Repression of the miR-143/145 cluster by oncogenic Ras initiates a tumor-promoting feed-forward pathway

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Although activating mutations in *RAS* oncogenes are known to result in aberrant signaling through multiple pathways, the role of microRNAs (miRNAs) in the Ras oncogenic program remains poorly characterized. Here we demonstrate that Ras activation leads to repression of the miR-143/145 cluster in cells of human, murine, and zebrafish origin. Loss of miR-143/145 expression is observed frequently in *KRAS* mutant pancreatic cancers, and restoration of these miRNAs abrogates tumorigenesis. miR-143/145 down-regulation requires the Ras-responsive element-binding protein (RREB1), which represses the miR-143/145 promoter. Additionally, *KRAS* and *RREB1* are targets of miR-143/miR-145, revealing a feed-forward mechanism that potentiates Ras signaling.

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Activating mutations in the *RAS* family of proto-oncogenes are among the most common in human malignancies and are particularly prominent in pancreatic ductal adenocarcinoma (PDAC), where mutations in *KRAS2* (hereafter referred to as *KRAS*) are found in 90%–95% of cases (Maitra and Hruban 2008). Ras activation stimulates a number of downstream effectors, including the mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) pathways, ultimately leading to enhanced cellular proliferation, survival, and motility (Hingorani and Tuveson 2003). The *KRAS* gene mutation principally encountered in pancreatic cancer and other malignancies is an alteration of codon 12 that prevents GTP hydrolysis, trapping Kras in the constitutively active configuration (Caldas and Kern 1995).

MicroRNAs (miRNAs) are 18- to 24-nucleotide (nt) ssRNAs that cause accelerated turnover and reduced translation of imperfectly complementary target messenger RNAs. Over the last decade, >700 human miRNAs have been identified and implicated in the regulation of a wide range of cellular processes, including differentiation, proliferation, and apoptosis (Ambros 2004). Accordingly, a large body of evidence has established an important role for miRNAs in cancer pathogenesis. Globally abnormal miRNA expression patterns are a ubiquitous feature of human cancers, including PDAC, and specific miRNAs have been shown to act as critical components of key oncogenic and tumor suppressor pathways (Bloomston et al. 2007; Lee et al. 2007; Szafranska et al. 2007; Lotterman et al. 2008; Kent et al. 2009). Moreover, delivery of antineoplastic miRNAs represents a highly effective therapeutic strategy in experimental cancer models (Esquela-Kerscher et al. 2008; Kumar et al. 2008; Kota et al. 2009).

Although both Ras signaling and miRNA activities can profoundly influence cancer cell behavior, the role of miRNAs in Ras-mediated phenotypes is poorly defined. To investigate a possible link between miRNA regulation and Ras-induced transformation, we characterized miRNA expression following expression of a constitutively active $KRAS^{G12D}$ allele in multiple model systems. We show that activated Kras signaling consistently leads to repression of the miR-143/145 cluster, and reduced expression of these miRNAs is necessary to maintain the tumorigenic potential of pancreatic cancer cells. Moreover, we elucidate an evolutionarily conserved regulatory pathway whereby Kras signaling activates Ras-responsive element-binding protein 1 (RREB1), which directly represses the miR-143/145 promoter. Both *KRAS* and *RREB1* transcripts are direct targets of these miRNAs, demonstrating the existence of a feed-forward pathway that potentiates Kras-mediated tumorigenesis.

Results and Discussion

Expression of activated Kras leads to down-regulation of the miR-143/145 cluster in diverse model systems

In order to identify miRNAs regulated by Kras signaling, we used a custom microarray to profile miRNA expression in a nontransformed pancreatic ductal epithelial cell line (HPNE) (KM Lee et al. 2003) stably overexpressing $Kras^{G12D}$ (Feldmann et al. 2008). Six miRNAs exhibited twofold or greater up-regulation (miR-34a, miR-199b, and miR-31) or down-regulation (miR-143, miR-145, and miR-27b) upon expression of $Kras^{G12D}$ (Supplemental Table S1). Of these, four miRNAs (miR-34a, miR-31, miR-143, and miR-145) showed similar expression changes when assessed by Northern blotting (Fig. 1A; data

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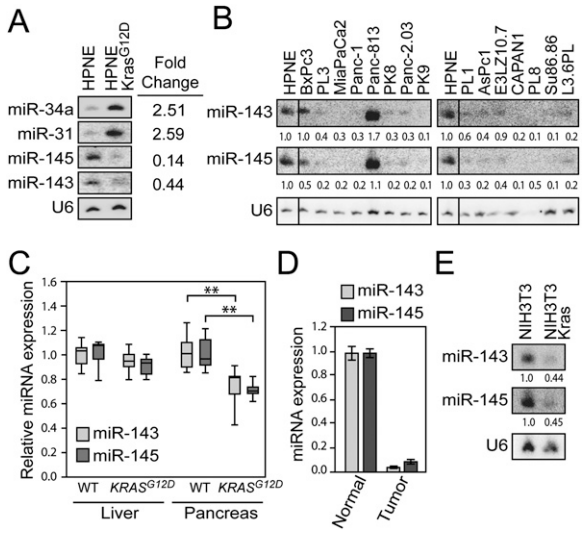

Figure 1. The miR-143/145 cluster is repressed by oncogenic Kras in multiple model systems. (*A*,*B*) Northern blot analysis of miRNA expression in HPNE cells (*A*) or PDAC cell lines (*B*). (*C*) Quantitative PCR (qPCR) analysis of relative miR-143/miR-145 expression in liver and pancreas of postnatal day 0 (P0) mice (n = 7 mice per genotype). Box plots show median (horizontal line), 25th and 75th percentiles (box), and full range of data (whiskers). (**) $P < 0.01$ (two-tailed t-test). (*D*) miR-143/miR-145 expression in normal zebrafish pancreas and pancreatic tumors induced by transgenic $Kras^{G12V}$ expression (Park et al. 2008). (*E*) miR-143/145 expression in NIH3T3 and *v-Kras*-transformed NIH3T3 cells.

not shown). miR-34a has been demonstrated previously to be a direct transcriptional target of p53 and is up-regulated under conditions that trigger oncogene-induced senescence, including expression of activated Ras (He et al. 2007), likely explaining its increased expression in HPNE-$Kras^{G12D}$ cells. miR-31 has been shown previously to be overexpressed in tumor types where activating mutations of *KRAS* are common (Liu et al. 2010). Of particular interest, and the focus of this study, was the Kras-mediated repression of miR-143 and miR-145, two cotranscribed miRNAs located on human chromosome 5q. Significant evidence indicates that these miRNAs possess tumor suppressor activity. Reduced miR-143/145 expression is a common attribute of several tumor types, most notably colorectal carcinoma (Michael et al. 2003; Akao et al. 2007). Moreover, expression of these miRNAs inhibits proliferation and activates apoptosis of cancer cells in vitro and in vivo (Chen et al. 2009; Clape et al. 2009; Sachdeva et al. 2009).

Pancreatic cancer cell lines frequently exhibit low expression of miR-143/145 (Fig. 1B). Of note, one of two examined lines with retained miR-143/145 expression was BxPc3, the only cell line with wild-type *KRAS* in the panel (Sipos et al. 2003). Primary human pancreatic adenocarcinoma cells harboring activating *KRAS* mutations passaged as xenografts in mice (Rubio-Viqueira et al. 2006) also showed strong down-regulation of these miRNAs compared with normal pancreas tissue (Supplemental Fig. S1). Importantly, deletion of the miR-143/145 locus is an infrequent event in pancreatic cancer cells, as revealed by a previously published analysis of genomic copy number variation that included 10 of 15 cell lines in our panel (Calhoun et al. 2006).

To further demonstrate that oncogenic Kras represses miR-143 and miR-145 in vivo, we examined miRNA levels in pancreata from newborn *PDX-1-Cre; LSL-*$KRAS^{G12D}$ mice, which harbor an activating mutation at the endogenous *KRAS* locus that is specifically expressed in pancreas and select other tissues (Hingorani et al. 2003). Repression of miR-143/145 was detectable in bulk pancreas in these animals relative to wild-type littermate controls (Fig. 1C). Moreover, tumors from a zebrafish model of pancreatic cancer induced by expression of oncogenic Kras (Park et al. 2008) exhibited nearly complete loss of expression of miR-143/145 relative to normal zebrafish pancreas (Fig. 1D). Finally, to determine if repression of miR-143/145 by Kras signaling is restricted to pancreas-derived cells, we examined their expression in the immortalized mouse fibroblast cell line NIH3T3 and its oncogenic *v-Kras*-transformed derivative, K:MoLV NIH3T3 (hereafter referred to as NIH3T3-Kras). miR-143 and miR-145 were strongly down-regulated by activated Kras in this setting as well (Fig. 1E). These experiments reveal the existence of an evolutionarily conserved pathway wherein activated Kras signaling leads to repression of the miR-143/145 cluster in diverse cell lineages.

Reduced expression of miR-143 and miR-145 is necessary for Kras-mediated transformation

In order to assess whether down-regulation of miR-143/145 is necessary for cellular transformation induced by oncogenic Kras expression, we used a retroviral construct to express the miRNA cluster at physiologic levels in two PDAC cell lines harboring activating *KRAS* mutations (MiaPaCa2 and Panc-1) and in HPNE-$Kras^{G12D}$ cells (Supplemental Fig. S2A; Supplemental Table S2). Despite normal rates of proliferation in culture (Supplemental Fig. S2B), cells expressing miR-143/145 exhibited greatly reduced anchorage-independent growth (Fig. 2A; Supplemental Fig. S2C). MiaPaCa2 and Panc-1 cells expressing these miRNAs were also unable to form tumors in immunocompromised mice (Fig. 2B; data not shown). These data document that expression of miR-143/145 fully abrogates the transformed phenotype of PDAC cells. Future studies will be necessary to elucidate the underlying mechanisms through which the disparate effects on cellular proliferation versus transformation are elicited.

We next determined whether the observed anti-tumorigenic effects of miR-143/145 expression could be attributed to either of the individual miRNAs in the cluster. miR-143 or miR-145 were expressed individually in HPNE-$Kras^{G12D}$, MiaPaCa2, Panc-1, or an additional PDAC cell line, Panc-2.03, at physiologically relevant levels (Supplemental Fig. S3A; Supplemental Table S2). Again, no effect on proliferation rates was observed (Supplemental Fig. S3B). Independent expression of miR-143 or miR-145 strongly inhibited soft agar colony formation in HPNE-$Kras^{G12D}$, MiaPaCa2, and Panc-2.03 cells but not in Panc-1 cells (Fig. 2C; Supplemental Fig. S3C). Likewise, miR-143 or miR-145 were able to independently block tumor formation by MiaPaCa2 cells (Fig. 2D). These observations indicate that both miRNAs in the miR-143/145 cluster exhibit tumor suppressor activity in pancreatic cancer cells, although in some settings both miRNAs must be coexpressed for the cluster to exert its full anti-tumorigenic potential.

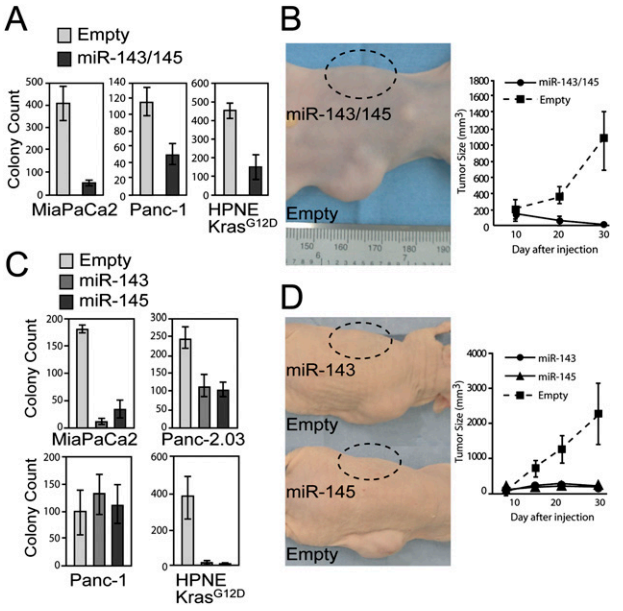

Figure 2. Expression of miR-143/145 inhibits transformation and tumorigenesis in pancreatic cancer cells. (*A*) Anchorage-independent growth of retrovirally infected cell lines (average of three independent experiments shown). Error bars for this and subsequent panels represent standard deviations. (*B*) Tumorigenesis assays with retrovirally infected MiaPaCa2 cells. Images depict a representative animal injected in each flank with cells infected with empty virus or miR-143/145 virus. The graph shows the average tumor volume (n = 5 mice per cell line). (*C*) Anchorage-independent growth of retrovirally infected cell lines expressing either miR-143 or miR-145. (*D*) Tumorigenesis assays with MiaPaCa2 cells infected with empty virus or virus expressing miR-143 or miR-145 (n = 5 mice per cell line).

Kras signaling leads to transcriptional repression of the miR-143/145 cluster in a RREB1-dependent manner

To investigate the mechanisms through which Kras signaling represses expression of the miR-143/145 cluster, we first elucidated the complete structure of the primary transcript (pri-miRNA) that encodes these miRNAs. An NCBI RNA reference sequence (RefSeq) transcript (LOC728264) encompasses the human miR-143/145 cluster. We confirmed the use of the RefSeq transcription start site (TSS) and polyadenylation signal using 5′ and 3′ rapid amplification of cDNA ends (RACE). However, RT–PCR revealed that HPNE cells express two major alternatively spliced transcripts with intron–exon structures distinct from the annotated RefSeq RNA (Fig. 3A). A similar series of experiments in NIH3T3 cells revealed that the genomic organization of the mouse miR-143/145 transcription unit is similar to the human locus, although the positions of a subset of the exons are unique to each species.

Close inspection of the highly conserved miR-143/145 proximal promoter using the University of California at Santa Cruz (UCSC) Genome Browser conserved transcription factor-binding site track revealed the presence of a high-scoring recognition site for RREB1 (Supplemental Fig. S4A), a transcription factor that has emerged from multiple screens as a likely oncogene (Uren et al. 2008; Starr et al. 2009). RREB1 is known to both activate and repress transcription of target genes in response to Ras pathway activity (Thiagalingam et al. 1996; Zhang et al. 1999; Date et al. 2004; Mukhopadhyay et al. 2007). These findings suggest that oncogenic Kras signaling represses transcription of these miRNAs through the direct action of RREB1 at the miR-143/145 promoter.

Consistent with transcriptional repression, pri-miR-143/145 was down-regulated in HPNE and NIH3T3 cells expressing Kras^{G12D} (Fig. 3B). Moreover, acute knockdown of *KRAS* expression using siRNA partially reverses repression of pri-miR-143/145 in cell lines with activating mutations in *KRAS* (Panc-2.03, PL3, and MiaPaCa2) but not in cell lines with wild-type *KRAS* (HPNE and BxPc3) (Fig. 3C; Supplemental Fig. S4B). Expression of Kras^{G12D} in HPNE and NIH3T3 cells also induced *RREB1* expression (Fig. 3D). *RREB1* knockdown in HPNE-Kras^{G12D} cells reversed the repression of pri-miR-143/145 and the mature miRNAs and inhibited soft agar colony formation (Fig. 3E; Supplemental Fig. S4C–E). These data establish an essential role for RREB1 in Kras-mediated repression of the miR-143/145 cluster.

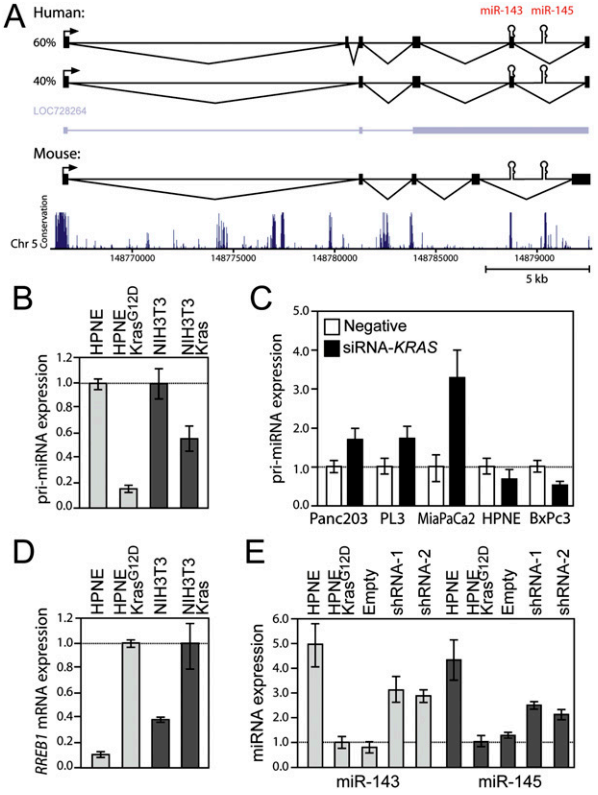

Figure 3. Kras-mediated repression of miR-143/145 occurs at the level of transcription and requires RREB1. (*A*) Structures of the experimentally determined human and mouse miR-143/145 primary transcripts and the annotated human RefSeq transcript (LOC728264). The plot depicted *below* the transcripts shows evolutionary conservation (UCSC Genome Browser 28 species conservation track, NCBI36/hg18 assembly). (*B*) qPCR analysis of pri-miR-143/145 expression in HPNE and NIH3T3-derived cell lines. Error bars for this and subsequent panels represent standard deviations from three independent measurements. (*C*) Pri-miR-143/145 abundance in cells transfected with negative control or *KRAS* targeting siRNAs. (*D*) *RREB1* expression in HPNE and NIH3T3-derived cell lines. (*E*) Mature miR-143 and miR-145 expression in HPNE-Kras^{G12D} cells following knockdown of *RREB1*.

Direct repression of the miR-143/145 promoter by RREB1

To determine whether RREB1 directly represses the miR-143/145 promoter, we constructed a luciferase reporter plasmid with a 1-kb human genomic segment encompassing the highly conserved TSS (the region shown in Supplemental Fig. S4A), which was active in multiple cell lines (Fig. 4A). Like the endogenous promoter, this reporter was repressed in NIH3T3-Kras relative to NIH3T3 cells, and mutations in the RREB1-binding site reversed this repression (Fig. 4B). Similarly, the mutant promoter construct was derepressed relative to the wild-type promoter when introduced into PDAC cell lines harboring activating mutations in *KRAS* (Fig. 4C). Additionally, shRNA-mediated *RREB1* knockdown in NIH3T3-Kras cells induced activity of the promoter (Fig. 4D). Lastly, we performed chromatin immunoprecipitation (ChIP), which documented that Flag-tagged RREB1 interacts directly with the endogenous miR-143/145 promoter in NIH3T3 cells (Fig. 4E). These experiments demonstrate that RREB1 directly represses the activity of the miR-143/145 promoter in cells expressing activated Kras.

A feed-forward circuit established by direct targeting of KRAS *and* RREB1 *by miR-143 and miR-145*

Interestingly, *KRAS* and *RREB1* are identified by TargetScan (Grimson et al. 2007) as potential miR-143 and miR-145 targets, respectively. Moreover, the direct targeting of *KRAS* by miR-143 in colon cancer cells was recently experimentally validated (Chen et al. 2009). Overexpression of miR-143 reduces *KRAS* mRNA and protein abundance in MiaPaCa2 and Panc-1 cells (Fig. 5A; Supplemental Fig. S5A). Conversely, inhibition of miR-143 increases *KRAS* expression in HPNE cells (Fig. 5B). These observations confirm that miR-143 regulates *KRAS* in pancreatic cancer cells.

RREB1 expression was reduced upon miR-145 overexpression in MiaPaCa2 and Panc-1 cells and was increased upon miR-145 inhibition in HPNE cells (Fig. 5C,D), suggesting that this transcript is a bona fide miR-145 target. The *RREB1* 3′ untranslated region (UTR) contains one predicted miR-145-binding site, which is highly conserved in vertebrates (Supplemental Fig. S5B). Reporter assays verified that this site, but not a mutant version, resulted in miR-145-mediated repression when placed in the 3′ UTR of luciferase (Supplemental Fig. S5C).

The ability of miR-143 and miR-145 to negatively regulate *KRAS* and *RREB1* expression suggests the existence of a feed-forward circuit through which Kras-mediated repression of these miRNAs further potentiates Kras signaling. Consistent with this model, miR-143 reduces *RREB1* expression in MiaPaCa2 cells (Fig. 5C), possibly indirectly through targeting of *KRAS*. Similarly, miR-145 expression results in reduced *KRAS* levels in this cell line (Fig. 5A), likely by inhibiting *RREB1*, thereby increasing miR-143, which can target *KRAS*. Interestingly, this circuit is not fully active in Panc-1 cells, since miR-143 did not repress *RREB1* and miR-145 did not repress *KRAS* (Fig. 5A,C). Although the molecular basis for this defect in the circuit in this cell line remains to be elucidated, this might explain why Panc-1 is insensitive to individual expression of the miRNAs (Fig. 2F).

To further test this model, we examined the activity of two major Kras effectors—the MAPK and the PI3K pathways—in pancreatic cancer cells with enforced miR-143/145 (Fig. 5E). Expression of these miRNAs reduced phosphorylation of ERK1/2 (indicative of MAPK signaling) and AKT (indicative of PI3K signaling). Thus, expression of miR-143/145 suppresses Ras pathway activity.

miR-143 and miR-145 have well-documented anti-proliferative and proapoptotic effects through their ability to negatively regulate a host of target genes, including *MYC*, *Insulin Receptor Substrate-1* (*IRS-1*), and *Extracellular Signal-Regulated Kinase 5* (*ERK5*) (Esau et al. 2004; Shi et al. 2007; Chen et al. 2009; Sachdeva et al. 2009). Repression of this miRNA cluster thereby contributes to Ras-mediated cellular transformation by promoting the coordinated activation of other important oncogenic signals. In addition, our studies uncovered the existence of a feed-forward loop established by the direct targeting of *KRAS* and *RREB1* by miR-143 and miR-145, respectively (Fig. 5F). This circuit is reminiscent of feed-forward loops involving miRNAs in other key oncogenic pathways that establish irreversible switches that drive cellular transformation. This is well illustrated by the recently described NF-κB/IL-6/Lin28/let-7 pathway (Iliopoulos et al. 2009), in which NF-κB signaling activates expression of Lin28, an inhibitor of let-7 biogenesis, thereby increasing expression of IL-6 (a let-7 target), which further stimulates NF-κB activity. Similarly, Myc is another let-7 target that activates Lin28 expression

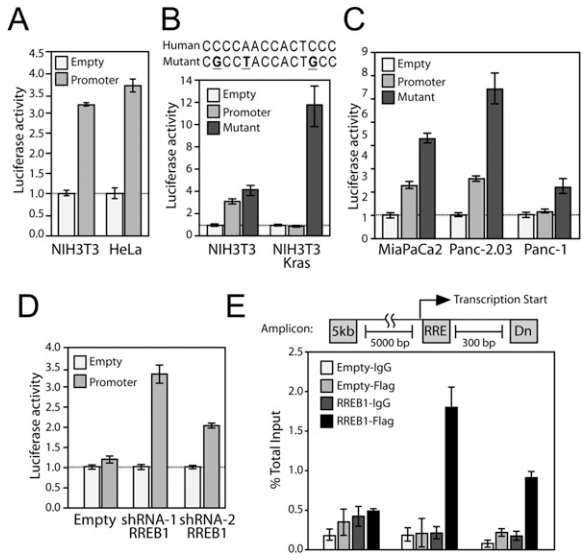

Figure 4. RREB1 negatively regulates the miR-143/145 promoter. (*A*) Activity of the miR-143/145 promoter reporter construct in NIH3T3 and HeLa cells. For this and subsequent panels, values represent firefly luciferase activity produced from each plasmid normalized to *renilla* luciferase activity produced from a cotransfected control vector. Error bars represent standard deviations from three independent transfections, each measured in triplicate. (*B*,*C*) Activity of the wild-type and mutant miR-143/145 promoter reporter constructs in NIH3T3 and NIH3T3-Kras cells (*B*) or PDAC cell lines (*C*). (*D*) *RREB1* knockdown reverses repression of the miR-145/145 promoter reporter construct in NIH3T3-Kras cells. (*E*) qPCR analysis of Flag-RREB1 chromatin immunoprecipitates in NIH3T3 cells. Signal obtained from the miR-143/145 promoter amplicon containing the RREB1-binding site (RRE), an amplicon 300 base pairs (bp) downstream (Dn), and a negative control amplicon 5 kb upstream (5kb) are shown. Error bars represent standard deviations derived from three independent measurements.

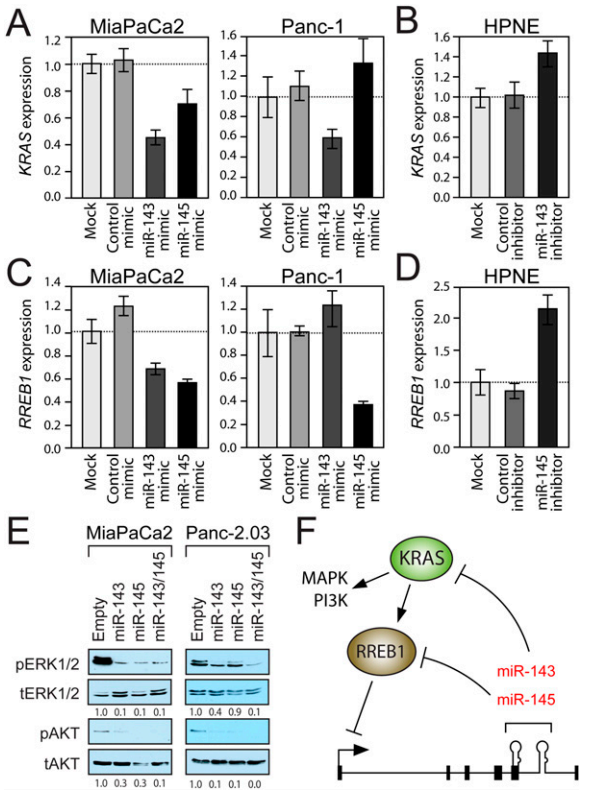

Figure 5. miR-143 and miR-145 target *KRAS* and *RREB1*, establishing a feed-forward circuit that potentiates Ras signaling. (*A–D*) qPCR analysis of *KRAS* (*A*,*B*) or *RREB1* (*C*,*D*) transcript abundance in cells transfected with negative control, miR-143, or miR-145 mimics (*A*,*C*) or inhibitors (*B*,*D*). Error bars represent standard deviations from three independent measurements. (*E*) Western blot analysis of phospho-ERK1/2 (pERK1/2), total ERK1/2 (tERK1/2), phospho-AKT^{S473} (pAKT), and total AKT (tAKT) in retrovirally infected cell lines. (*F*) Model of feed-forward regulatory circuit in which Kras signaling, via RREB1, represses miR-143/145 transcription, thereby relieving *KRAS* and *RREB1* negative regulation and potentiating signaling through Ras effector pathways.

(Chang et al. 2009; Dangi-Garimella et al. 2009), constituting an intersecting positive feedback loop. These observations demonstrate that major oncoproteins—including Ras, Myc, and NF-κB—use similar regulatory circuitry to establish stable cellular transformation programs, and suggest that use of analogous positive feedback loops may be a widespread property of oncogenic pathways.

The ability of miR-143/145 expression to completely abrogate tumorigenesis by pancreatic cancer cells is likely the result of repression of a large set of oncogenic targets. Nevertheless, the ability of these miRNAs to robustly suppress downstream Ras signaling, as evidenced by suppression of the MAPK and PI3K pathways, likely plays a central role in suppressing pancreatic tumorigenesis, since activating *KRAS* mutations are nearly ubiquitous in this setting (Caldas and Kern 1995). In light of the importance of loss of miR-143/145 in sustaining Kras pathway activity and the exquisite sensitivity of pancreatic cancer cells to restored miR-143/145 expression, delivery of these miRNAs represents a promising therapeutic approach for this tumor type.

Materials and methods

Cell culture

Proliferation rates were measured using the CKK-8 kit (Dojindo). For anchorage-independent growth assays, cells were plated in 4% agarose in culture media; for tumorigenesis assays, 5×10^6 cells were subcutaneously injected into immunodeficient (BALB/c nu/nu) mice.

Measurement of miRNA expression

A custom microarray was used to profile miRNA expression as described previously (Chang et al. 2009). Following *DROSHA* knockdown (HPNE cells) (Y Lee et al. 2003) or *Dgcr8* knockdown (NIH3T3 cells) with siRNA, the GeneRacer kit (Invitrogen) was used to characterize the miR-143/145 primary transcript.

Luciferase assays

Cells were transfected with pGL3-IRES-promoter reporter constructs or pGL3-control 3′ UTR reporter constructs using Lipofectamine 2000 (Invitrogen), and luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega). Where appropriate, control or miR-145 mimics (Dharmacon) were cotransfected with 3′ UTR reporters at 15 nM final concentration.

ChIP

A mouse N-terminal Flag-tagged RREB1 cDNA expression construct was transfected into NIH3T3 cells using PolyFECT (Qiagen), and ChIP was performed as described (Boyd et al. 1998). Immunoprecipitation was performed with anti-Flag-M2 antibody (Sigma) or control mouse IgG1 antibody (Dako).

Additional Materials and Methods are provided in the Supplemental Material.

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