ZEB1 drives prometastatic actin cytoskeletal remodeling by downregulating miR-34a expression

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Metastatic cancer is extremely difficult to treat, and the presence of metastases greatly reduces a cancer patient’s likelihood of long-term survival. The ZEB1 transcriptional repressor promotes metastasis through downregulation of microRNAs (miRs) that are strong inducers of epithelial differentiation and inhibitors of stem cell factors. Given that each miR can target multiple genes with diverse functions, we posited that the prometastatic network controlled by ZEB1 extends beyond these processes. We tested this hypothesis using a mouse model of human lung adenocarcinoma metastasis driven by ZEB1, human lung carcinoma cells, and human breast carcinoma cells. Transcriptional profiling studies revealed that ZEB1 controls the expression of numerous oncogenic and tumor-suppressive miRs, including miR-34a. Ectopic expression of miR-34a decreased tumor cell invasion and metastasis, inhibited the formation of promigratory cytoskeletal structures, suppressed activation of the RHO GTPase family, and regulated a gene expression signature enriched in cytoskeletal functions and predictive of outcome in human lung adenocarcinomas. We identified several miR-34a target genes, including Arhgap1, which encodes a RHO GTPase activating protein that was required for tumor cell invasion. These findings demonstrate that ZEB1 drives prometastatic actin cytoskeletal remodeling by downregulating miR-34a expression and provide a compelling rationale to develop miR-34a as a therapeutic agent in lung cancer patients.

Introduction

Metastasis currently represents a tipping point in a cancer patient’s likelihood of achieving long-term survival. Metastatic deposits cannot be eradicated with any standard treatment options and are the most common cause of death from epithelial malignancies (1). Understanding the basic biological processes that govern metastasis represents a critical barrier to attaining long-term survival for patients afflicted with epithelial cancer.

In one working hypothesis, metastasis is initiated by a population of tumor cells that undergo epithelial-to-mesenchymal transition (EMT) in response to extracellular cues, leading to loss of polarized features, detachment from neighboring cells, increased motility, and invasion into surrounding matrix (2). Context-dependent cellular plasticity has been identified in side populations of established human cancer cell lines and in cell lines derived from mouse models of human epithelial cancers; these cells are marked by increased expression of aldehyde dehydrogenase or prominin-1 (CD133) (3, 4). The unique biological features of these cells and their dearth within primary tumors has led to the belief that they originate from rare populations of pluripotent cells or have uncommon gain-offunction somatic mutations (5).

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2012;122(9):3170–3183. doi:10.1172/JCI63608.

EMT is driven by several families of transcriptional repressors (ZEB, SNAIL, and basic helix-loop-helix factors) (6). ZEB factors contain 2 widely separated clusters of zinc fingers that bind to paired CAGGTA/G E-box–like promoter elements. They induce EMT by downregulating the expression of epithelial genes, including E-cadherin (7, 8). During development, ZEB expression is upregulated in cells that undergo EMT and migrate by extracellular signals such as TGF-β and Notch ligands (9, 10). Beyond its physiological roles, ZEB1 is overexpressed in many human cancers (e.g., prostate, colon, breast, and pancreatic) and has been implicated in metastasis and cellular events thought to precede it, including reduced expression of basement membrane components and induction of EMT (9). Increased ZEB1 levels correlate with poor prognosis in a variety of epithelial tumor types (11). In tumor cells, ZEB1 represses the expression of certain microRNAs (miRs) — including miR-183, miR-203, and miR-200 family members (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) — that function not only as strong inducers of epithelial differentiation, but also as inhibitors of stem cell properties through repression of the stem cell factors SOX2, BMI1, and KLF4 (9). Reciprocally, miR-200 family members directly target the ZEB factors (ZEB, SNAIL, and basic helix-loop-helix factors) (6). ZEB factors are induced by upstream transcriptional repressors (p53, β-catenin, and Notch ligands) (9, 10). Beyond its physiological roles, ZEB1 is overexpressed in many human cancers (e.g., prostate, colon, breast, and pancreatic) and has been implicated in metastasis and cellular events thought to precede it, including reduced expression of basement membrane components and induction of EMT (9). Increased ZEB1 levels correlate with poor prognosis in a variety of epithelial tumor types (11). In tumor cells, ZEB1 represses the expression of certain microRNAs (miRs) — including miR-183, miR-203, and miR-200 family members (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) — that function not only as strong inducers of epithelial differentiation, but also as inhibitors of stem cell properties through repression of the stem cell factors SOX2, BMI1, and KLF4 (9). Reciprocally, miR-200 family members directly target the ZEB1 3′-untranslated region (3′-UTR); hence, ZEB1 and miR-200 are interconnected through a double-negative feedback loop (12–15). The relevance of these findings to metastasis is supported by findings in a mouse model of human lung adenocarcinoma driven by expression of Trp53R172HAG.
miRs regulated by ZEB1

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393P_ZEB1 and 393P_vector cells subjected to global miR profiling (Figure 1, A and B) to identify upregulated (>2-fold) or downregulated (<0.5-fold) miRs. A P value (2-tailed Student’s t test) less than 0.01 was considered significant. aGenomic cluster with chromosomal loci Chr6:30,115,918–30,119,737. bGenomic cluster with chromosome loci Chr8:139,663,032–139,863,295. cGenomic cluster with chromosome loci Chr3:45,428,014–45,429,859. dGenomic cluster with chromosome loci Chr10:397,969–10,437,548. eGenomic cluster with chromosome loci Chr1:93,667,932–124,668,408. fGenomic cluster with chromosome loci Chr15:50,402,580–50,407,231. gGenomic cluster with chromosome loci Chr1:50,402,580–50,407,231. hGenomic cluster with chromosome loci Chr1:502,160,866–502,167,125. iGenomic cluster with chromosome loci Chr1:139,663,032–139,863,295. jGenomic cluster with chromosome loci Chr8:86,702,615–86,702,860.
$P = 2.9 \times 10^{-9}$; Figure 2, A and B); similar findings were observed in a panel of 39 human lung cancer cell lines (Figure 2C).

Although located on a separate chromosome from miR-34a, expression of the miR-34b/c cluster is frequently coregulated with miR-34a (27, 28). However, TaqMan PCR assays confirmed the evidence from our microarray studies (Figure 1) that ZEB1 downregulated the expression of miR-34a, but not that of miR-34b or miR-34c (Supplemental Figure 2). Furthermore, miR-34a levels did not correlate with miR-34b or miR-34c in the panel of human lung cancer cell lines (Figure 2C). Collectively, these findings suggest that miR-34a expression is regulated through mechanisms different from those controlling the miR-34b/c cluster in the human and murine lung adenocarcinoma cells examined in this study.

**Figure 1**
ZEB1 regulates the expression of numerous miRs. (A) Volcano plot depiction of findings from microarray analysis showing the miRs differentially expressed in 393P_vector and 393P_ZEB1 cells. The $-\log_{10}$ of $P$ values (y axis) is plotted against the log$_2$ of fold change between 2 groups (x axis). The size of the circle for each probe is proportional to the miR detection rate for the entire experiment. Each symbol is color coded according to average expression of the probe across the 2 groups (scale at right). Dotted lines delineate the cutoffs for miRs significantly downregulated (left) or upregulated (right) in 393P_ZEB1 cells. (B) Heat map depiction of miRs differentially expressed in 393P_ZEB1 cells (ZEB1), using 393P_vector cells (vec) as reference. (C) Taqman microRNA assays (Q-PCR) to confirm miRs differentially expressed by microarray. Data are mean ± SD (n = 3 samples). $P$ values are indicated (2-tailed Student’s $t$ test).
was not different between 2 KP cell lines with high and low miR-34a levels (Figure 3A), which indicates that differential p53 activity does not contribute to relative miR-34a levels in KP cells. As an alternative intermediary, we turned our attention to ΔN isoforms of p63 (ΔNp63), which lack the transactivation domain and are transcriptional targets of ZEB1 in 393P cells (Supplemental Figure 3 and ref. 30). ΔNp63 acts in a dominant-negative fashion against p53, TAp63, and TAp73, but has been shown to function as a transcriptional activator of specific genes such as T and T2 (31). Murine embryonic fibroblasts (MEFs) deficient in all p63 isoforms had reduced miR-34a levels (Figure 3B), which suggests that p63 positively regulates miR-34a expression. On the basis of these findings in MEFs, we sought to determine whether ΔNp63 serves as an intermediate in ZEB1-induced miR-34a repression in KP cells. Basal levels of ΔNp63 transcriptional target genes T and T2 were higher in low-ZEB1 cell line 393P than they were in high-ZEB1 cell line 344SQ, and introduction of ZEB1 into 393P cells decreased T and T2 levels (Figure 3C), which demonstrated that ZEB1 repressed ΔNp63 transcriptional activity. Ectopic ZEB1 expression downregulated ΔNp63 mRNA levels in 393P cells (Figure 3D), and siRNA-mediated knockdown of ZEB1 upregulated ΔNp63 mRNA levels in 344SQ cells (Figure 3E). The activity of a ΔNp63 promoter fragment (−1,128 to +109) was repressed by exogenous ZEB1, but not SNAI1 or TWIST1 (Figure 3F). Promoter deletion studies demonstrated that ZEB1 repressed the activity of a minimal ΔNp63 promoter fragment (−482 to +109) containing 2 putative ZEB1-binding sites (E-boxes; Figure 3G), and site-directed mutagenesis of the most proximal E-box element abrogated ZEB1-induced repression of the ΔNp63 promoter (Figure 3H).

We next examined whether ΔNp63 regulates miR-34a expression and acts directly on the miR-34a gene promoter in KP cells. Ectopic ΔNp63β expression in 344SQ cells upregulated miR-34a, but not miR-34b or miR-34c (Supplemental Figure 4), and increased the activity of a WT promoter, but not a mutant miR-34a promoter lacking a p53-binding site (Figure 3I). Binding of endogenous p63 to the miR-34a promoter was examined by performing ChIP assays using an antibody that recognizes all p63 isoforms. In 344SQ cells, total p63 binding roughly reflected ΔNp63 binding, because ΔN isoforms were 67.0-fold more abundantly expressed than were TA isoforms (Supplemental Figure 5A). Murine keratinocytes were included as a positive control. The percentage of total p63 that bound to the p53/p63 site and to a nonspecific site in the miR-34a promoter was 0.19% and 0.06%, respectively (P = 0.08; Supplemental Figure 5B). To more specifically examine ΔNp63 binding to that site, ChIP assays were performed on 393P cells stably transfected with Myc-tagged ΔNp63. Using an anti-Myc antibody, we found the percentage of ectopic ΔNp63 that bound to the p53/p63 site and to a nonspecific site in the miR-34a promoter to be 0.39% and 0.17%, respectively (P = 0.002; Figure 3J). We concluded that ΔNp63 serves as an intermediate in ZEB1-induced miR-34a repression in KP cells.

miR-34a abrogates tumor cell invasion and metastasis and induces transcriptional changes that are prognostic in human lung adenocarcinomas. To examine the biological role of miR-34a repression, miR-34a was...
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A. Relative luciferase activity for Mdm2, 14-3-3σ, and Generic with 393P, 344SO, 393P+p53, and 344SO+p53 treatments.

B. miR-34a expression in WT and p63−/− mice with 393P and 344SO treatments.

C. mRNA expression for T and T2 mice with 393P, 344SO, and 393P_vec treatments.

D. mRNA expression for Zeb1 and ΔNp63 with 393P_vec and 393P_ZEB1 treatments.

E. mRNA expression for con and Zeb siRNA with Zeb1 and ΔNp63 treatments.

F. Relative luciferase activity for vec, ZEB1, SMN1, and TWIST1 with ΔNp63 treatments.

G. E-boxes with Lucerase activity at -1128, -735, and -482 positions with +1 and +109 treatments.

H. Luciferase activity with E-boxes in WT, Mut-a, Mut-b, and Mut-a/b with + vec and + ZEB1 treatments.

I. Murine miR-34a promoter (GenBank: EF606691) with Exon 1 and Exon 2 luciferase activity at p53/63 site and NS with p53/63 site, GL3, and ΔNp63β treatments.

J. % p63 bound with vec and ΔNp63β treatments with 393P and p53/63 site.
exogenously expressed under the control of a doxycycline-inducible promoter in 344SQ cells, which have high ZEB1 and low miR-34a expression (Figure 4A). Grown in monolayer, 344SQ_miR-34a cells proliferated at a rate similar to that of 344SQ_vector cells and demonstrated no biochemical evidence of apoptosis (Figure 4B and Supplemental Figure 6A). However, when grown in suspension, 344SQ_miR34a cells did not proliferate and demonstrated evidence of apoptosis (Supplemental Figure 6B, B and C), which suggests that miR-34a enhanced the susceptibility of 344SQ cells to anoikis. 344SQ_miR34a cells exhibited reduced migration and invasion in Boyden chambers (Figure 4, C and D) and generated flank tumors in syngeneic mice that were smaller and metastasized to the lung less frequently (Figure 4E). Conversely, transfection of miR-34a hairpin inhibitor into 393P cells, which have low ZEB1 and high miR-34a expression, induced a 84% decrease in endogenous miR-34a levels and 1.6- and 1.5-fold increases in cell migration and invasion, respectively (Supplemental Figure 7, A–C). In MDA-MB-231 human breast cancer cells and H1299 human lung cancer cells, which have high basal ZEB1 expression and undergo EMT in response to TGF-β (13, 32), overexpression of miR-34a antagonized migration and invasion (Figure 4F and Supplemental Figure 8, A–C), which was not a consequence of apoptosis or reduced proliferation (Supplemental Figure 8, D and E). There was no evidence of EMT reversal on the basis of expression of epithelial and mesenchymal markers in 344SQ cells and MDA-MB-231 cells (Figure 4, G and H). Thus, miR-34a downregulation was required for ZEB1-induced metastatic properties and apparently mediated these actions through EMT-independent mechanisms.

To gain insight into the biologic processes induced by miR-34a repression, microarray-based interrogation was carried out on RNA from tumor samples (344SQ_miR34a and 344SQ_vector), which revealed a total of 805 genes that were differentially expressed (fold change greater than 1.5, P < 0.01; Supplemental Figure 9; GEO accession no. GSE38341). Q-PCR analysis of the same RNA samples confirmed differential expression of 22 of 24 genes sampled (Supplemental Figure 10). The 512 downregulated genes were enriched in, among other Gene Ontology terms mitosis (P = 3.80 × 10–24, Fisher exact test), cell cycle (P = 3.40 × 10–23), mitochondrial mRNA processing (P = 4.72 × 10–19), and cytokoskeleton (P = 3.04 × 10–7), whereas the 293 upregulated genes were enriched in protein dimerization activity (P = 7.55 × 10–7) and glutathione transferase activity (P = 1.12 × 10–8). Human lung adenocarcinomas for which both gene expression and clinical outcome data are publicly available (33–35) were scored based on the presence or absence of this 805-gene signature (high or low t score, respectively), as described previously (36); the absence of this expression signature in primary lung tumors correlated with poor prognosis in 3 independent cohorts of lung cancer patients (Figure 4I). We conclude that the genes regulated by miR-34a are functionally diverse and have prognostic value in lung adenocarcinoma patients.

did not yet publications
miR-34a regulates multiple biological properties of tumor cells. (A–D) 344SQ_miR-34a cells and 344SQ_vector cells were cultured in the presence or absence of doxycycline (Dox). (A) Q-PCR analysis of miR-34a levels. (B) Cell numbers in monolayer. Migrating (C) and invading (D) cells in Boyden chambers were photographed and counted. Scale bars: 100 μm. (E) Primary tumor weight and total lung metastases from flank tumors in syngeneic mice (mean ± SD, n = 5). P values were determined by 2-tailed Student's t test. (F) MDA-MB-231 cells were transiently transfected with a random sequence miR precursor molecule control or with pre–miR-34a precursor. Shown are Q-PCR analysis of miR-34a levels, expressed relative to control transfectants (set at 1.0), and migration and invasion assays in Boyden chambers. (G and H) Q-PCR analysis of epithelial (Cdh1 and Scrib) and mesenchymal (Cdh2 and Vim) markers and their transcriptional regulators (Zeb1, Zeb2, Snai1, Snai2, and Twist1) in 344SQ_vector and 344SQ_miR-34a cells (G) and in MDA-MB-231 cells transiently transfected with pre-miR control or pre–miR-34a precursor (H). Results are expressed relative to control transfectants (set at 1.0). Data are mean ± SD (n = 3). *P < 0.01. (I) Kaplan-Meier analysis of 3 independent cohorts of lung cancer patients (33–35), comparing the differences in risk between tumors with high (>0) or low (<0) scores (36), reflecting the presence or absence, respectively, of overlap with the murine miR-34a signature. P values from log-rank (differences between arms) and univariate Cox (gene signature score as a continuous variable) tests are shown.
Arhgap1 is a miR-34a target gene required for the regulation of RHO GTPase activity and tumor cell invasion. We next examined whether miR-34a inhibits RHO GTPases at the level of GTP loading or upstream of the level of focal adhesion kinase (FAK), which is auto-phosphorylated at Tyr397 and initiates RHO GTPase activation after recruitment to ligand-bound EGFR (39). Arguing against the latter possibility, EGF-induced FAK-Tyr397 phosphorylation was unchanged by exogenous miR-34a, as determined by Western blot analysis (data not shown). At the level of GTP loading, RHO GTPases cycle from inactive (GDP-bound) to active (GTP-bound) states by binding to guanine nucleotide exchange factors (GEFs), and in the reverse direction by binding to GAPs (37). To examine whether miR-34a directly targets GEFs or GAPs, we used a prediction algorithm (TargetScan; http://www.targetscan.org) to scan the genome for putative miR-34a binding sites and discovered sites in the 3′-UTR of a RHOGAP (Arhgap1) and a CDC42 downstream effector (Cdc42se1) (Figure 7A). Reporter assays were carried out to determine whether miR-34a binds directly to these 3′-UTRs as well as those of other predicted miR-34a target genes identified in our analysis that are not directly involved in RHO GTPase regulation, but are potentially important in tumorigenesis (Figure 7B). Relative to its effect on a negative control 3′-UTR (Flt1), cotransfection of miR-34a precursors repressed 3′-UTRs of Arhgap1 by 59%, Satb2 (a regulator of chromatin structure; ref. 40) by 47%, Lef1 (a regulator of Wnt signaling; ref. 41) by 44%, and Hnf4a (a known miR-34a target; ref. 42) by 37%, but did not affect other reporters (Figure 7B). Site-directed mutagenesis of 2 predicted binding sites in the Arhgap1 3′-UTR with differing P values (0.71 and <0.1) revealed that miR-34a suppressed Arhgap1 3′-UTR reporter activity through the conserved binding site, but not the nonconserved site (Figure 7C).

In the KP cell line panel, ARHGAP1 and miR-34a levels correlated negatively (R = −0.7692; P = 0.0021, 1-tailed Spearman rank correlation test; Figure 7D), and exogenous miR-34a decreased ARHGAP1 levels in 344SQ cells (Figure 7E). Conversely, transfection of miR-34a hairpin inhibitor into 393P cells induced a 2.0-fold increase in ARHGAP1 expression (Supplemental Figure 11A). In MDA-MB-231 and H1299 cells, ectopic miR-34a downregulated ARHGAP1 (Figure 7F and Supplemental Figure 11B). To determine whether ARHGAP1 downregulation recapitulates the effects of miR-34a, ARHGAP1 was depleted by introduction of shRNAs (Figure 8A), which increased basal levels of GTP-bound CDC42 and RAC1 (Figure 8B), attenuated EGF-induced levels of GTP-bound CDC42 and RAC1 (Figure 8B), reduced invasive projections on tumor spheres after TGF-β treatment (Figure 8C), and increased cell adhesion (Figure 8D).

The anti-invasive effect of Arhgap1 shRNA was paradoxical, given that RHO is required for proinvasive actin cytoskeletal remodeling. We performed experiments to confirm this finding and to determine whether ARHGAP1 is required for miR-34a-induced phenotypic features. ARHGAP1 expression was reconstituted in 344SQ_mir-34a cells through stable transfection of an Arhgap1 cDNA expression vector (Figure 8E), and the 344SQ_mir-34a/ARHGAP1 double transfectants were compared with 344SQ_mir-34a/vector cells from the standpoint of basal and cytokine-induced RHO activity and invasive activity. ARHGAP1 reconstitution repressed basal GTP-bound CDC42 and rescued TGF-β-induced sphere invasion in Matrigel (Figure 8, F and G), which suggests that miR-34a represses TGF-β-induced tumor cell invasion by down-regulating ARHGAP1. However, ARHGAP1 reconstitution did not restore RHO activation in response to EGF or TGF-β treatment (Figure 8F) or rescue tumor growth or metastasis (data not shown), which suggests that these phenotypic effects of miR-34a are mediated through other target genes.
Figure 6
miR-34a regulates actin cytoskeletal remodeling and RHO family GTPase activity. (A–C) Cells imaged at leading edge of scratch-wound confluent cultures. (A) Filopodia (arrows) formed in 34SQ_vector, but not 34SQ_miR-34a, cells. Brackets denote lamellipodia. (B) Focal adhesions in anti-vinculin–stained cultures, counted per defined surface area of confluent cells (circles) using ImageJ. Data are mean ± SD (n = 10). Red, phalloidin; green, vinculin. (C) Cells were outlined (white lines), and their surface areas were measured using ImageJ. Data are mean ± SD (n = 20 [34SQ_vector]; 12 [34SQ_miR-34a]). (D) Attached cells were quantified 1, 2, or 3 hours after seeding by optical densitometry (595 nm) of cells stained with crystal violet. Data are mean ± SD (n = 3). *P < 0.01. (E) Cells seeded in soft agar were stained with nitrotetrazolium blue 3 weeks after seeding, and colonies larger than 100 μm in diameter were counted. Data are mean ± SD (n = 3). (F) Attached cells were quantified by optical densitometry 3 hours after seeding and expressed relative to miR-34a levels from Figure 2A. Correlation (R and P, 1-tailed Pearson’s correlation test) is indicated. (G) MDA-MB-231 cells transiently transfected with control, miR-34a, or miR-200b precursors and imaged under fluorescence (blue, DAPI; red, phalloidin; green, anti–ZO-1). As a comparison, miR-200b–transfected cells demonstrated mesenchymal-to-epithelial transition. (H and I) Western blot analysis of GTP-bound (CDC42-GTP) and total (CDC42) RHO family GTPases. Phospho-ERK1/2 (H) and phospho-SMAD3 (I) were included as positive controls for EGF- and TGF-β–induced signaling, respectively. Scale bars: 50 μm (A, B, and G); 100 μm (C); 500 μm (E). See complete unedited blots in the supplemental material.

Discussion
Studies using experimental tumor models have established a strong link between high levels of EMT activators and loss of cell polarity, reduced expression of basement membrane components, and increased propensity for metastasis (43–47). The discovery that EMT activators endow epithelial tumor cells with pluripotency led to the current belief that metastatic propensity is directly related to plasticity in response to extracellular cues (12, 48–50). Here, positing that the scope of prometastatic biological processes controlled by ZEB1 extends beyond EMT and stem-ness, we discovered that ZEB1 drove promigratory cytoskeletal processes and metastasis by downregulating the expression of miR-34a. Exogenous miR-34a decreased tumor cell invasion and metastasis, inhibited the formation of promigratory cytoskeletal structures, suppressed activation of the RHO GTPase family, and regulated a gene expression signature that was enriched in cytoskeletal functions and prognostic in human lung adenocarcinomas. Biological reprogramming of this magnitude supports a central role for miR-34a in metastasis regulation by ZEB1.

RHO family members play key parts in the regulation of actin cytoskeletal remodeling and tumorigenesis. RAC1 is required for the development of primary lung adenocarcinomas in mice that express mutant KRAS (51). The activities of RHO, RAC1, and CDC42 are coordinated to regulate membrane protrusions and cell-matrix adhesions at the leading edge of migrating cells to control forward movement (52). Effectors of RHO GTPases include RHO-associated protein kinase, focal adhesions, and membrane protrusions, which together mediate cell adhesion to extracellular matrix, link matrix attachments to intracellular signaling pathways, and drive actomyosin contractility and cell locomotion (52). Beyond these roles, a large body of evidence implicates RAC1 in the assembly, disassembly, and maintenance of adherens junctions and tight junctions, which play a central role in the regulation of apical-basal polarity (52). Tightly regulating these processes is a miR network that targets RHO GTPases and their associated GEFs and GAPs (53). Examples include RHOA (miR-31, miR-133, and miR-155), RHOC (miR-138 and miR-10b), CDC42 (miR-29), TIA1 (miR-10b), and ARHGDI (miR-151) (53). Here we showed that miR-34a inhibited cytokine-induced RHO family GTPase activation and discovered that a RHOGAP, Arhgap1, was a miR-34a target gene. ARHGAP1 reconstitution in miR-34a–overexpressing cells did not rescue RHO activation in response to EGF or TGF-β treatment, which was expected, given that RHOGAPs inhibit RHO GTPase activity. However, TGF-β–induced invasion was abrogated in metastasis-prone tumor cells by ARHGAP1 depletion and was rescued in miR-34a–overexpressing cells by ARHGAP1 reconstitution. The proinvasive effect of ARHGAP1 was paradoxical, given that RHO GTPase activity stimulates the formation of actin cytoskeletal structures that drive cell migration. Although the mechanism is unclear, ARHGAP1 binds to a number of proteins other than RHO family GTPases — including BNI2, SRC, UBC, and PIK3R1 (BioGRID; http://thebiogrid.org) — that regulate diverse biological processes and may have contributed to the proinvasive effect of ARHGAP1 through RHO GTPase–independent mechanisms. Collectively, these findings suggest that ARHGAP1 mediates some, but not all, of the biological effects of miR-34a (Figure 8H).

We discovered that ZEB1 regulated a larger number of miRs than had previously been reported (12, 13, 15). This multiplicity was due in part to 19 miRs clustered within 7 genomic loci that are transcribed and processed together. ZEB1 downregulated certain miRs and upregulated others, which could be related either to the capacity of ZEB1 to function as a transcriptional repressor or activator (54–56) or to indirect regulation of miRs by ZEB1. In support of the latter possibility, we found that ZEB1 indirectly repressed miR-34a through ΔNp63. The reported biological functions of the 46 miRs were diverse, encompassing hypoxic response (miR-210), cell differentiation (miR-326), proliferation (e.g., miR-224, miR-206, miR-542-3p, and miR-126), apoptosis (miR-96, miR-193a, and miR-181a), and migration (miR-206, miR-503, and miR-181b), among other functions (Supplemental Table 1), which indicates that ZEB1 might control a number of biological processes by regulating the expression of these miRs.

The p63 transcription factor family plays a central role in the regulation of embryonic development, normal adult tissue homeostasis, and malignancy (57). The tumor-suppressive properties of TAp63 are exerted through the upregulation of a wide variety of miRs, including let-7, miR-15/16a, miR-145, miR-129, miR-26, miR-30, and miR-146a (57). Senescence in keratinocytes is activated through ΔNp63-induced downregulation of miR-138, miR-181a, miR-181b, and miR-130b (58). TAp63 is also a transcriptional activator of Dicer, an endoribonuclease required for miR biogenesis (29). The findings presented here build on this growing body of evidence that miRs are central mediators of the diverse biological actions of p63 by showing that miR-34a was upregulated by ΔNp63 and was a potent tumor suppressor in a Kras/Trp53-driven lung adenocarcinoma model. Furthermore, our finding that ΔNp63 served as a downstream mediator of ZEB1 complete a feedback circuit initiated by p63, which transcriptionally activates the miR-200b/a/429 cluster (59) and, in turn, directly targets ZEB1 (9, 13, 14), thereby relieving the ZEB1-induced repression of ΔNp63 shown here. There are numerous other p63/miR circuits, including one involving miR-193-5p, which targets p63 and is directly repressed by p63 (60). Thus, miR homeostasis is tightly regulated through multiple mechanisms involving p63 and ZEB1.
miRs locally or systemically to the tumor tissue where they regulate their target genes (62, 63). Physical and chemical moieties of the particles that facilitate the targeted distribution and the controlled and sustained release of miRs are under clinical investigation (64). External moieties, such as aptamers and ligands that enhance miR uptake by cancer cells, are being developed to direct the particles to a particular tissue (65, 66). Moreover, efforts are underway to initiate clinical trials that deliver miRs into patients with advanced cancer.

Methods

Antibodies and plasmid constructs. Antibodies against ERK, phospho-ERK, SMAD3, phospho-SMAD3, PARP, cleaved caspase-3 (Cell Signaling Technology), ACTIN (Sigma-Aldrich), p63 (Abcam), and ARHGAP1 were purchased (Santa Cruz Biotechnologies). Doxycycline (Sigma-Aldrich), EGF (Invitrogen), and TGF-β (Calbiochem) were purchased. Human SNAI1 cDNA (catalog no. 16218), murine Twist1 cDNA (catalog no. 1783; Addgene), murine Arhgap1 cDNA, and murine Arhgap1 shRNA (Origene) were purchased. To construct the miR-34a overexpression vector, a tet operator–H1 promoter fusion (tH1) was cloned into the XmnI and BamHI sites of pENTR2B (Invitrogen). A 487-nt fragment containing miR-34a (∼200 nt either side of the mature miR) was amplified from human cDNA by PCR and directionally cloned into the BamHI and EcoRI sites of pENTR2B-tH1.

The evidence presented here that miR-34a is a potent repressor of tumor growth and metastasis in a mouse model of human lung cancer bolsters evidence from other mouse models that miR-34a is a promising therapeutic agent. Delivery of miR-34a oligomers systemically by tail vein inhibits tumor growth in mice bearing lung adenocarcinomas, suppresses metastasis to the lung and other organs, and prolongs the survival of mice bearing orthotopic human prostate carcinomas (61). The mechanisms by which miR-34a exerts its therapeutic effects are tumor cell type specific. For example, in the lung adenocarcinoma metastasis model shown here, miR-34a downregulation enhanced promigratory cytoskeletal processes, but was not required for stem cell features, based on formation of polarized epithelial spheres, whereas it targets the stem cell marker CD44 in prostate cancer cells and represses stem-ness in prostate, glioblastoma, pancreatic, and gastric cancer cells (18, 49). The distinct mechanisms by which miRs exert tumor suppressor functions in a given tumor type might be leveraged to create combinatorial treatment approaches. In metastatic KP cells, the miR-200 family members and miR-34a are all sharply downregulated, and ectopic expression of the miR-200b/a/429 cluster locks KP cells into an epithelial state and abrogates metastasis (17). Thus, combined delivery of miR-34a and miR-200 family members might be complementary in these cells. Safe and efficient approaches using lipid-based nanoparticles (neutral or charged) have been developed that deliver miRs locally or systemically to the tumor tissue where they regulate their target genes (62, 63). Physical and chemical moieties of the particles that facilitate the targeted distribution and the controlled and sustained release of miRs are under clinical investigation (64). External moieties, such as aptamers and ligands that enhance miR uptake by cancer cells, are being developed to direct the particles to a particular tissue (65, 66). Moreover, efforts are underway to initiate clinical trials that deliver miRs into patients with advanced cancer.

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EF-1a promoter (67). Gateway technology (Invitrogen) was used for the transfer of miR-34a into the pLV711G vector to create a single lentiviral vector enabling doxycycline-responsive expression of miR-34a.

**Cell culture studies.** Murine (307P, 344LN, 344P, 344SQ, 393LN, 393P, 412P, 531LN1, 531LN2, 531LN3, 531P1, 531P2, and 713P) and human lung cancer cells (H2009 and H1299) were cultured in RPMI 1640 (Mediatech) with 10% FBS (Sigma-Aldrich). WT and p63–/– murine embryonic fibroblasts (MEFs) were maintained in DMEM (Mediatech) with 10% FBS. MDA-MB-231 cells were cultured in Leibovitz's L-15 (Mediatech) with 10% FBS. Cells were transfected using Dharmafect-DUO (Dharmacon). For soft agar assays, $5 \times 10^4$ cells (in 0.3% agar) were seeded into 6-well plates layered with 0.8% agar, and colonies were stained with 0.5 mg/ml nitrotetrazolium blue (Sigma-Aldrich) 21 days later. For migration and invasion assays, $1 \times 10^5$ cells were cultured in the upper wells of Transwell and Matrigel chambers, respectively (BD Biosciences), and allowed to migrate toward 10% FBS in the bottom wells. After 16 hours of incubation, migrating or invading cells were stained with 0.1% crystal violet, photographed, and counted. Cellular proliferation was measured in anchorage-dependent and -independent conditions by counting cells seeded onto high- or low-adherence plates, respectively (Greiner Bio-One), using the Countess automated cell counter (Invitrogen). For immunocytochemistry, cells were cultured on collagen-coated coverslips and then stained with DAPI (Sigma-Aldrich), Alexa Fluor 568–conjugated phalloidin (Invitrogen), and anti-vinculin (Millipore) antibody. Cells were cultured in 3D Matrigel cultures (BD Biosciences) and stained with immunofluorescently tagged antibodies, as described previously (17). A Zeiss LSM 510 confocal microscope was used to

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**Figure 8**

ARHGAP1 mediates specific phenotypic effects of miR-34a. (A) Western blot analysis of ARHGAP1 in 344SQ cells stably transfected with scrambled shRNA (scr) or 1 of 4 Arhgap1 shRNAs (shA, shB, shC, or shD). ACTIN was used as loading control. (B) Western blot analysis of GTP-bound (GTP) and total RHO family GTPases. (C) Spheres formed by scrambled shRNA– and Arhgap1 shB–transfected 344SQ cells after 10 days in 3D Matrigel cultures in the presence or absence of TGF-β (10 ng/ml). Scale bars: 100 μm. (D) Attached cells were quantified by optical densitometry 3 hours after seeding. Data are mean ± SD (n = 3). P values were determined by 2-tailed Student’s t test. (E) Western blot analysis of 344SQ_miR-34a cells stably transfected with Flag-tagged ARHGAP1 cDNA or empty vector using antibodies against ARHGAP1 (top). Flag (middle), or ACTIN as a loading control (bottom). Arrows at top denote locations of endogenous (ARHGAP1) and ectopic (Flag-ARHGAP1) ARHGAP1. (F) Western blot analysis of GTP-bound and total CDC42 in 344SQ_mir34a/vector and 344SQ_mir34a/ARHGAP1 cells treated for 10 minutes with or without EGF or TGF-β. (G) 344SQ_mir34a/vector and 344SQ_mir34a/ARHGAP1 cells were cultured for 10 days in Matrigel in the presence or absence of TGF-β and photographed under phase-contrast microscopy. TGF-β-induced loss of lumen formation in both transfectants, but invasive projections formed only in 344SQ_mir34a/ARHGAP1 cells. Scale bars: 50 μm. (H) Proposed model illustrating the ARHGAP1-dependent and -independent phenotypic effects of miR-34a. See complete unedited blots in the supplemental material.
capture fluorescent images of Matrigel cultures. Fluorescence-stained slides depicting filopodia, lamellipodia, and focal adhesions were imaged on a Nikon Ti-microscope equipped with a CoolSnap HQ2 camera (Photometrics). Image stacks of 100-nm sections were then deconvolved with Autoquant (Media Cybernetics). For attachment assays, 1 × 10⁵ cells were seeded on 24-well plates and incubated for 1–3 hours. After washing with PBS twice, attached cells were stained with 0.1% crystal violet, and optical density was measured at 595 nm.

miR expression profiling. Total RNA was isolated from 393P vector and 393Pavg cells, profiled using the DiscoArray platform by Asuragen, and analyzed as described previously (17). The platform included all but 91 of the 1,086 probes in the Sanger miRBase version 9.2 covering humans, rats, and mice and an additional 12,894 exploratory probes covering multiple other species. Transcriptomic data sets were deposited in GEO (accession no. GSE38386). Heat maps were generated using Cluster and TreeView software (http://rana.lbl.gov/EisenSoftware.html).

Quantitative RT-PCR (Q-PCR). Total RNA was isolated from the cells using TRIzol (Invitrogen) according to the manufacturer’s protocol. To analyze miRNA levels, Q-PCR assays were performed after reverse transcription with Superscript III reverse transcriptase (Invitrogen) using a SYBR-Green-based system (Applied Biosystems). miRNA levels were normalized on the basis of mRNA for ribosomal protein L32 (Rpl32). See Supplemental Table 2 for primer sequences. miR levels were quantified using Taqman microRNA assays (Applied Biosystems) according to the manufacturer’s protocol and normalized on the basis of snoRNA-135.

Luciferase reporter assay. Cells were seeded on 24-well plates (1 × 10⁵ cells/well) 1 day before transfection, and then transfected with 500 ng luciferase reporter plasmids and 50 ng hRL-control vector. After 48 hours, luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega). A murine miR-34a promoter region was isolated by PCR from TC-1 murine ES cell genomic DNA and ligated into the pgLL3-basic vector (Promega). p53-reporter plasmids (Mdm2, I3-3-ct, and generic promoters) were gifts from M.-H. Lee (University of Texas MD Anderson Cancer Center, Houston, Texas, USA), and the ΔNp63 and TAp63 promoter reporters were gifts from I. Shachar (Weizmann Institute of Science, Rehovot, Israel). For the 3′-UTR assay, murine 3′-UTRs were amplified by PCR from genomic DNA and ligated into pCI-neo-hRL vector (13). 3′-UTR reporters (500 ng) and pgLL3-control (50 ng; Promega) were cotransfected into 344SQ cells seeded on 24-well plates (1 × 10⁵ cells/well) in the presence or absence of pre-miR-34a precursor (5 nM; Ambion). A PCR-based site-directed mutagenesis strategy was carried out to generate mutant constructs.

ChIP assays. 393P cells were transiently transfected with Myc-ΔNp63β or empty vector. Cells were cross-linked with 1% formaldehyde and then incubated in lysis buffer (50 mM Tris-HCl, pH 8.1; 1% SDS; 10 mM EDTA; and protease inhibitor cocktail) on ice for 10 min. After sonication (Cole-Parmer GEX-130 sonicator; 50% power, pulse on for 10 s, pulse off for 10 s, 20 cycles), samples were immunoprecipitated with anti-Myc tag antibody (Millipore) or anti-mouse IgG (Santa Cruz). DNA was eluted and purified with PCR purification kit (Qiagen), and quantitative PCR was carried out with specific primers to amplify the p53/63-binding region of the miR-34a promoter (forward, 5′-CAGCCTGGGAGAGGTACGA-3′; reverse, 5′-TCCCAAGCCCCCAATCT-3′) on a nonspecific region within exon 2 as a negative control (forward, 5′-AAGCCTGTCTGAGATCTTCTCAG-3′; reverse, 5′-TGAGCCCTCTAAACAGTGGTCCCT-3′). To detect binding of endogenous p63 to miR-34a promoter elements, cellular proteins from 393P cells and murine primary keratinocytes were cross-linked to DNA using 1% formaldehyde, and chromatin was prepared as described earlier (68), p63-DNA complexes were diluted 10-fold in ChIP dilution buffer and incubated overnight at 4°C with 2 μg anti–pan-p63 antibody (4A4; Abcam) or 2 μg IgG. Resulting chromatin was resuspended in 300 μl double-distilled H₂O. The percent DNA bound was calculated as (2^ΔΔCt)/(2^ΔΔCt + 1) × 100%.

Western blotting. Cells were lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease/phosphatase inhibitors (Sigma-Aldrich). Cell lysates were separated by SDS-PAGE, transferred onto PVDF membrane, and then incubated with primary antibodies and HRP-conjugated secondary antibodies. Protein bands were visualized with Pierce ECL Western Blotting substrate (Thermo). RAC1, CDC42, and RHO activation assay kits (Upstate) were used for GTPhase assays.

Affymetrix gene expression profiling. Total RNA was extracted from primary tumors from mice injected with 344SQ vector and 344SQ miR-34a cells using RiboPure kit (Ambion), and then hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 array (Affymetrix). Data processing and determination of differentially expressed genes were carried out essentially as described previously (69). Transcriptomic data sets were deposited in GEO (accession no. GSE38341).

Statistics. With the exception of miRNA and miR profiling, data were analyzed using 2-tailed Student’s t test and Spearman rank correlation test. A P value less than 0.05 was considered significant.

Study approval. All mouse studies were approved by the IACUC at the University of Texas MD Anderson Cancer Center. Mice received standards of care and were euthanized according to the standards set forth by the IACUC.

Acknowledgments

This work was supported by R01 CA157450 (to J.M. Kurie), J.M. Kurie is the Elza and Ina A. Shackelford Endowed Professor in Lung Cancer Research. D.L. Gibbons was supported by NCI R08 CA151651, an International Association for the Study of Lung Cancer Fellow Grant, and received financial support from Dr. Waun Ki Hong (MD Anderson Cancer Center). C.J. Creighton was supported by P30 CA125123. D. Chakravarti was funded by a CPRIT training grant (RP101502). Z.H. Rizvi was supported by HHMI-Medical Research Fellows Program. A. Pertsemidis was funded by R01 CA129632 and P50 CA70907 (the UT Southwestern/MD Anderson Cancer Center Lung Specialized Program of Research Excellence). E.R. Flores was funded by R01CA134796 and is a Leukemia and Lymphoma of America Scholar. G.J. Goodall was supported by NHRMC project grant 1008327. We thank Suraya Roslan for technical assistance.

Received for publication February 28, 2012, and accepted in revised form June 14, 2012.

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