ml of ice-cold 70% ethanol and kept at 4 °C for 24 hours. The next day the cells were washed with 1xPBS and treated with 1 mg/ml Ribonuclease A for 90 min at 37°C in 0.5ml PBS. Before cell cycle analysis cells were stained in 5 μg/ml propidium iodide (PI) protected from light. Flow cytometry was performed with a FACScan apparatus (Becton Dickinson, Franklin Lakes, NJ). A minimum of 15,000 cells per sample were collected and the DNA histograms were further analyzed using FlowJo software (Tree Star, Inc., Ashland, OR) for cell cycle analysis.

Annexin-V apoptosis assay

Cells (50–60% confluent) were treated with or without etoposide (40 μM, 30min) before trypsinization and washed with 1xPBS twice. Apoptosis was assessed using an ApoTarget™ Annexin-V FITC Apoptosis Kit (Invitrogen, Carlsbad, CA). This assay employs fluorescein-labeled Annexin-V in concert with propidium iodide (PI) to detect the cells undergoing apoptosis. Briefly, cells were washed with 1xPBS twice and suspended at 2-3 x 10^6 cells/mL in 1x Annexin-V Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin-V FITC and Propidium Iodide Buffer were added to the cells, which were then incubated at room temperature for 15 minutes in the dark. Cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) within 1 hr of staining using the FL1 (FITC) and FL3 (PI) lines.

miRNA microarray

RNA was sent to LC Sciences (http://lcsciences.com/) where the RNA samples were labeled either with Cy3 or Cy5 and were hybridized with dual-color miRNA microarray chips: the Human miRNA Detection Array (MRA-1001, LC Sciences). The array contains probes to detect mature miRNA sequences as well as pre-miRNAs in the Sanger miRNA registry 7.0 (http://microrna.sanger.ac.uk/sequences/). Each human miRNA on the chip contains seven redundancies for each sequence to increase sensitivity. Microarray analysis was performed by LC Sciences including background subtraction and data normalization to the statistical median of all detectable transcripts.

NF-κB DNA binding and chromatin occupancy assays

For electrophoretic gel mobility shift assay (EMSA), the nuclear extracts from 293T cells were prepared and 10 μg of nuclear protein was incubated with binding buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM MgCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 1 μg/reaction poly (dI–dC) with 10^5 cpm of the γ-p^{32}-ATP labeled oligonucleotide probe, which contains the specific recognition sequence for NF-κB (AGT TGA GGG GAC TTT CC CAG GC), for 30 min at room temperature. In supershift studies, 1 μl of the indicated affinity-purified mouse anti-peptide antibodies (Santa Cruz) was incubated with the extracts for 15 min prior to the addition of the probe. The protein–DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel. The gel was dried and autoradiographed using a Storm 860 Imaging system (Molecular Dynamics, Sunnyvale, CA) and signal densities were analyzed using ImageQuant 5.1.

A commercially available kit was utilized for the NF-κB and p53 chromatin immunoprecipitation (ChIP) assay (Active Motif, Carlsbad, CA). Antibodies against NF-κB RelA (ab7970, ChIP grade, Abcam Inc., Cambridge, MA) or p53 (antibody #39334, ChIP grade, ActiveMotif) were used for immunoprecipitation. 293T cells were treated with or without 10ng/ml of TNFα or 40μM of etoposide for 30 min. DNAs were purified from the NF-κB or p53 ChIP eluates as well as input lysate using phenol-chloroform extraction followed by ethanol precipitation. The purified DNA was resuspended and subjected to qPCR with SYBR Green I using primers flanking the NF-κB site in the human miR-301a promoter (forward primer, 5'-TCA CGC CTG TAA TCC CAG CAC TTT, and reverse primer, 5'-TAG GCT GGA GTA CAG TGG CAC AAT; product 208bp). An NF-κB positive control was lin28b, a known NF-κB target gene (forward primer, 5'-TTA CCC CAA TAC TGG GCA TT; reverse primer, 5'-GTG TGG GCG GTA CAA AAC AT; product 103bp) (Iliopoulos et al, 2009). The negative control uses primers to amplify a noncoding region between GAPDH and CNAP1 gene (forward primer, 5'-ATG GTT GCC ACT GGG GAT CT; reverse primer, 5'-TGC CAA AGC CTA GGG GAA GA; product 173bp; ActiveMotif). The p53 positive control was miR-34a, a known p53 target gene (forward primer, 5'-ACG CTT GTG TTT CTC AGT CCG; reverse primer, 5'-TGG TCT AGT TCC CGC CTC CT; product 73bp) (Raver-Shapira et al, 2007). TF occupancy (fold enrichment) was represented by $2^{(Ct,input-Ct,elute)}$ before the ΔCt values were normalized to that of the negative control.

***In situ* hybridization (ISH) and IHC with tissue microarrays**

Pancreatic cancer tissue microarrays (TMA) (Lot Number: ARY-HH0118-001-01) were purchased from Folio Biosciencies (Columbus, OH), which contain sixty pancreatic cancer specimens along with 10 normal adjacent tissues and 10 normal pancreas tissues. For the IHC and ISH studies, fifty eight adenocarcinoma specimens were chosen, while one acinic cell carcinoma and one squamous cell carcinoma were excluded. ISH was performed using antisense oligonucleotide probes for miR-301a (Exiqon Inc, MA) with Scramble-miR (GTGTAACACGTCTATACGCCCA) serving as a negative control. After the sections were deparaffinized, hydrated and deproteinated, prehybridization was performed in hybridization buffer for 2 hrs in a humidified chamber at 55°C. Hybridization was then performed by applying 20 nM of probe in hybridization buffer to the

array slides covered with nescofilm overnight at 55°C in a humidified chamber. Hybridized probes were detected by incubation with anti-digoxigenin – alkaline phosphatase conjugate at 37°C for 30 minutes followed by substrate NBT/BCIP to develop a blue color. Finally, the cells were counterstained with nuclear fast red for 3-5 min and mounted on slides. RelA and Nkrf staining was carried out using the DAKO EnVision™+System Kit (DAKO Corporation, Carpentaria, CA). After the sections were deparaffinized and hydrated, they were rinsed with a TRIS-buffer, and peroxidase blocking was performed for 5 min. Monoclonal mouse RelA antibody (1: 100) (sc-71677, Santa Cruz Biotechnology Inc, CA) or polyclonal rabbit Nkrf antibody (1: 100) (HPA001476, Sigma, St. Louis, MO) was then applied for 30 min. For other staining, Rabbit antibodies against human VEGF-C (ab9546,Abcam), rabbit antibodies against human PCNA (sc-7907, Santa Cruz), and rabbit antibodies against mouse CD31 (sc-28188, Santa Cruz) were used. The slides were incubated with labeled polymer for 30 min at room temperature. The substrate diaminobenzidine was added as a visualization reagent. Finally, the tissue microarray slides were counterstained with Haematoxylin. A negative control was included in each run. The IHC slides were evaluated histologically by two independent, blinded observers, and the gradation was scored from 0 to 3 according to the intensity of staining (0, negative; 1, weak; 2, moderate; 3, strong).

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miR-301a as a NF-κB Activator in Pancreatic Cancer Cells:

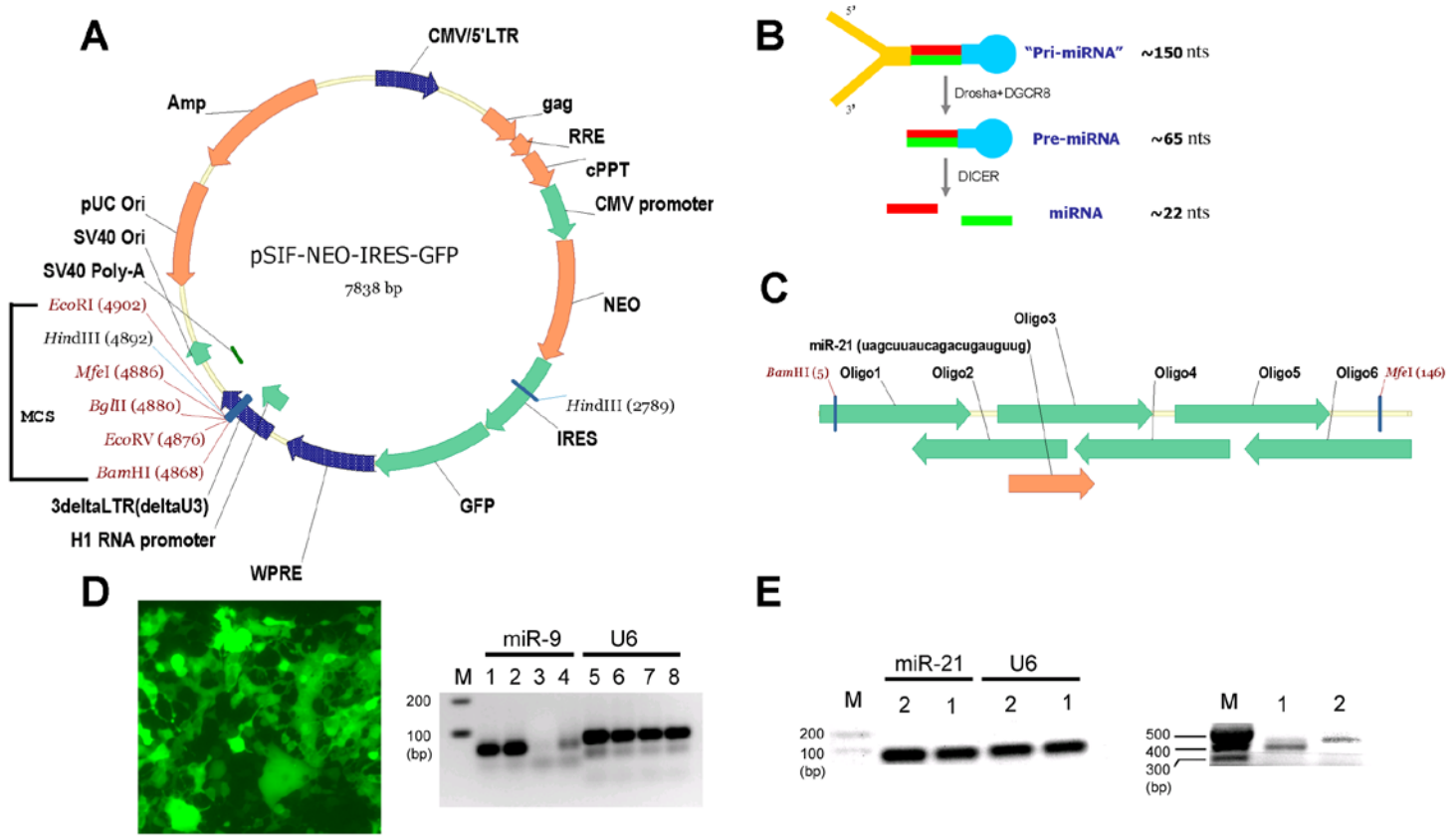

Figure S1 The miRNA library construction. (A) The modification of a cloning and expression lentiviral vector. We optimized the pSIF-H1-copGFP vector with three modifications: (1) the addition of a MCS (*Bam*HI, *Eco*RV, *Bgl*II, *Mfe*I and *Eco*RI) in the expression cassette; (2) the addition of G418 resistant gene NEO; and (3) the addition of IRES allowing NEO and GFP expression with one promoter. Other features of this vector: H1 expression cassette provides constitutive and efficient RNA polymerase III-dependent transcription of RNA transcripts in wide range of cell lines; CMV promoter promotes high level of expression GFP and NEO for detection and selection of transduced cells; Hybrid CMV-5'-LTR promoter provides a high level of expression of the full-length viral construct in 293T cells; Genetic elements (cPPT, GAG, LTRs) necessary for packaging, transducing, and stable integration of the viral expression construct into genomic DNA of host cells; SV40 origin for stable propagation of the plasmid in 293T producer cells and other cells with T-antigens; The pUC origin for high copy replication and maintenance of the plasmid and the Ampicillin-resistance gene for selection in *E. coli* cells; WPRE element enhances stability and translation of the CMV-driven transcripts; and the SV40 polyadenylation signal enables efficient termination of transcription and processing of the transcripts. (B) Experimental design for miRNA minigenes based on miRNA biosynthesis. miRNA can come from either end of the processed pre-miRNA. There are 267 from the 5' part in red and 272 from 3' part in green (67 pre-miRNAs produce two miRNAs) in miRBase 9.2 (Griffiths-Jones et al., 2006). (C) DNA synthesis of miRNA minigenes using *mir-21* as an example. The oligonucleotides are designed to cover the complete sequence (for *mir-21* minigene in this figure) of both strands, and the full-length molecule is generated progressively in a single reaction by overlap extension PCR (OE-PCR), followed by amplification in a separate tube by PCR with two outer primers (D) The expression of miR-9 in 293T cells with transient transfection. The miR-9 is an extremely low abundant miRNA in 293T cells (our laboratory and Ambion miRNA Research Guide). The mature miR-9 level in 293T cells transfected with the *mir-9* construct was ~690 fold of the control as determined by a Taqman stem-loop real-time PCR method with U6 RNA as a control (Applied Biosystems). Image on the left is fluorescence from cells transfected with miR-9 construct; image on the right is an agarose gel eletrophoresis analysis of the PCR products after 30 cycles. Lane 1, 2, 5 & 6, cells transfected with miR-9 construct; Lane 3, 4, 7 & 8, cells transfected with the control pSIF. (E) The expression of the miR-21 in A549 cells with pseudoviral transduction. The miR-21 is a miRNA with high abundance in lung carcinoma including A549 cells (Yanaihara et al., 2006). The miR-21 construct or the control plasmid pSIF is packaged into pseudoviral particles with pFIV-34N and pVSV-G. A549 cells were transduced and selected for 3 weeks with 400 μg/ml of G418. The chromosomal integration of the miR-21 minigene into the chromosome of A549 is examined by PCR amplification using genomic DNAs (right). The PCR products (right, ~400 bp in lane 1 and ~500 bp in lane 2) using primers (GTAACCATTATAAGCTGCAA for SV40 Poly-A site & GCCCTGCAATATTTGCATGTCGCT for H1 promoter) are confirmed with DNA sequencing. Lane 1, cells transduced with the control vector; Lane 2, cells with miR-21 construct. The miR-21 level in transduced A549 cells with the miR-21 construct is ~2 fold that of the control as determined by Taqman quantitative real-time PCR (Applied Biosystems).

SI Table S1 A summary miRNAs dysregulated in pancreatic cancer

ID	Expression	Folds	P Value	Methods	Samples	Target	Reference	NFkB reporter	Dysregulated in other tumors
hsa-miR-301	**Up**	**34.2**	**0.000011**	**Microarray; real-time quantitative PCR assay; RT *in situ* PCR**	**28 tumors, 15 adjacent benign tissues, 4 chronic pancreatitis specimens, 6 normal pancreas tissues and 9 pancreatic cancer cell lines**	**NKRF**	**(Lee et al., 2007)**	5.0	**Hepatocellular carcinomas (Jiang et al., 2008)**
		2	NA	Microarray	Tissue from normal pancreas (n=7), chronic pancreatitis (n=7) and PDAC (n=10); 6 tumor cell lines		(Szafranska et al., 2007)		
hsa-miR-203	Up	5	0.000206	Microarray	Tissue from normal pancreas (n=7), chronic pancreatitis (n=7) and PDAC (n=10); 6 tumor cell lines	IKKB	(Szafranska et al., 2007)	0.43	Hematopoietic tumors(Bueno et al., 2008); Oral Cancer (Kozaki et al., 2008); Esophageal cancer (Feber et al., 2008); Colon adenocarcinoma (Schetter et al., 2008); Ovarian cancer (Iorio et al., 2007); Bladder cancer (Gottardo et al., 2007)
		2.63	NA	Microarray	28 tumors, 15 adjacent benign tissues, 4 chronic pancreatitis specimens, 6 normal pancreas tissues and 9 pancreatic cancer cell lines		(Lee et al., 2007)		
hsa-miR-99a/b	Up	2.08	NA	Microarray	patients with ductal adenocarcinoma of the pancreas (n=65) or chronic pancreatitis (n=42)	NKRF	(Bloomston et al., 2007)	1.00 /1.26	Ovarian Carcinoma (Iorio et al., 2007; Nam et al., 2008); Squamous Cell Carcinoma of Tongue (Wong et al., 2008); Hodgkin lymphoma (Navarro et al., 2008)
		2.44	0.0114	Microarray	Tissue from normal pancreas (n=7), chronic pancreatitis (n=7) and PDAC (n=10);		(Szafranska et al., 2007)		

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					6 tumor cell lines				
		4.45	NA	Microarray	28 tumors, 15 adjacent benign tissues, 4 chronic pancreatitis specimens, 6 normal pancreas tissues and 9 pancreatic cancer cell lines		(Lee et al., 2007)		
		4.35; 5.91	NA	Microarray	12 nontumor pancreas and 44 pancreatic primary tumors		(Roldo et al., 2006)		
hsa-miR-196	Up	75% of tumors high expression of *miR-196a-2* was found to predict poor survival.		Microarray	patients with ductal adenocarcinoma of the pancreas (n=65) or chronic pancreatitis (n=42)	IKBIA	(Bloomston et al., 2007)	1.31	Development related (Hornstein et al., 2005)
		14	0.00001	Microarray; qRT-PCR	Tissue from normal pancreas (n=7), chronic pancreatitis (n=7) and PDAC (n=10); 6 tumor cell lines		(Szafranska et al., 2007)		
		6.76	NA	Microarray	28 tumors, 15 adjacent benign tissues, 4 chronic pancreatitis specimens, 6 normal pancreas tissues and 9 pancreatic cancer cell lines		(Lee et al., 2007)		
hsa-miR-199a	Up	2.46	NA	Microarray	patients with ductal adenocarcinoma of the pancreas (n=65) or chronic pancreatitis (n=42)	IKKB	(Bloomston et al., 2007)	1.4	Ovarian Carcinoma (Iorio et al., 2007; Nam et al., 2008; Yang et al., 2008); Oral Cancer (Kozaki et al., 2008); Hepatocellular carcinomas (Jiang et al., 2008); Hodgkin lymphoma (Navarro et al., 2008); Colorectal Cancer (Bandres et al., 2006)
		2.72	0.000263	Microarray	Tissue from normal pancreas (n=7), chronic pancreatitis (n=7) and PDAC (n=10); 6 tumor cell lines		(Szafranska et al., 2007)		
hsa-miR-100	Up	2.49	NA	Microarray	patients with ductal adenocarcinoma of the pancreas (n=65) or	NKRF	(Bloomston et al., 2007)	0.52	Ovarian Carcinoma (Iorio et al., 2007; Nam et al., 2008; Yang et al., 2008); Squamous Cell Carcinoma of Tongue

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					chronic pancreatitis (n=42)				(Wong et al., 2008); Hepatocellular carcinoma (Varnholt et al., 2008)
		36.9	0.000004	Microarray; real-time quantitative PCR assay; Northern blotting	28 tumors, 15 adjacent benign tissues, 4 chronic pancreatitis specimens, 6 normal pancreas tissues and 9 pancreatic cancer cell lines		(Lee et al., 2007)		
		3.25	0.00223	Microarray	Tissue from normal pancreas (n=7), chronic pancreatitis (n=7) and PDAC (n=10); 6 tumor cell lines		(Szafranska et al., 2007)		
		9.71	NA	Microarray	12 nontumor pancreas and 44 pancreatic primary tumors		(Roldo et al., 2006)		
hsa-miR-146a	Up	2.44	NA	Microarray	patients with ductal adenocarcinoma of the pancreas (n=65) or chronic pancreatitis (n=42)	TRAF6 & IRAK1	(Bloomston et al., 2007)	0.48	Oral Cancer (Kozaki et al., 2008); Prostate cancer (Lin et al., 2008); Breast cancer (Bhaumik et al., 2008);
		4.29	NA	Microarray	12 nontumor pancreas and 44 pancreatic primary tumors		(Roldo et al., 2006)		
		8	0.000012	Microarray	Tissue from normal pancreas (n=7), chronic pancreatitis (n=7) and PDAC (n=10); 6 tumor cell lines		(Szafranska et al., 2007)		
hsa-miR-181c	Up	2.36	NA	Microarray	patients with ductal adenocarcinoma of the pancreas (n=65) or chronic pancreatitis (n=42)	NKRF	(Bloomston et al., 2007)	1.45	Squamous Cell Carcinoma of Tongue (Wong et al., 2008); Glioblastoma (Ciafre et al., 2005); Leukemia (Yu et al., 2006); Brain tumor (Liu et al., 2007); Hodgkin lymphoma (Navarro et al., 2008)
		18.6	0.000831	Microarray; real-time quantitative PCR assay	28 tumors, 15 adjacent benign tissues, 4 chronic pancreatitis specimens, 6 normal pancreas tissues and 9 pancreatic cancer cell lines		(Lee et al., 2007)		
		2.38	NA	Microarray	12 nontumor pancreas and 44 pancreatic primary tumors		(Roldo et al., 2006)		

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hsa-miR-200c	Up	3.08	NA	Microarray	12 nontumor pancreas and 44 pancreatic primary tumors	IKKB	(Roldo et al., 2006)	0.44	Oral Cancer (Kozaki et al., 2008); Ovarian cancer (Iorio et al., 2007; Nam et al., 2008); Hepatocellular tumors (Ladeiro et al., 2008); Colorectal Cancer (Bandres et al., 2006; Xi et al., 2006)
hsa-miR-34	Down	2.38	NA	Microarray	12 nontumor pancreas and 44 pancreatic primary tumors	IKBIA	(Roldo et al., 2006)	1.3	Oral Cancer (Kozaki et al., 2008); Squamous Cell Carcinoma of Tongue (Wong et al., 2008); Hodgkin lymphoma (Navarro et al., 2008)
		>2	NA	northern blotting	Two nontransformed pancreatic ductal epithelial cell lines (HPNE and HPDE) as well as 15 pancreatic cancer cell lines		(Chang et al., 2007)		
hsa-miR-96	Down	1.77	NA	Microarray	patients with ductal adenocarcinoma of the pancreas (n=65) or chronic pancreatitis (n=42)	IKKB	(Bloomston et al., 2007)	0.33	Oral Cancer (Kozaki et al., 2008); Hepatocellular tumors (Ladeiro et al., 2008); Hodgkin lymphoma (Navarro et al., 2008); Colorectal Cancer (Bandres et al., 2006)
		5	0.000009	Microarray	Tissue from normal pancreas (n=7), chronic pancreatitis (n=7) and PDAC (n=10); 6 tumor cell lines		(Szafranska et al., 2007)		
		6.7	NA	Microarray;	28 tumors, 15 adjacent benign tissues, 4 chronic pancreatitis specimens, 6 normal pancreas tissues and 9 pancreatic cancer cell lines		(Lee et al., 2007)		

NA: Not Available

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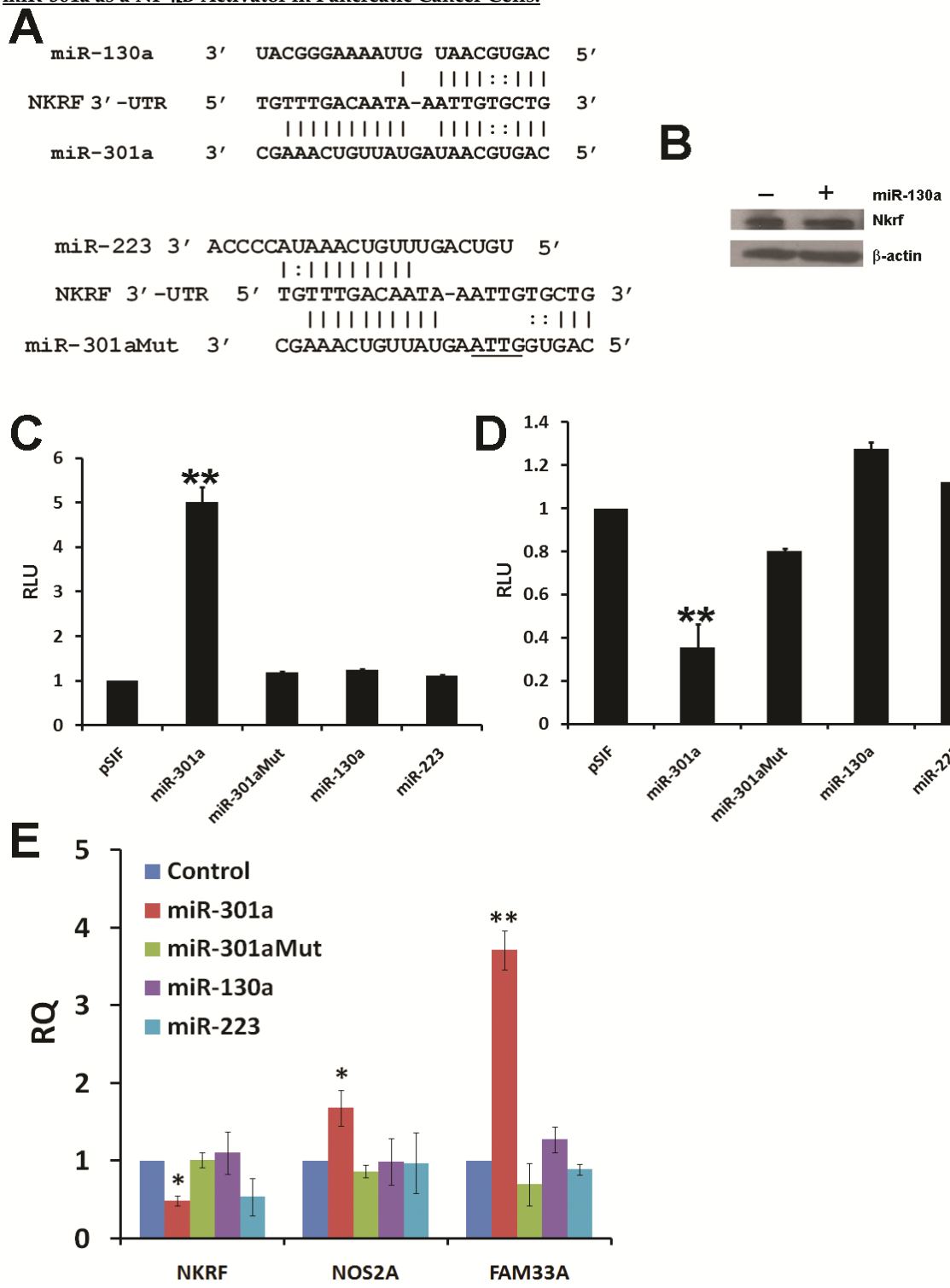

Figure S2 The importance of compensatory 3′ pairing and the seed sequence for Nkrf: miR-301a interaction. (A) miR-130a and miR-301a differ in their 3′-parts but have the same seed sequences, while miR-223 and miR-301Mut have 10 base pairs but do not have a minimal of 6 base pairs in the seed sequences. (B) 293T cells with the expression construct for miR-130a (+) or the parental vector pSIF (-). 48 hrs post-transfection, proteins were extracted and Western blotting was analyzed. (C) The first assay to demonstrate that miR-301a upregulates NF-kB-dependent reporter expression. (D) The second assay to demonstrate miR-301a down-regulate NKRF 3′-UTR-dependent reporter expression. (E) qPCR to demonstrate that miR-301a but not its variants down-regulates NKRF mRNA levels and upregulates NOS2A and FAM33A. *, P value ≤0.05; **, P value ≤0.01.

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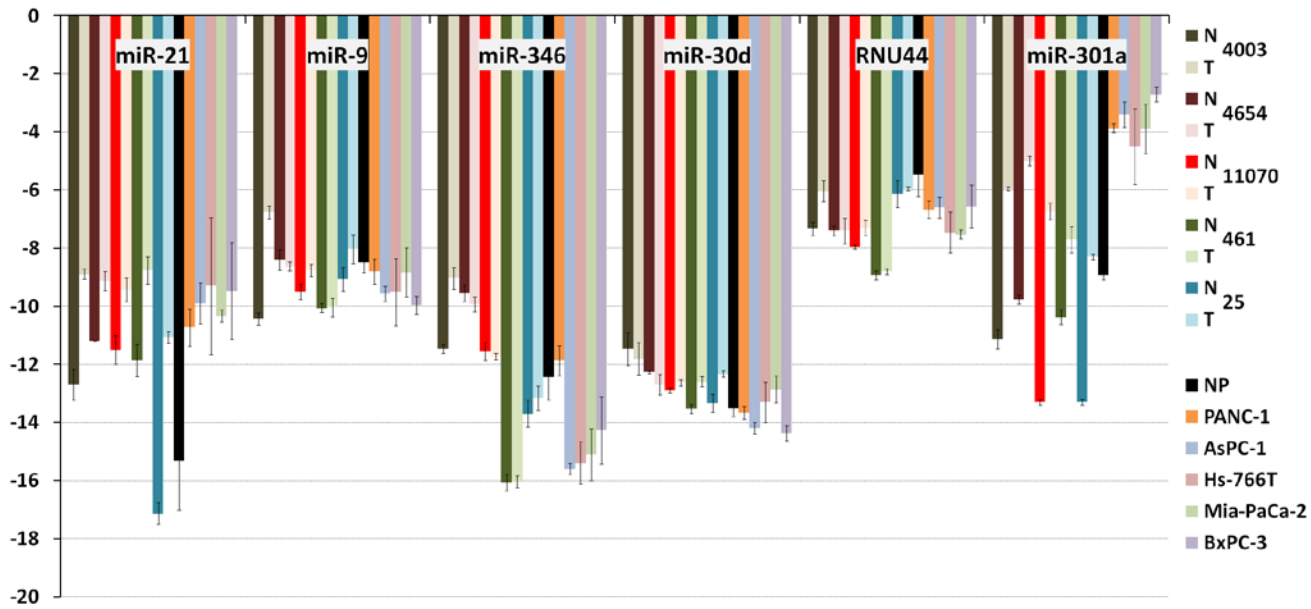
A



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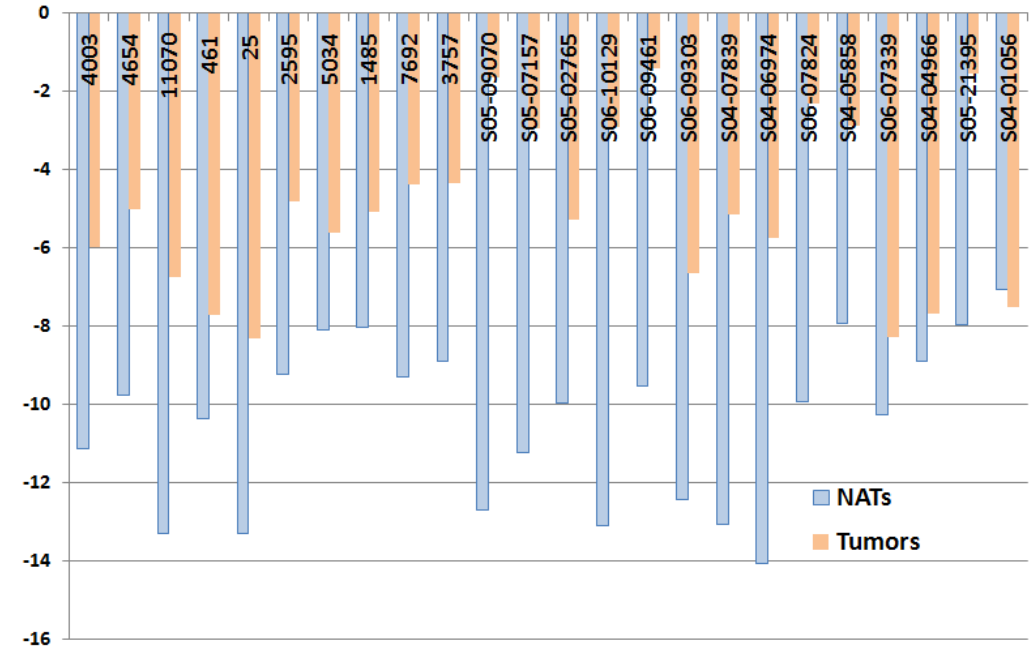

Figure S3 qPCR of miR-301a in pancreatic cancer tissues and cell lines. (A) was used to determine whether U6, U24, or other miRNAs can be used as a reference gene and we found U6, U24, and miR-30d varied little between NP, NAT, Tumors, and cell lines. In (B), U6 was used as a reference. (A) Taqman® miRNA expression assays of miR-301a, U6 (as a reference), miR-9, miR-346, miR-30d, and RUN44 (U24 sno RNA), and miR-21 (as a positive control) were performed using RNAs from five pancreatic cancer cell lines, normal pancreas (NP, a pool of total RNAs from pancreases of 10 healthy donors, Ambion, Inc.), and 5 pancreatic tumors (T) and paired normal adjacent tissues (N). Y axis denotes ($Ct_{(U6)}$-$Ct_{(target)}$). Except NP, all total RNAs were prepared using the Trizol reagent (Invitrogen). T, pancreatic cancer tissues; N, NATs. (B) Taqman® miRNA expression assays of miR-301a and U6 (as a reference) of 24 pancreatic tumor sections (Tumors) and paired normal adjacent tissues (NATs). Y axis denotes ($Ct_{(U6)}$-$Ct_{(target)}$). Patient specimens with an ID starting with a digit (e.g. "4003") were from University of Louisville and those with "S0" from University of Wisconsin.

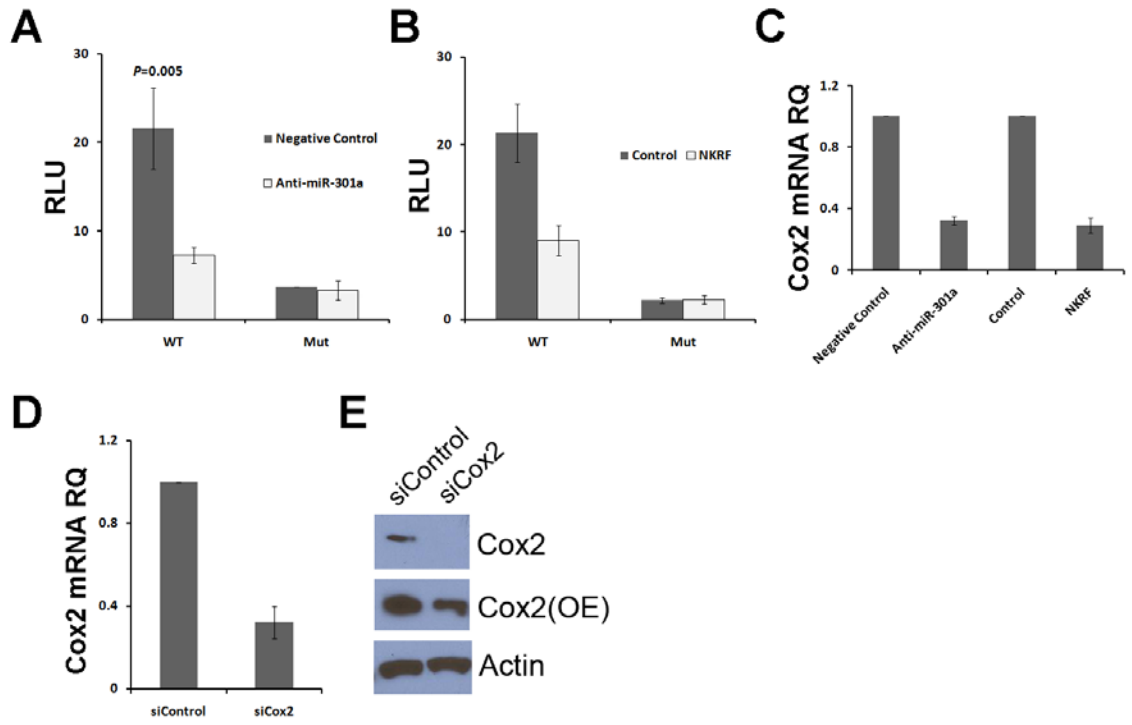

Figure S4 **Regulation of Cox2 by NF-κB, Nkrf, and miR-301a.** PANC-1 cells were transfected with anti-miR-301a (A) or Nkrf expression construct (B), the *luc* reporter construct, and a renilla luciferase construct. Dual-luciferase assay was performed to determine whether the promoter of Cox2 or its NF-κB-specific mutant was regulated by anti-miR-301a/Nkrf or its respective control. Cox2 mRNA levels in PANC-1 cells transfected with anti-miR-301a, Nkrf (C), or a siRNA against Cox2 (siCox2) (D) were determined by qPCR. The protein levels of Cox2 were determined by Western blotting in (E). "OE", over-exposure.

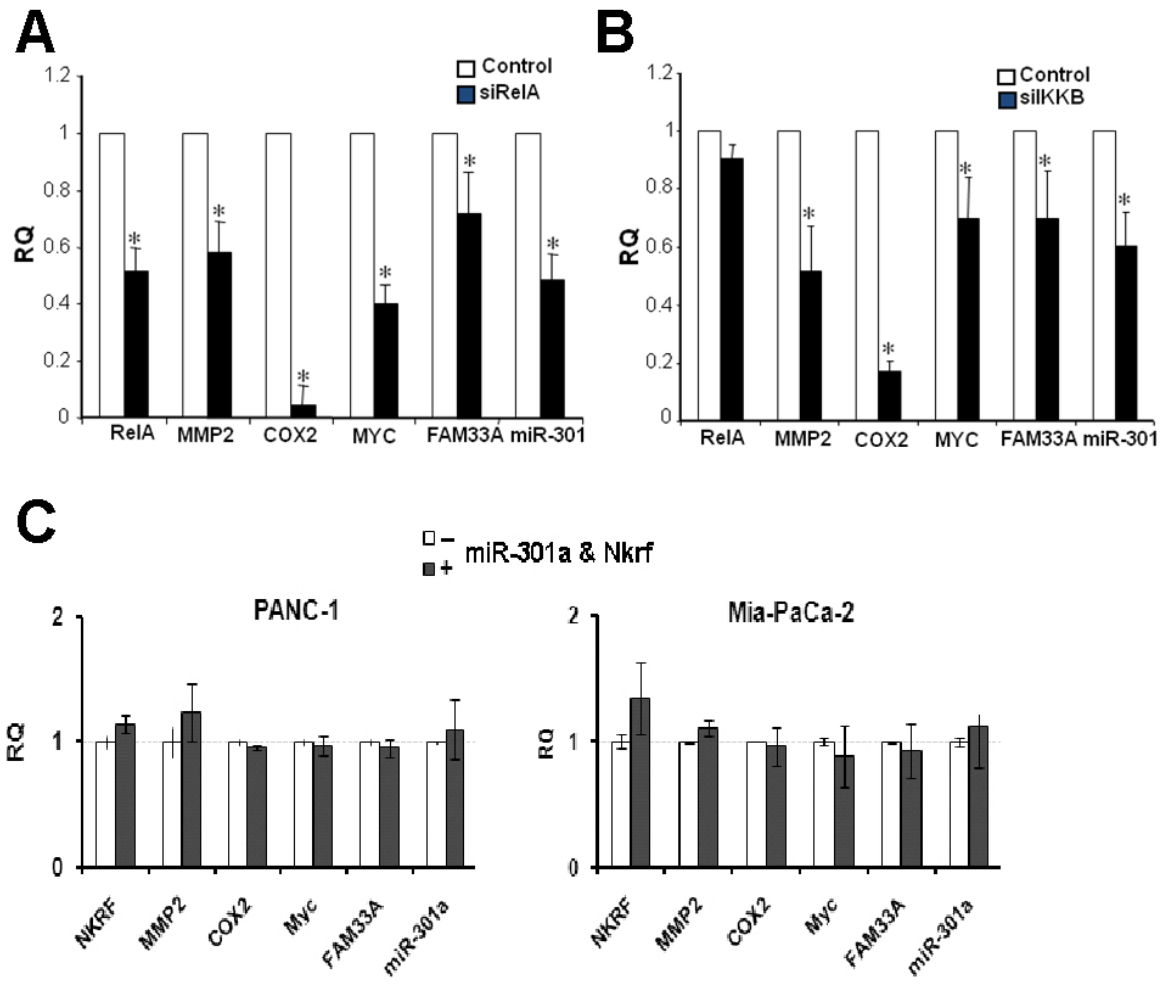

Figure S5 Down-regulation of RelA or IKKβ expression reduces miR-301a expression (A, B) and co-expression of miR-301a and Nkrf (C) in pancreatic cancer cells. (A) siRNA against the human RelA gene (siRelA). PANC-1 cells were transfected with siRNAs or Control (ON-TARGETplus Non-Targeting Pool, Dharmacon Inc., Lafayette, CO). 48 hrs post-transfection, total RNAs were extracted and Taqman® real-time PCR performed. U6 RNA or β-Actin mRNA was used for normalization. RQ indicates the relative quantity of respective mRNA or miRNA. (B) siRNA against the human IKKβ gene (siIKKB). (C) Overexpression of miR-301a and Nkrf simultaneously abolished the regulation of NF-κB target genes in pancreatic cancer cells by Nkrf overexpression alone. PANC-1 and Mia-PaCa-2 cells were co-transfected with either pSIF and pCMV-SPORT6 (1:1 ratio) or pSIF-miR-301a and pCMV-SPORT6-Nkrf (1:1 ratio). 48 hrs post-infection, total RNAs were extracted and qRT-PCR performed with β-actin (for protein-coding genes) and U6 snRNA (for miR-301a) as references. There are no statistical differences in expression levels of these NF-κB target genes. *, $P \leq 0.05$ with n=3.

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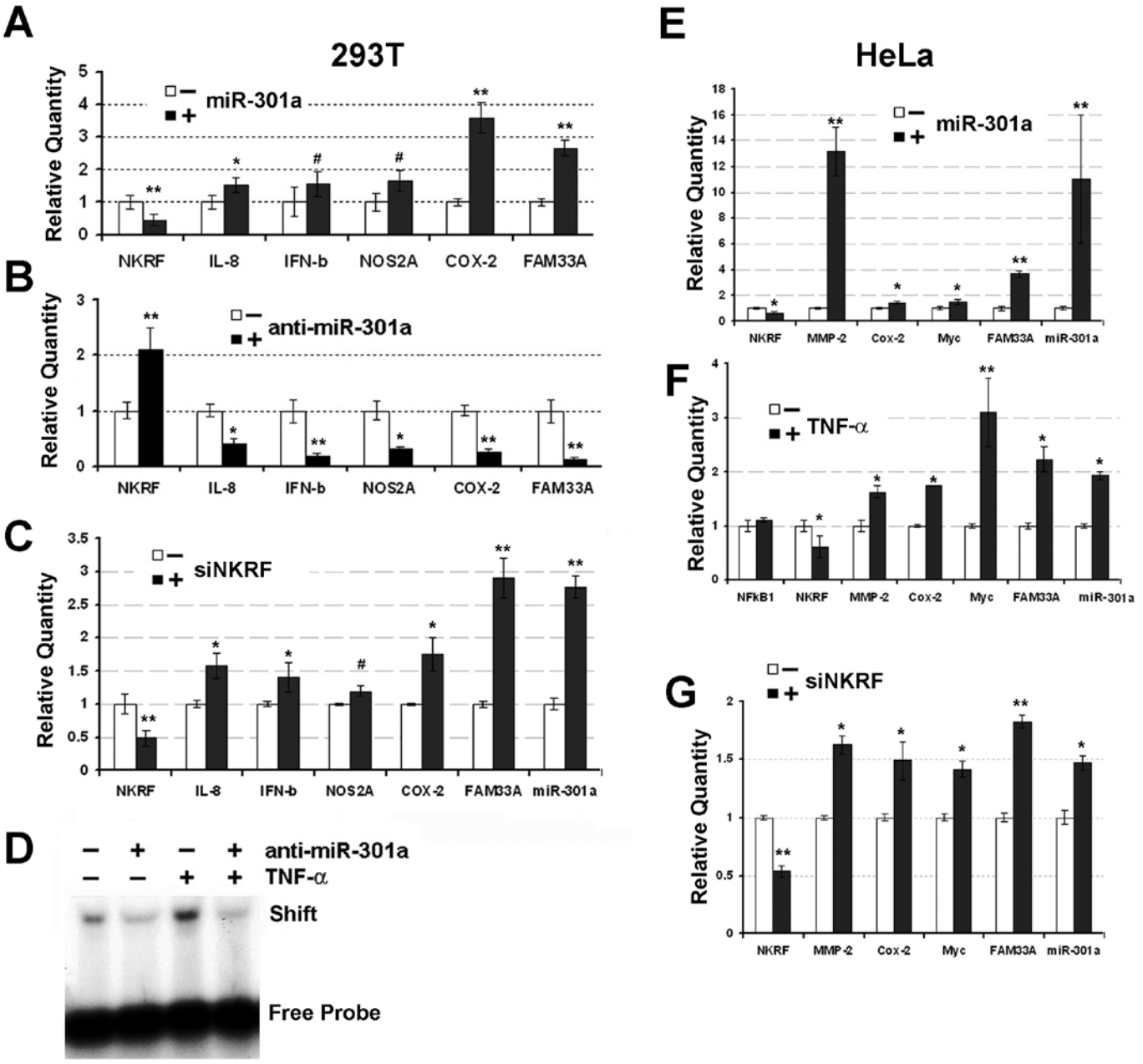

Figure S6 Testing miR-301a-mediated NF-κB activation in 293T and HeLa cells.
(A) Overexpresssion of miR-301a down-regulated Nkrf expression and promoted the expression of NF-κB target genes including FAM33A in 293T cells. (B) Inhibiting miR-301a expression up-regulated Nkrf expression and reduced the expression of NF-κB target genes in 293T cells. mRNA amount was normalized to that of Negative control #1 (Ambion), which was set to 1.0. (C) Down-regulation of NKRF expression by siNkrf increased the expression of NF-κB target genes including FAM33A and miR-301a in 293T cells. (D) Inhibiting miR-301a expression by anti-miR-301a reduced both basal and induced NF-κB activation in 293T cells as determined by EMSA. (E), (F), and (G) are similar to (A), (B), and (C) but in HeLa cells. "Relative Quantity" in (A-C, E-G) is the relative expression level of genes determined by qPCR when referenced by that of β-actin or U6 snRNA; #, P≤ 0.05; *, P≤ 0.01; **, P≤0.001 with n=3-6 compared with respective controls.

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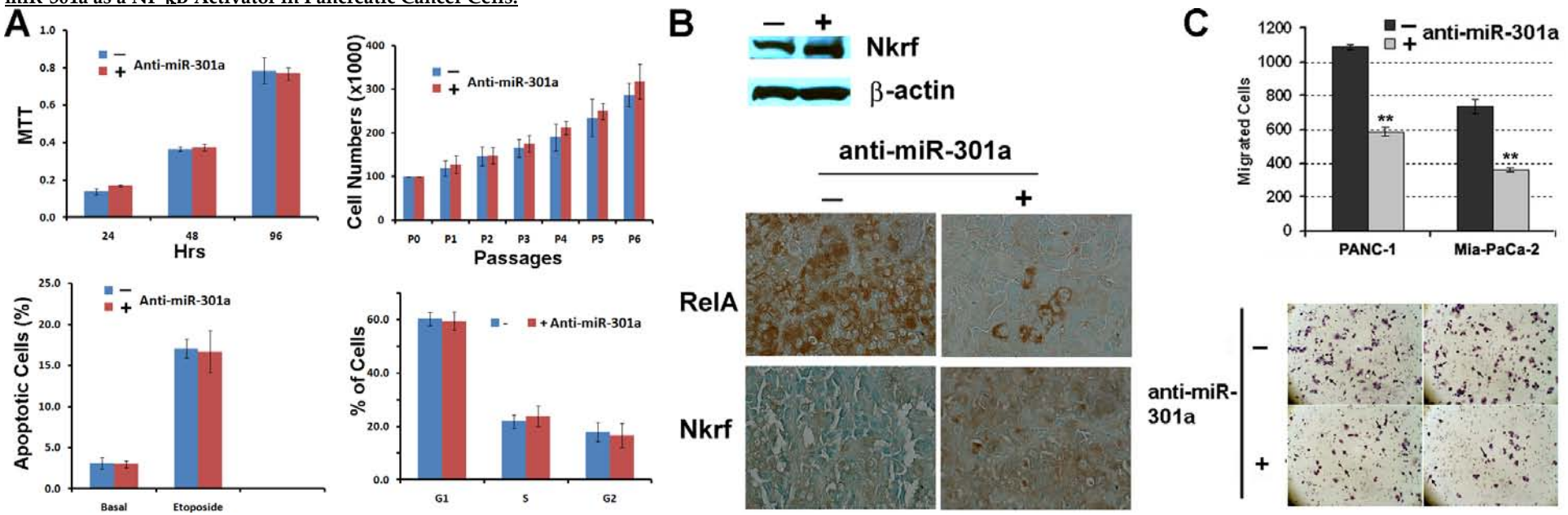
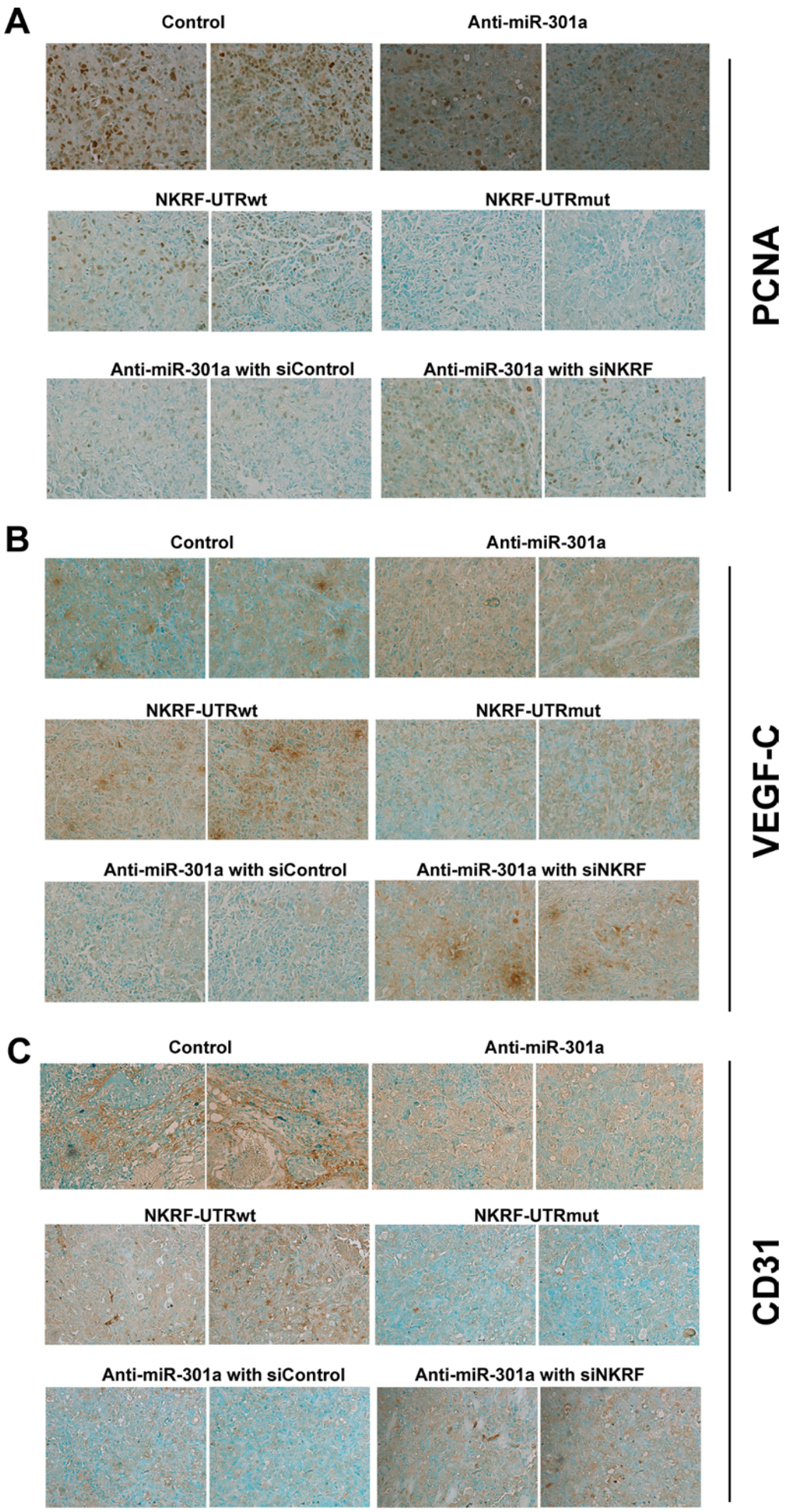


Figure S7 TuD:Anti-miR-301a The TuD:anti-miR-301a expression cassette (**gacggcgcuaggaucaucaac** gcuuugacaauacuauugcacug **caa guauucug guca cagaauac aac** gcuuugacaauacuauugcacug **caagaugauccuagcgccguc (bold: antisense to miR-301a; underlined: stem structure)** was inserted downstream of the H1 Promoter of pSIF-H1-copGFP (System Biosciences Inc.). PANC-1 and Mia-PaCa-2 cells were transduced with TuD:anti-miR-301a or the parental vector. 4 days post-infection, cells were sorted and GFP-positive cells were expanded for Western blotting, cellular assays, and xenografts. **(A) miR-301a inhibition does not affect cell proliferation, apoptosis, and cell cycle of PANC-1 cells.** MTT assay: 3,000 PANC-1 cells with the parental vector or Anti-miR-301a (Fig. 7A) were seeded into 96-well plates; After 24, 48, or 96 hrs, MTT assays were performed. Cell Counting: Long-term cell proliferation using a cell counter (Countess®, Invitrogen). 100,000 cells were seeded in 6-well plates and cells were split every week for 6 weeks with one-third of the total cells were counted. Apoptosis Assay: 100,000 cells were seeded in 6-well plates and 24 hrs later treated with or without etoposide for 30min. Cell Cylce: cell cycle analysis was performed using flow cytometry. N=4 in each experiments. **(B) Inhibiting miR-301a increases Nkrf expression and reduces RelA staining.** Upper Panel: Western blotting analyses of Nkrf expression levels in PANC-1 cells with TuD:anti-miR-301a. Bottom Panel: IHC staining of RelA and Nkrf in tumor sections from PANC-1 mouse xenografts with TuD:anti-miR-301a. **(C) Cell migration assay of PANC-1 and Mia-PaCa-2 cells with miR-301a inhibition.** Upper Panel: The bar graph summarizes the number of migrated cells counted in 24-well plates ± s.d. (**, P≤0.01 with n=6). Bottom Panel: Representative pictures taken at × 20 magnification (arrows pointing to migrated cells).



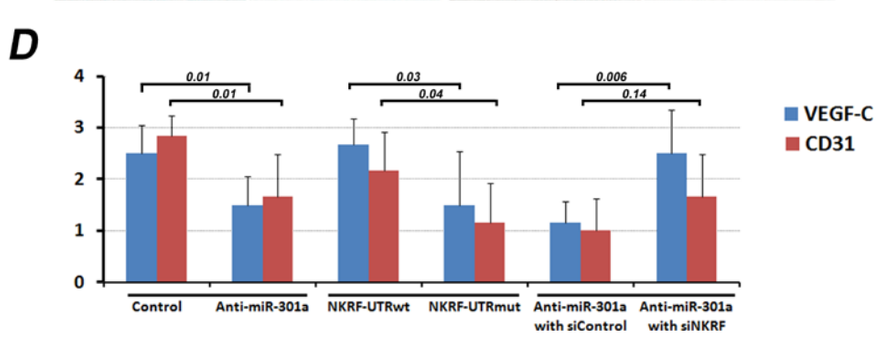


Figure S8 PCNA, VEGF-C, and CD31 staining of xenograft tumor sections. Tissues sections from six groups of xenografts were stained with antibodies against (A) PCNA, (B) VEGF-C, and (C) CD31. These are PANC-1 cells with a Control or TuD:Anti-miR-301a; PANC-1 cells with NKRF-UTRwt or NKRF-UTRmut; and PANC-1 expressing TuD:Anti-miR-301a with siControl or siNKRF. Rabbit antibodies against mouse CD31 (sc-28188, Santa Cruz), human PCNA (sc-7907, Santa Cruz), or human VEGF-C (ab9546,Abcam) were used. (D) Histological scores are provided for VEGF-C and CD31 staining (n=6; two sections from three tumors from each group). P values are listed.