Supporting Information

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SI Text

Generation of microRNA 21 (miR-21) knockout mice. The gene targeting of miR-21 in mice was performed by inGenious Targeting Laboratory, Inc. An approximately 7.49-kb region used to construct the targeting vector was first subcloned from a positively identified C57BL/6 (RPCI-23: 49B19) BAC clone using a homologous recombination-based technique. The region was designed such that the short homology arm (SA) extends 2.0 kb 3′ to *miR-21*. The long homology arm is located on the 5′ side of miR-21 and is approximately 5.39 kb long. The *NEO* cassette replaces 93bp of precursors to miR-21 (pre-miR-21). The targeting vector was confirmed by restriction analysis after each modification step and by DNA sequencing. The BAC was subcloned into an approximately 2.4-kb (pSP72, Promega) backbone vector containing an ampicillin selection cassette for retransformation of the construct prior to electroporation. A pGK-gb2 loxP/FRT-flanked neomycin cassette was inserted into the gene as described in Fig. 1*B*. The total size of the targeting construct (including vector backbone) is approximately 11.49 kb.

Ten micrograms of the targeting vector was linearized by NotI and then transfected by electroporation of iTL1 BA1 (C57BL/6 × 129/SvEv) hybrid embryonic stem cells. After selection with G418 antibiotic, surviving clones were expanded for PCR analysis to identify recombinant ES clones. Screening primers A1 and A2 were designed downstream of the SA outside the 3′ region used to generate the targeting construct. PCR reactions using A1 or A2 with the F3 primer (located within the Neo cassette; Table S1) amplify 2.24- and 2.38-kb fragments, respectively. The control PCR reaction was performed using the internal targeting vector primers AT1 and AT2, which are located at the 3′ and 5′ ends, respectively, of the SA. This amplified a product 1.89 kb in size. Individual clones from positive pooled samples were screened using the A2 and F3 primers. Positive recombinant clones were identified by a 2.38-kb PCR fragment. PCR reaction controls were performed with screening and internal primers. The negative control was indicated by a (–) in Fig. 1*E*. Positive SA PCR clones were sequenced for integration using the OUT1 primer. Secondary confirmation of positive clones identified by PCR was performed by Southern Blotting analysis using a probe generated by PCR with primers PB1 and PB2. DNA from C57Bl/6 (B6), 129/SvEv (129), and BA1 (C57Bl/6 × 129/SvEv) (Hybrid) mouse strains were used as wild-type controls (Fig. 1*D*). Targeted iTL BA1 hybrid embryonic stem cells were microinjected into C57BL/6 blastocysts. Resulting chimeras with a high percentage agouti coat color were mated to wild-type C57BL/6 mice to generate F1 heterozygous offspring with germline transmission. Tail DNA was analyzed from pups with agouti or black coat color. For following genotyping, primers P1, P2, and P3 were used.

Mouse Primary Cell Preparation. Mouse embryonic fibroblasts (MEFs) were prepared according to an established protocol (1). For keratinocytes, 2- to 3-d-old mice (three per group) were euthanized by exposure to ice for 1 h. Dead mice were washed with water, 70% ethanol, and 1×PBS. Mice were skinned using sterile forceps and skin tissues were incubated in 1 × antibiotic-antimycotic solutions for 20 min and then washed with 1×PBS. Skins from three mice were pooled in one 10-cm dish and flattened with the dermis facing down. Skins were incubated overnight (15 h) at 4 °C in 12 mL of 0.25% trypsin solution. Forceps were used to separate the epidermis from the dermis. Flattened epidermis was then put into 1×PBS in a new petri dish and cells were scraped using a slide before seeded onto collagen-coated dishes with Defined Keratinocytes–Serum-Free Medium (Invitrogen Corp.) and incubated at 37 °C with 5% CO_2. Medium was changed daily and cells were used 4–10 d after plating (2).

RNA Preparation for Microarray and qPCR. Total RNAs from keratinocytes were extracted using Trizol (Invitrogen) and run on the Agilent Bioanalyzer 2100 to ensure A260/A280 ratio in the range of 1.8–2.2, rRNA ratio (28S/18S) >0.9 and RNA Integrity Numbers value >8.0. Aliquots of RNAs were stored at −80 °C. At least 50 μg of total RNAs were sent to Beckman Coulter Genomics, Inc., a member of the Microarray Quality Control (MAQC) Consortium (3). A two-color (Cy3 and Cy5) gene expression microarray analysis was performed. Briefly, Beckman Coulter Genomics converted total RNAs into cDNA before an in vitro T7 transcription to obtain labeled cRNA with nucleotides coupled to a fluorescent dye (either Cy3 or Cy5) using the Agilent Low RNA Input Linear Amplification Kit Plus, Two-Color. Equal amounts of Cy3 and Cy5-labeled cRNA from two different samples were hybridized to the Agilent Whole Mouse Genome 44 K Oligonucleotide Microarray for 17 h at 65 °C. The hybridized microarrays were washed using the manufacturer's recommended conditions and scanned using an Agilent G2565BA scanner. Data were extracted from the scanned image using Agilent Technologies' Feature Extraction software version 8.5 (FE8.5). Duplicates were run and data were finally represented as the average. qPCR was run using Taqman® gene expression assays in a 96-well format with all reagents listed in SI Table S2 (Applied Biosystems, Inc.).

Histology, Immunohistochemistry, and TUNEL (TdT-Mediated dUTP Nick End). Skin and skin papillomas were collected and fixed overnight in 10% neutral-buffered formalin. Fixed tissue was paraffin embedded, sectioned (5 μm), and stained with hematoxylin and eosin (H&E) for morphological analyses. For immunohistochemistry, Ki-67 primary antibody (Abcam) was used to assess epithelial proliferation. Deparaffinized and rehydrated sections were boiled in Na-citrate buffer (10 mM, pH6.0) for 10 min for antigen retrieval. Tissue sections labeled with Ki67 were developed by UltraVision Detection System Anti-Rabbit (Thermo Fisher Scientific Inc.). About 800–1,000 cells were counted in each group to calculate the percentage of positive Ki-67 staining cells. For TUNEL assay, deparaffinized sections or fixed keratinocytes were incubated with 20 μg/mL Protease K for 15 min at room temperature, washed with PBS, and then incubated with TUNEL reaction mixture (Roche Applied Science) for 60 min at 37 °C in humidified atmosphere in the dark. Tissue sections were directly visualized using a fluorescence microscope. For keratinocytes, treated cells were incubated with converter-POD (peroxidase with antifluorescein antibody) and subsequently with the DAB (3,3′-diaminodbenzidine) substrate. All images were acquired using an Olympus IX51 microscope and processed using the cellSens Dimension software (Olympus America Corp.).

Western Blotting. Cells were lysed with radio-immunoprecipitation assay buffer (Cell Signaling Technology) supplemented with protease inhibitor (EMD Chemicals) and phosphatase inhibitor (Thermo Scientific). Soluble proteins were subjected to SDS-PAGE and then transferred to PVDF membranes. The following antibodies were used: anti-phospho-Akt (Ser473, 1:1,000), anti-phospho-SAPK/JNK (Thr183/Thr185, 1:1,000), anti-phospho-p44/42 MAPK (ERK) (Thr202/Tyr204, 1:1,000), anti-Pdcd4

(D29C6, 1:1,000), and anti-Pten (D4.3, 1:1,000), all of which were obtained from Cell Signaling. Anti-Spry1 (RR-15, 1:1,000) was obtained from Santa Cruz Biotechnolgy, Inc. and anti-Spry2 (ab50317, 1:2,400) was obtained from Abcam. Monoclonal antibody against β-actin was obtained from Sigma-Aldrich.

FACS Analysis of Apoptosis. Keratinocytes were plated in six well plates with the density of 2×10^5 per well and cultured in medium until 80% confluence. Cells were transfected with anti-miR-21 inhibitors, pre-miR-21 precursors, or the control reagents (Ambion) using Lipofectamine LTX (Invitrogen) according to the manufacturers' protocols. After 48 h, cells were collected and incubated with Alexa Fluor® 488 Annexin V and PI by using Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen). Cells were analyzed on flow cytometer (BD LSR II, BD Biosciences), and data were processed with the FACSDiva software (BD Biosciences).

Statistical Analysis. Average data were expressed as mean and standard deviation. Statistical analysis was carried out using the SPSS16.0 software (SPSS Inc.). The one-way ANOVA test was performed and statistical significance was set at $P \leq 0.05$. Differences in tumor multiplicity were analyzed using nonparametric Mann–Whitney U test (i.e., Wilcoxon rank-sum test). Fisher's exact test was used to compare tumor incidence between the two groups (4). For microarray data, raw data signals were preprocessed, normalized (linear scaling and Lowess normalization), and filtered according to Agilent's protocol and MAQC principles and guidelines, all of which were performed by Beckman Coulter Genomics (3). Differentially expressed genes were identified after dye swap results were averaged. A one sample t test of $\log_2$ (Sample B/SampleA) ratio of triplicates that differ from 0 was performed. Genes that are differentially expressed with P values of ≤ 0.05 were candidates for further analyses (3). For qPCR, three independent experiments were performed using *Gapdh* as an internal reference; a gene is considered up-regulated or down-regulated only when all three runs show ≥20% up-regulation or ≥20% down-regulation compared to the control (*miR*-21$^{+/+}$ in Fig. 5 *A* and *B*; *miR*-21$^{-/-}$ transfected with pre-Contl in Fig. 5*C*).

1. Xu J (2005) *Preparation, Culture, and Immortalization of Mouse Embryonic Fibroblasts* (John Wiley, Hoboken, NJ). Current Protocols in Molecular Biology. UNIT 28.1; 10.1002/0471142727.mb2801s70.
2. Munoz P, Blanco R, Flores JM, Blasco MA (2005) XPF nuclease-dependent telomere loss and increased DNA damage in mice overexpressing TRF2 result in premature aging and cancer. *Nat Genet* 37:1063–1071.
3. Patterson TA, et al. (2006) Performance comparison of one-color and two-color platforms within the Microarray Quality Control (MAQC) project. *Nat Biotech* 24:1140–1150.
4. Abel EL, Angel JM, Kiguchi K, DiGiovanni J (2009) Multi-stage chemical carcinogenesis in mouse skin: Fundamentals and applications. *Nat Protoc* 4:1350–1362.

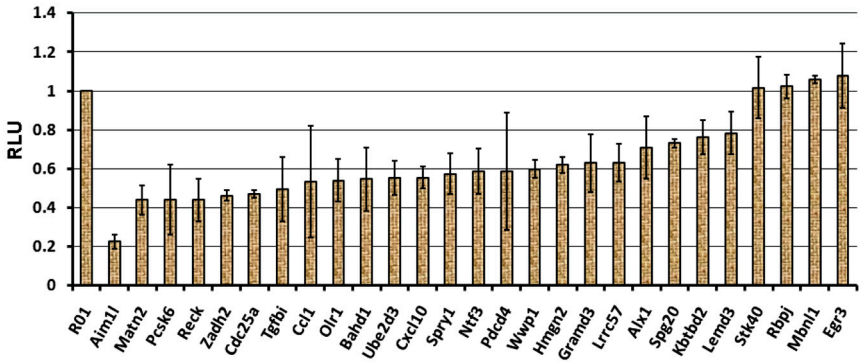

Fig. S1. The UTR reporter assay. UTR reporter constructs, in which a firefly luciferase (*luc*) is placed upstream, were obtained from SwitchGear Genomics. For each construct, two transfection experiments (each with triplicates) were performed in 293T cells in 96-well plates: one with miR-21 expression plasmid and pRL-TK (Promega), the other (control) with parental vector and pRL-TK. The Renilla luciferase (*Rluc*) from pRL-TK was used to normalize transfection efficiency and total protein synthesis. The Y axis denotes the relative luminescent units (RLU, luc/Rluc) normalized to the control (luc/Rluc of R01). "R01" (a randomized genomic fragment, Switchgear Genomics) represents a negative control for UTRs. All UTRs are from human and have one or more miR-21 targeting sites according to TargetScan.

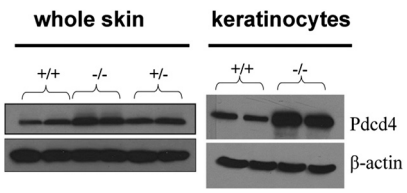

Fig. S2. The expression of Pdcd4 determined by Western blotting using lysates from whole skin or from epidermis. Two animals of each genotype were used.

Table S1. Primers used for *miR-21* gene targeting

Primers	Sequences (5′ → 3′)
A1	GCT GGA ATG TAG CCT CAG CAC
A2	CTG ACA GGC AGA AGC TGT TCT TG
AT1	CTG GTG CAC TTG CTA CAT TAT GAC G
AT2	GGT CCT TAT GTC TTG TGA GAC TTG G
OUT1	AGC TAA ACC CGG GCT TCC ACA T
F3	GCA TAA GCT TGG ATC CGT TCT TCG GAC
PB1	GTC CAT TCG TCT GTC CTT AGT GCT C
PB2	GGC TTG CGT CAT TTT GTT CTC AGA TC
P1	TTG CTT TAA ACC CTG CCT GAG CAC
P2	AAG GGC TCC AAG TCT CAC AAG ACA
P3	ACT TCC ATT TGT CAC GTC CTG CAC

Table S2. Taqman® reagents (from Applied Biosystems Inc.) used for qPCR

Gene	Taqman® reagent	Gene	Taqman® reagent
Gapdh	Mm99999915_g1	*Pom121*	Mm00455744_m1
THRB	Mm00437044_m1	*Arhgef12*	Mm00804321_m1
WWP1	Mm01210682_m1	*Bcl11b*	Mm00480516_m1
SOX7	Mm00776876_m1	*CXCL10*	Mm99999072_m1
Ccl1	Mm00441236_m1	*ZADH2*	Mm00524960_s1
GRAMD3	Mm00509320_m1	*MBNL1*	Mm00490954_m1
Spg20	Mm00523212_m1	*STK40*	Mm00512138_m1
CNTFR	Mm00516697_m1	*CDK6*	Mm00438163_m1
Egr3	Mm00516979_m1	*Spry1*	Mm01285700_m1
CDC25A	Mm00483162_m1	*Spry2*	Mm00442344_m1
FNIP1	Mm00620486_m1	*MATN2*	Mm00489626_m1
YOD1	Mm00769424_m1	*Hmgn2*	Mm01705384_g1
KLF3	Mm00627090_m1	*ARHGAP24*	Mm00525303_m1
CD44	Mm01277163_m1	*TGFBI*	Mm00493634_m1
ATPAF1	Mm00619286_g1	*TIMP3*	Mm00441827_m1
DNAJC16	Mm00552376_m1	*ALX1*	Mm00553295_m1
NFAT5	Mm00467257_m1	*Ntf3*	Mm00435413_s1
Klhdc5	Mm00723437_m1	*OLR1*	Mm00454586_m1
Thbd	Mm00437014_s1		
PCSK6	Mm01319135_m1		
Dcun1d3	Mm00557801_m1		
LEMD3	Mm03024122_m1		
LRRC57	Mm00712359_m1		
Pdzd2	Mm01308962_m1		
UBE2D3	Mm00787086_s1		
Notch2	Mm00803077_m1		
Unkl	Mm00840627_m1		
Terf2	Mm01253555_m1		
RBPJ	Mm00524960_s1		
RMND5A	Mm00662449_m1		
RECK	Mm00443829_m1		
Pcbp1	Mm00478712_s1		
KBTBD2	Mm00524223_s1		
GPR64	Mm00724545_m1		
**DUSP8*	Mm00456230_m1		
**PRPF4B*	Mm00443401_m1		
**KRIT1*	Mm00459502_m1		
Rhob	Mm00455902_s1		