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Xiaochao Tan, …, Ignacio I. Wistuba, Jonathan M. Kurie

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Epithelial tumor cells undergo epithelial-to-mesenchymal transition (EMT) to gain metastatic activity. Competing endogenous RNAs (ceRNAs) have binding sites for a common set of microRNAs (miRs) and regulate each other’s expression by sponging miRs. Here, we address whether ceRNAs govern metastasis driven by the EMT-activating transcription factor ZEB1. High miR-181b levels were correlated with an improved prognosis in human lung adenocarcinomas, and metastatic tumor cell lines derived from a murine lung adenocarcinoma model in which metastasis is ZEB1-driven were enriched in miR-181b targets. ZEB1 relieved a strong basal repression of $\alpha_1$ integrin (ITGA1) mRNA, which in turn upregulated adenylyl cyclase 9 mRNA (ADCY9) by sponging miR181b. Ectopic expression of the ITGA1 3’-untranslated region reversed miR-181b–mediated metastasis suppression and increased the levels of adenylyl cyclase 9 protein (AC9), which promoted tumor cell migration and metastasis. In human lung adenocarcinomas, ITGA1 and ADCY9 levels were positively correlated, and an AC9-activated transcriptomic signature had poor-prognostic value. Thus, ZEB1 initiates a miR-181b–regulated ceRNA network to drive metastasis.
The epithelial-to-mesenchymal transition activator ZEB1 initiates a prometastatic competing endogenous RNA network

Xiaochao Tan,1 Priyam Banerjee,1 Xin Liu,1 Jiang Yu,1 Don L. Gibbons,1,2 Ping Wu,3,4 Kenneth L. Scott,3,4 Lixia Diao,5 Xiaofeng Zheng,5 Jing Wang,5 Ali Jalali,5 Milind Suraokar,7 Junya Fujimoto,5 Carmen Behrens,1,7 Xiuping Liu,8 Chang-gong Liu,8 Chad J. Creighton,4,5 Ignacio I. Wistuba,1,7 and Jonathan M. Kurie1

1Department of Thoracic/Head and Neck Medical Oncology and 1Department of Molecular Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA. 3Department of Molecular and Human Genetics and 4Department of Medicine and Dan L. Duncan Cancer Center, Baylor College of Medicine, Texas, USA. 1Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA. 4Department of Neurosurgery, Baylor College of Medicine, Houston, Texas, USA. 5Department of Translational Molecular Pathology, Division of Pathology and Laboratory Medicine, and 6Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA. 7Department of Translational Molecular Pathology, Division of Pathology and Laboratory Medicine, and 8Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA.

Introduction

Metastasis is the primary cause of cancer-related death (1) and is driven by tumor cells that are uniquely capable of switching reversibly between epithelial and mesenchymal states in response to extracellular cues (2). During epithelial-to-mesenchymal transition (EMT), tumor cells switch their axis of polarity from apical-basal to front-rear, detach from neighboring cells, and become more motile and invasive and more resistant to cytotoxic chemotherapy (3–6). EMT is activated by several transcription factor families (ZEB, SNAIL, TWIST, FOXO, and others) (7). In a pancreatic cancer model driven by PDX1-Cre–mediated activation of mutant Kras and p53 alleles, multiple EMT-activating transcription factors (EMT-TFs) are upregulated, and metastasis is ablated by inactivation of ZEB1 but not SNAIL or TWIST (8, 9), suggesting variable prometastatic activities. In breast and lung adenocarcinomas, high ZEB1 expression levels confer tumor-initiating and metastatic activities and are correlated with a poor prognosis (10). ZEB1 downregulates the expression of epithelial polarity genes and microRNAs (miRs; e.g., miR-34a, miR-148a, and miR-200 family members) that promote epithelial differentiation and inhibit tumor initiation and metastasis by regulating actin cytoskeletal remodeling, Golgi compaction, and polarized vesicle trafficking (4, 11, 12). Thus, miRs are critical downstream effectors of EMT-TFs.

Competing endogenous RNAs (ceRNAs) have binding sites for a common set of microRNAs (miRs) and regulate each other’s expression by sponging miRs. Here, we address whether ceRNAs govern metastasis driven by the EMT-activating transcription factor ZEB1. High miR-181b levels were correlated with an improved prognosis in human lung adenocarcinomas, and metastatic tumor cell lines derived from a murine lung adenocarcinoma model in which metastasis is ZEB1-driven were enriched in miR-181b targets. ZEB1 relieved a strong basal repression of α integrin (ITGA1) mRNA, which in turn upregulated adenyl cyclase 9 mRNA (ADC9) by sponging miR181b. Ectopic expression of the ITGA1 3′-untranslated region reversed miR-181b–mediated metastasis suppression and increased the levels of adenyl cyclase 9 protein (AC9), which promoted tumor cell migration and metastasis. In human lung adenocarcinomas, ITGA1 and ADC9 levels were positively correlated, and an AC9-activated transcriptomic signature had poor-prognostic value. Thus, ZEB1 initiates a miR-181b–regulated ceRNA network to drive metastasis.

Epithelial tumor cells undergo epithelial-to-mesenchymal transition (EMT) to gain metastatic activity. Competing endogenous RNAs (ceRNAs) have binding sites for a common set of microRNAs (miRs) and regulate each other’s expression by sponging miRs. Here, we address whether ceRNAs govern metastasis driven by the EMT-activating transcription factor ZEB1. High miR-181b levels were correlated with an improved prognosis in human lung adenocarcinomas, and metastatic tumor cell lines derived from a murine lung adenocarcinoma model in which metastasis is ZEB1-driven were enriched in miR-181b targets. ZEB1 relieved a strong basal repression of α integrin (ITGA1) mRNA, which in turn upregulated adenyl cyclase 9 mRNA (ADC9) by sponging miR181b. Ectopic expression of the ITGA1 3′-untranslated region reversed miR-181b–mediated metastasis suppression and increased the levels of adenyl cyclase 9 protein (AC9), which promoted tumor cell migration and metastasis. In human lung adenocarcinomas, ITGA1 and ADC9 levels were positively correlated, and an AC9-activated transcriptomic signature had poor-prognostic value. Thus, ZEB1 initiates a miR-181b–regulated ceRNA network to drive metastasis.

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miR-181b–regulated ceRNA network involving α and remain in an epithelial state (18). The findings presented here metastatic KP cells do not exhibit such differentiation plasticities that are driven by high ZEB1 expression levels, whereas poorly metastatic KP cell lines undergo a reversible EMT in response to miR-181b. To identify miRs that govern an EMT-regulated, prometastatic effects, we first screened for miRs that are correlated with an improved prognosis and were not silenced during EMT, have targets that are enriched in the ZEB1 transcriptome, and function as suppressors of metastases (Figure 1B). Thus, miR-181b functioned as a metastasis suppressor in the context of a ZEB1-driven lung adenocarcinoma model.

We reasoned that if ZEB1 increases metastasis by activating a miR-181b–regulated ceRNA network, then high ZEB1 levels should antagonize the prometastatic activity of antagoniR-181b. To test this idea, we compared the effects of antagoniR-181b on KP cells with high (344SQ, 344P) and low (393P, 307P) ZEB1 levels. Although antagoniR-181b prominently decreased miR-181b levels in both groups (Supplemental Figure 3A), prometastatic effects were observed only in low-ZEB1 KP cells, which demonstrated enhanced cell migration (Figure 2A), reduced anoikis (Figure 2, B and C), increased colony formation in soft agar (Figure 2D), and increased miR-181b target gene expression (Figure 2, E, F). Importantly, ectopic ZEB1 expression attenuated the sensitivity of low-ZEB1 tumor cells (393P, 307P, HCC827) to antagoniR-181b (Figure 2G and Supplemental Figure 3, B and C). These findings supported further studies to determine whether ZEB1 activates a miR-181b–regulated ceRNA network.

To identify ZEB1-upregulated miR-181b targets that might function as ceRNAs, we queried RNA-Seq data from 393P_ZEB1 cells and 393P vector cells and found that the genes upregulated at least 2-fold by ectopic ZEB1 expression were enriched in predicted targets of the 6 miRs, we used a prediction algorithm (www.targetscan.org) to query RNA-Seq data from 393P_ZEB1 cells and 393P vector cells and found that the genes upregulated at least 2-fold by ectopic ZEB1 expression were enriched in predicted targets of the 6 miRs.

Figure 1. miRs not repressed by ZEB1 have metastasis suppressor activity. (A) Quantitative reverse transcription PCR (qPCR) analysis of miRs in 393P_ZEB1 cells (ZEB1) and 393P_vector cells (Vec). Results expressed relative to 393P_vector cells. miR-200b included as positive control. (B) Enrichment of ZEB1-upregulated genes in miR targets as assessed by a hypergeometric test. (C) qPCR assays quantify antagoniR-mediated depletion of miRs in 393P cells. Results expressed relative to control antagoniRs (anti-NC). (D) Boyden chamber assays on antagoniR- and control-transfected cells. Migratory and invasive cells were photographed (images) and counted (bar graphs). Scale bar: 100 μm. (E) Relative densities of transfectants on plastic. (F) Primary tumor weights (left) and numbers of lung metastases (right) in mice injected subcutaneously with 344SQ cells that express ectopic miR-181b or vector (Vec). (G) Fluorescence microscopic images of lung metastases in mice injected by tail vein with red fluorescent protein–labeled (RFP-labeled) 344SQ cells transfected with miR-181b or control mimics. Scatter plot: numbers of lung metastases per mouse (dots). (H) Numbers of viable 344SQ and H1299 transfecants on low-adhesion plates. n = 4. (I) Western analysis detects cleaved PARP as a marker of apoptosis in transfectants on adhesive or low-adhesion (suspension) plates. β-Actin is loading control. (J) Quantitative reverse transcription PCR (qPCR) analysis of miRs compared with scrambled control transfectants, antagoniR-181b–transfected 344SQ cells and H1299 cells underwent more anoikis in suspension cultures (Figure 1, H and I) and formed fewer colonies in soft agar (Figure 1J) but exhibited no differences in proliferation in monolayer culture (Supplemental Figure 2C). Conversely, compared with scrambled control transfectants, antagoniR-181b–transfected 393P cells formed more lung colonies following tail vein injection (Figure 1K and Supplemental Figure 2D). Thus, miR-181b functioned as a metastasis suppressor in the context of a ZEB1-driven lung adenocarcinoma model.

Results

ZEB1 upregulates ITGA1 to inactivate the metastasis suppressor miR-181b. To identify miRs that govern an EMT-regulated, prometastatic ceRNA network, we first screened for miRs that are correlated with an improved prognosis in lung cancer, are not silenced during EMT, have targets that are enriched in the ZEB1 transcriptome, and function as suppressors of metastases (Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI97225DS1). To validate these findings, we performed quantitative reverse transcription PCR (qPCR) analysis on KP cell lines that have been classified as mesenchymal or epithelial (11) and on 393P_ZEB1 cells and 393P_vector cells, which confirmed that 6 of the 8 miRs were not silenced during EMT (Figure 1A and Supplemental Figure 1B). To determine whether the ZEB1 transcriptome is enriched in predicted targets
Figure 2. ZEB1 suppresses the prometastatic activity of antagomiR-181b. (A) Boyden chamber assays on KP cells with low endogenous ZEB1 levels (393P and 307P) or high endogenous ZEB1 levels (344SQ and 344P) after transfection with antagomiR-181b or control antagomirs (anti-NC). Migratory cells were photographed (images) and counted (bar graph). Results expressed relative to controls (anti-NC). Scale bars: 200 μm. (B) Numbers of viable antagomiR-181b- and anti-NC-transfected cells seeded on low-adhesion plates and counted 24–48 hours later. n = 4. (C) Western analysis to detect cleaved PARP in transfectants seeded on adhesive or low-adhesion (suspension) plates. β-Actin included as loading control. (D) Soft agar colony formation by antagomiR-181b- and anti-NC-transfected cells. Colonies were photographed (images) and counted (bar graph). Results expressed relative to controls. Scale bar: 100 μm. (E) 3′-UTR activity assays of a known miR-181b target, BCL2. 393P and 344SQ cells were transfected with a BCL2 3′-UTR luciferase reporter and antagomiR-181b or anti-NC. Luciferase activity was normalized and expressed as a ratio (antagomiR-181b/anti-NC). ZEB1 3′-UTR included as a negative control. n = 4. (F) Western analysis of BCL2 levels in 393P and 344SQ cells transfected with antagomiR-181b or anti-NC. The numbers indicate the normalized expression levels of BCL2 protein. (G) Boyden chamber assays on antagoniR-181b- and anti-NC-transfected cells. Migratory and invasive cells were photographed (images) and counted (bar graph). Results expressed relative to anti-NC. Scale bars: 200 μm. **P < 0.01 and ***P < 0.001. Values are mean ± SD. n = 3, unless otherwise indicated. P values, 2-tailed Student’s t test. Results were replicated (n ≥ 2 experiments).
fold by ectopic ZEB1 expression (Figure 3A). qPCR analysis of the most highly upregulated miR-181b targets in the KP cell line panel showed that ITGA1 was the only miR-181b target that was differentially expressed between mesenchymal and epithelial KP cells (Supplemental Figure 4A). In The Cancer Genome Atlas human lung adenocarcinoma database (https://gdc.cancer.gov/) (n = 1,016 tumors), ZEB1 and ITGA1 mRNA levels were positively correlated (Supplemental Figure 4B). In 344SQ cells, ITGA1 and miR-181b were approximately equimolar (~300 copies per cell) (Figure 3B), an important determinant of ceRNA functionality (21). ITGA1 contains 3 predicted miR-181b–binding sites in the coding sequence and 1 site in the 3′-untranslated region (3′-UTR) (www.targetscan.org). Relative to empty vector transfecants, miR-181b–transfected 344SQ cells had lower activities of reporters containing the ITGA1 3′-UTR but not reporters containing the ITGA1 coding sequence or a mutant ITGA1 3′-UTR lacking the predicted miR-181b site (Figure 3C). A negative control miR (miR-181c) did not suppress ITGA1 3′-UTR activity (Figure 3C). To address whether miR-181b binds to the ITGA1 3′-UTR, we performed R17/MS2 coat protein–based (MS2-based) RNA immunoprecipitations (22). Compared with MS2 RNA and a negative control miR (miR-200b), an MS2-ITGA1 3′-UTR fusion transcript was significantly enriched in miR-181b (Figure 3D). A mutation in the predicted miR-181b–binding site in the ITGA1 3′-UTR abrogated the enrichment (Figure 3D).

ITGA1 encodes α1 integrin, which is a component of a heterodimeric collagen receptor (αβ1) that stimulates actin cytoskeletal remodeling and cell motility (23) but has no known role in EMT-driven metastasis. To determine whether it promotes metastasis through a noncoding RNA function, we used shRNAs to generate ITGA1-deficient 344SQ cells, reconstituted them with full-length ITGA1 or with ITGA1 constructs that lack 3′-UTR or translation start codon sequences, and injected the transfecants subcutaneously or intravenously into syngeneic, immunocompetent mice. Compared with ITGA1-replete cells, ITGA1-deficient 344SQ cells formed flank tumors that were less metastatic to the lungs following subcutaneous injection (Supplemental Figure 4, C and D) and generated fewer lung colonies following tail vein injection (Supplemental Figure 4, E and F). Lung colonization was partially restored by ITGA1 constructs that lack 3′-UTR or start codon sequences and completely restored by full-length ITGA1 (Figure 3E and Supplemental Figure 5, A and B). Thus, ITGA1 was prometastatic in part through its 3′-UTR.

To determine whether miR-181b–binding sites in the ITGA1 3′-UTR are required for metastasis, we introduced WT or mutant ITGA1 3′-UTR constructs lacking miR-181b–binding sites into 393P cells, which have relatively low endogenous ITGA1 levels (Supplemental Figure 4A). Compared with control transfecants, 393P cells transfected with the WT ITGA1 3′-UTR had increased migratory activity in Boyden chambers (Figure 3F) and reduced anoikis in suspension cultures (Figure 3G) and generated more lung colonies following tail vein injection (Figure 3H), whereas a mutant ITGA1 3′-UTR that lacks the miR-181b–binding site did not increase these metastatic properties (Figure 3, F–H). Similarly, compared with ITGA1-replete cells, ITGA1-deficient H1299 cells had reduced migratory activity in Boyden chambers, and the migratory activity of the ITGA1-deficient H1299 cells was restored by vectors that express WT ITGA1 3′-UTR but not a mutant ITGA1 3′-UTR that lacks the miR-181b–binding site (Figure 3D). Furthermore, ectopic miR-181b expression diminished the ITGA1 3′-UTR–induced increase in 393P cell migratory activity (Figure 3J), and antagomiR-181b partially rescued the migratory and invasive activities of ITGA1-deficient 344SQ cells (Figure 3K). Thus, the ITGA1 3′-UTR promoted metastasis through its miR-181b–binding activity.

ZEB1 upregulates ITGA1 through multiple intermediates. On the basis of the above evidence that ITGA1 is a prometastatic miR-181b target, we performed additional studies to elucidate the way in which ZEB1 upregulates ITGA1 expression. Ectopic ZEB1 expression increased RNA polymerase II binding to the ITGA1 gene promoter (Supplemental Figure 6A), indicating that ZEB1 increases ITGA1 gene transcription. However, ZEB1 functions primarily as a transcriptional repressor (24), which led us to reason that ZEB1 increases ITGA1 transcription through intermediates. To test this idea, we examined the kinetics of ITGA1 mRNA upregulation in 393P cells that express ZEB1 under the control of a doxycycline-inducible promoter. ITGA1 mRNA levels increased 72 hours after the initiation of doxycycline treatment, whereas the levels of miR-200b, a ZEB1 target (25), decreased within 24 hours (Supplemental Figure 6B). To determine whether the delay was the result of ZEB1-induced epigenetic regulation of the ITGA1 genomic locus, we used a prediction algorithm (http://www.urogene.org/methprimer/) and identified a CpG island in the ITGA1 gene locus (+186 to +400: +1 refers to the first nucleotide of the 5′-UTR) that bisulfite sequencing revealed to be more methylated in epithelial than in mesenchymal KP cells and that was demethylated in response to ectopic ZEB1 expression (Figure 4A). Treatment with the DNA methyltransferase inhibitor 5-azaacytidine increased ITGA1 mRNA levels in epithelial (393P, 307P, HCC827) but not mesenchymal (344SQ, 393P_ZEB1) lung cancer cells (Figure 4B and Supplemental Figure 6C). Relative to empty vector transfecants, ZEB1-transfected 393P cells had higher mRNA levels of ten-eleven translocation 2 gene (TET2) and lower levels of the DNA methyltransferases DNMT1 and DNMT3B (Figure 4C). In the KP cell line panel, mesenchymal differentiation status was positively correlated with TET2 levels and negatively correlated with DNMT1 and DNMT3B levels (Supplemental Figure 6C). In TET2 and DNMT1/3B gain- and loss-of-function studies, ITGA1 levels were upregulated by TET2 (Figure 4, D and E) but not DNMT1 or DNMT3B (Supplemental Figure 6, D–F). Thus, ZEB1 upregulated TET2 to relieve an epigenetic repression of ITGA1.

Because CpG island demethylation facilitates transcription factor binding to promoter elements (26), we examined whether ITGA1 is a target of ZEB1-upregulated transcription factors. Relative to empty vector transfecants, ZEB1-transfected 393P cells had higher ITGA1 promoter reporter activity (Supplemental Figure 6G), which was attenuated by mutation of an ARNT-binding site but not predicted binding sites for other transcription factors (Figure 4F). ARNT bound to the ITGA1 promoter in chromatin immunoprecipitation assays (Figure 4G). ITGA1 mRNA levels (Figure 4H) and ITGA1 promoter reporter activity (Figure 4I) were lower in ARNT siRNA–transfected cells than in scrambled controls. ARNT mRNA and protein levels were higher in mesenchymal-

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Figure 3. ITGA1 3′-UTR promotes metastasis through its miR-181b-binding site. (A) RNA-Seq analysis of predicted miR-181b targets in 393P_ZEB1 cells and 393P_vector cells expressed as a ratio (ZEB1/Vec). (B) miR-181b and ITGA1 mRNA copy numbers. (C) EGFP reporters fused to ITGA1 coding sequence (CDS) or 3′-UTR containing wild-type (WT) or mutated (MT) miR-181b-binding sites. Western analysis (bottom) of EGFP in 344SQ cells cotransfected with EGFP reporters and miR-181b, miR-181c, or vector (Vec). (D) Constructs containing M52 binding sites (12X) fused downstream of a WT (UTR-WT-M52) or mutated (UTR-MT-M52) ITGA1 3′-UTR. M52-based RIP (bottom). M52-UTR-associated miR-181b and miR-200b (negative control) quantified as fold enrichment relative to M52. (E) Fluorescence microscopically images of lung metastases in mice injected with the 344SQ_RFP reporter lacking the miR-181b–binding site but not a mutant ITGA1 3′-UTR that lacks the miR-181b–binding site (Figure 5D).

By the 3′-UTRs of ADCY9 or ONECUT2 were lower in miR-181b–transfected 344SQ cells than in negative control transfectants, and loss of the miR-181b-binding sites in the ADCY9 and ONECUT2 3′-UTRs ablated the miR-181b–induced suppression of both reporters (Figure 5B), which is consistent with a prior report on ADCY9 (27).

To determine whether predicted miR-181b targets identified from the above screen can compete with ITGA1 for binding to miR-181b, we subjected ITGA1-deficient and -replete 344SQ cells to Argonaute 2 (AGO2) immunoprecipitation assays. ITGA1 depletion led to a significant increase in the incorporation of ADCY9 but not TGFBR3, ONECUT2, or BHLHE41 into the RNA-induced silencing complex (Figure 5C). Incorporation of ADCY9 into the RNA-induced silencing complex was reduced by ectopic expression of the ITGA1 3′-UTR

On the basis of the above findings, we examined whether ITGA1 regulates ADCY9 expression through competition for binding to miR-181b. ADCY9 mRNA levels were higher in ITGA1 3′-UTR-transfected 393P cells than in controls, whereas a mutant ITGA1 3′-UTR that lacks the miR-181b–binding site did not increase ADCY9 levels (Figure 5E). Conversely, ADCY9 levels were lower in ITGA1 3′-UTR siRNA-transfected 344SQ cells than in scrambled controls and were not rescued by cotransfection of ITGA1 coding sequences (Supplemental Figure 7, B and C). ADCY9 3′-UTR reporter activity was higher in ITGA1 3′-UTR-transfected 393P cells than in controls and was reduced by loss of the miR-181b–binding sites in either 3′-UTR (Figure 5F). Conversely, relative to scrambled controls, ITGA1 3′-UTR siRNA-transfected 344SQ cells had lower activity of a WT ADCY9 3′-UTR reporter but not a mutant ADCY9 3′-UTR reporter lacking the miR-181b–binding sites (Supplemental Figure 7D). To determine whether ZEB1-induced upregulation of ADCY9 is ITGA1-dependent, we depleted ITGA1 from 393P_ZEB1 cells and found that ITGA1 depletion suppressed ZEB1-induced ADCY9 mRNA and protein levels (Supplemental Figure 7, E and F).

On the basis of the above evidence that ITGA1 regulates ADCY9 levels through competition for miR-181b, we reasoned that ADCY9 and ITGA1 could interact bidirectionally through this competition. To address this possibility, we first depleted ADCY9 from 344SQ cells and found that, relative to control oligomer-transfected cells, ADCY9 siRNA–transfected cells had reduced ITGA1 mRNA levels (Supplemental Figure 7G). To determine whether ADCY9 upregulates ITGA1 levels through competition for miR-181b, we ectopically expressed the ADCY9 3′-UTR in 393P cells and found that ITGA1 mRNA levels were higher in ADCY9 3′-UTR-transfected cells than in enhanced green fluorescent protein–transfected (EGFP-transfected) controls, whereas a mutant ADCY9 3′-UTR that lacks miR-181b–binding sites did not have this effect (Supplemental Figure 7H). Moreover, in the KP cell line panel, ITGA1 and ADCY9 levels were positively correlated (Supplemental Figure 7I). Thus, ITGA1 and ADCY9 are components of a miR-181b–regulated ceRNA network.

To determine whether these findings are broadly applicable to human cancer, we mined RNA-Seq data from The Cancer Genome Atlas, the largest publicly available cohort of human cancer transcriptomes. Lung adenocarcinomas (n = 541) were grouped on the basis of high or low expression (defined as top and bottom quartiles, respectively) of ITGA1 and ADCY9. ADCY9 levels were significantly higher in the ITGA1-high group than in epithelial KP cell lines (Supplemental Figure 6A). Activities of reporters driven by the 3′-UTRs of ADCY9 or ONECUT2 were lower in miR-181b-transfected 344SQ cells than in negative control transfectants, and loss of the miR-181b-binding sites in the ADCY9 and ONECUT2 3′-UTRs ablated the miR-181b–induced suppression of both reporters (Figure 5B), which is consistent with a prior report on ADCY9 (27).

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than in the ITGA1-low group (Figure 5G), and ITGA1 levels were significantly higher in the ADCY9-high group than in the ADCY9-low group (Figure 5H). Moreover, by pairwise comparisons, ZEB1, ITGA1, and ADCY9 were positively correlated with each other in 16 of 32 different cancer types examined (Figure 5I and Supplemental Table 1).

**ADCY9 mediates ZEB1-driven metastasis.** ADCY9 encodes 1 of 9 transmembrane adenyl cyclase (AC) protein isoforms (AC1–9) that catalyze the synthesis of cAMP from ATP and are regulated primarily by heterotrimeric G protein–coupled receptors (28). As a second messenger molecule, CAMP binds and activates multiple effector proteins that have been studied extensively in cancer (28), but whether AC proteins regulate metastasis remains unclear.

To address this question, we first quantified cAMP levels in the KP cell line panel. cAMP levels were higher in mesenchymal KP cells than in epithelial KP cells (Supplemental Figure 8A), and they were higher in 393P_ZEB1 cells than in 393P_vector cells (Supplemental Figure 8B). Next, we treated 393P cells with the membrane-permeable cAMP analog 8-bromo-cAMP or the AC activator forskolin (Supplemental Figure 8C). Both treatments increased 393P cell migration and invasion in Boyden chambers (Figure 6A and Supplemental Figure 8D). These findings show that ZEB1 increased cAMP production, which enhanced the metastatic properties of tumor cells.

On the basis of the above findings, we examined whether ZEB1-driven metastasis requires ADCY9. Relative to scrambled control transfecants, ADCY9 siRNA-transfected 344SQ cells (Figure 6B) had lower cAMP levels (Figure 6C), were less migratory and invasive in Boyden chambers (Figure 6D), exhibited more anoikis in suspension cultures (Figure 6E), formed fewer colonies in soft agar (Figure 6F), and generated fewer lung colonies after tail vein injection (Figure 6G and Supplemental Figure 8E). Conversely, compared with empty vector transfecants, 393P cells that ectopically express ADCY9 (Figure 6H) had higher cAMP levels (Figure 6I) and were more migratory (Figure 6J). To determine whether ADCY9 is sufficient to reverse miR-181b’s metastasis suppressor activity, we restored ADCY9 levels in 344SQ and H1299 cells that ectopically express miR-181b and showed that, in Boyden chambers, migratory activity was higher in ADCY9-restored cells than in empty vector-transfected cells (Supplemental Figure 8F). To confirm that ADCY9 mRNA promoted tumor cell migration through its protein product rather than its 3′-UTR, we reconstituted ADCY9-deficient 344SQ cells with protein-coding or 3′-UTR sequences of ADCY9 (Supplemental Figure 8G) and showed that, in Boyden chambers, the migratory activity of ADCY9-deficient cells was restored to a greater extent by protein-coding sequences than by 3′-UTR sequences (Supplemental Figure 8H).

To determine whether ADCY9 is the only AC isoform through which ZEB1 activates cAMP production, we quantified the mRNA levels of all 9 transmembrane ADCY isoforms. Relative to 393P_vector cells, 393P_ZEB1 cells had higher mRNA levels of ADCY3, ADCY7, and ADCY9 (Supplemental Figure 9A). Compared with scrambled control transfecants, ADCY7 siRNA-transfected 344SQ cells (Supplemental Figure 9B) had lower cAMP levels (Supplemental Figure 9I) and reduced migratory activity in Boyden chambers (Supplemental Figure 9C) but exhibited no change in cell proliferation (Supplemental Figure 9D). In contrast, ADCY3 siRNA-transfected 344SQ cells (Supplemental Figure 9B) had no detectable change in cAMP levels (Supplemental Figure 9C) or 344SQ cell migration (Supplemental Figure 9F) or proliferation (Supplemental Figure 9G). Thus, ZEB1 activates cAMP production and promotes 344SQ cell migration through ADCY7 and ADCY9.

On the basis of the above evidence that ADCY9 promotes metastasis in a ZEB1-driven lung adenocarcinoma model, we examined the prognostic value of ADCY9 in human lung adenocarcinomas. For this purpose, we probed a compendium of clinically annotated human lung adenocarcinomas (n = 1,586) with a signature of 1,421 genes that were differentially expressed (601 up, 820 down; P < 0.01 by t test, fold change > 1.4) in ectopic ADCY9–expressing and empty vector–transfected 393P cells (Supplemental Table 2). qPCR analysis confirmed that 19 of 20 genes sampled were differentially expressed in 393P_ADCY9 cells and 393P_vector cells (Supplemental Figure 10A). Enrichment analysis showed that the differentially expressed genes have diverse gene functions (Supplemental Table 2) but none related to EMT, which we confirmed by qPCR analysis of EMT markers (Supplemental Figure 10, B and C). Using a “z score” approach described previously (29), we identified a manifestation of the 1,421-gene signature in human lung adenocarcinomas that was correlated with a significantly shorter duration of survival (log-rank P = 7.6 × 10−6) (Supplemental Figure 10D).

**Discussion**

We have identified a ceRNA network that plays a key role in ZEB1-driven lung adenocarcinoma metastasis. ITGA1 and ADCY9 competed for binding to miR-181b, and ZEB1 upregulated ITGA1
Figure 5. ITGA1 and ADCY9 are miR-181b–regulated ceRNAs. (A) qPCR of mRNAs in 393P transfectants (antagomiR-181b [anti-181b] or control [anti-NC]). Levels of 32 predicted miR-181b targets that were approximately equimolar with ITGA1 in 393P_ZEB1 cells expressed as a ratio (anti-181b/anti-NC). Red, significantly upregulated more than 2-fold. *P < 0.05 and **P < 0.001. (B) Schema. 3′-UTRs of ADCY9, ONECUT2, TGFBR3, and BHLHE41 with locations of predicted miR-181b–binding sites. Bar graph: luciferase activity in 344SQ cells cotransfected with miR-181b or control (miR-NC) and a 3′-UTR reporter with WT or mutant (MT) miR-181b–binding sites. Bar graph: luciferase activity in 344SQ cells cotransfected with miR-181b or control (miR-NC) and a 3′-UTR reporter with WT or mutant (MT) miR-181b–binding sites. Bar graph: luciferase activity in 344SQ cells cotransfected with miR-181b or control (miR-NC) and a 3′-UTR reporter with WT or mutant (MT) miR-181b–binding sites. Bar graph: luciferase activity in 344SQ cells cotransfected with miR-181b or control (miR-NC) and a 3′-UTR reporter with WT or mutant (MT) miR-181b–binding sites. Bar graph: luciferase activity in 344SQ cells cotransfected with miR-181b or control (miR-NC) and a 3′-UTR reporter with WT or mutant (MT) miR-181b–binding sites.

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Methods

Antibodies, plasmids, and oligomers. Antibodies against α, integrin (Santa Cruz Biotechnology, sc-271034), β-actin (Cell Signaling Technology, 4970), RNA polymerase II (Millipore, 05-623, clone CTD4H8), BCL2 (Cell Signaling Technology, 3498), MYC-tag (Cell Signaling Technology, 2272), PARP-1 (Cell Signaling Technology, 9542), ARNT (Cell Signaling Technology, 3444), EGFP (Sigma-Aldrich, G1544), ADCY9 (Abcam, ab191423), and AGO2 (Abcam, ab57113) were used. The ARNT (Cell Signaling Technology, 2272), PARP-1 (Cell Signaling Technology, 9542), ARNT (Cell Signaling Technology, 3444), EGFP (Sigma-Aldrich, G1544), ADCY9 (Abcam, ab191423), and AGO2 (Abcam, ab57113) were used. The following gene-specific shRNAs, siRNAs, and miR mimics were purchased from Sigma-Aldrich: mouse shITGA1 nos. 1-5 (TRCN0000025418, TRCN00000265481, TRCN00000254182, TRCN00000254180, and TRCN00000254181); mouse shTET2 nos. 1-3 (TRCN00000250895, TRCN00000250892, and TRCN00000217530); mouse siITGA1 nos. 1 and 2 (SASI_Mm01_00102775 and SASI_Mm02_00288356); human siITGA1 nos. 1 and 2 (SASI_Hs01_00067020 and SASI_Hs01_00067021); mouse siDNMT1 (SASI_Mm01_00024007); mouse siDNMT3 (SASI_Mm02_00286413); mouse siADCY9 (SASI_Mm01_000744401 and SASI_Mm01_000744441); mouse siADCY3 (SASI_Mm01_00139448 and SASI_Mm01_00139449); mouse siADCY7 (SASI_Mm02_00315533 and SASI_Mm02_00315534); and miR mimics (HMIO237 and HMIO270) and inhibitors (HSTUD0016, HSTUD0270, and HSTUD0387). The TET2 expression vector pcDNA3-Tet2 was a gift from Yi Zhang (Boston Children’s Hospital, Boston, Massachusetts, USA) (Addgene plasmid 60939) (39). pcDNA3/Myc-DNMT1 (Addgene plasmid 36399) and pcDNA3/Myc-DNMT3B1 (Addgene plasmid 35522) were gifts from Arthur Riggs (City of Hope, Duarte, California, USA) (40). pMS2-GFP (Addgene plasmid 27121) (41), and pSL-MS2-12X (Addgene plasmid 27119) (42) were gifts from Robert Singer (Albert Einstein College of Medicine, Bronx, New York, USA). pSL-MS2-12X was double-digested with EcoRI and XhoI, and the MS2-12X fragment was subcloned downstream of the ITGA1 3′-UTR in the pcDNA3.1 vector. The ITGA1 and ADCY9 coding sequences and pre-miR-181b were amplified from a cDNA isolated from 344SQ cells and subcloned into the pcLX-puro vector (Clontech). ITGA1 promoter fragments were amplified from the genomic DNA of 344SQ cells and subcloned into the pGL3-Basic vector (Promega). Transcription binding site mutations were created with a PCR-based site-directed mutagenesis method. The PCR primers are listed in Supplemental Table 3.

miRNA expression profiling. Murine lung cancer cell lines (713P, 307P, 344LN, 393LN, 393P, 412P, 344SQ, 344P, 531LN1, 531LN2, 531P1, and 531P2) were derived from KP mice as described previously (18). Human lung cancer cells (HCC827 and H1299) were purchased from the American Type Culture Collection and cultured in RPMI 1640 (Corning) supplemented with 10% FBS (Gibco). 393P_vector cells, 393P_ZEB1 cells, 307P_vector cells, 307P_ZEB1 cells, HCC827_vector cells, and HCC827_ZEB1 cells were described previously (11, 12, 45). Cells were transfected using jetPRIME Versatile DNA/siRNA transfection reagent (Polyplus). Stable cell transfectants were selected with puromycin (InvivoGene) or G418 (Corning). When indicated, 2 μM 5-aza-2′-deoxycytidine (Sigma-Aldrich), 1 μg/ml doxycycline (Sigma-Aldrich), 1 μg/ml forskolin (Sigma-Aldrich), or 200 μM 8-bromo-cAMP (Sigma-Aldrich) was added to the culture medium. Two thousand cells were plated in 96-well plates, and the WST-1 reagent (Roche) was used according to the manufacturer’s instructions to measure cellular proliferation. For anoikis assays, cells were plated in low-adhesion plates (Corning) and counted each day for 2 days. For soft agarose colony formation assays, 5 × 104 cells suspended in 0.4% agarose were seeded into 6-well plates layered with 0.8% agarose, and colonies were stained with 0.005% crystal violet after 2–3 weeks. For migration and invasion assays, 2 × 104 cells were cultured in the upper wells of Transwell or Matrigel chambers (BD Biosciences), respectively, and allowed to migrate toward 10% FBS in the bottom wells. After 8–10 hours of incubation, migrating or invading cells were stained with 0.1% crystal violet, photographed, and counted. For the cAMP

Figure 6. AC9 is essential for EMT-driven metastasis. (A) Boyden chamber assays of 393P cells treated with 8-bromo-cAMP or vehicle (C). Migratory cells were photographed (images) and counted (bar graph). Scale bar: 200 μm. (B) qPCR (bar graph) and Western analyses (gels) of AC9 expression in 344SQ cells stably transfected with scrambled (siCTL) or 1 of 2 AC9 siRNAs. (C) CAMP levels in 344SQ cells transfected with siCTL or siAC9. (D) Boyden chamber assays of 344SQ cells transfected with siAC9 or siCTL. Scale bar: 200 μm. (E) Bar graph: numbers of viable 344SQ transfectants seeded on low-adhesion plates and counted 24 hours later. n = 4 samples per condition. Gels: Western analysis to detect cleaved PARP as a marker of apoptosis in transfectants seeded on adhesive or low-adhesion (supplemental) plates. β-Actin included as loading control. (F) Soft agarose colonies were photographed (images) and counted (bar graph). Scale bar: 100 μm. (G) Fluorescence microscopic images of lung metastases in mice injected with RFP-labeled 344SQ cells transfected with siAC9 or siCTL. Scatter plot: numbers of lung metastasis per mouse (dots). (H) qPCR and Western analyses of AC9 expression in 393P_AC9 cells (AC9) and 393P_vector cells (Vec). (I) CAMP levels in 393P_AC9 cells and 393P_vector cells. (J) Boyden chamber assays of 393P_AC9 cells and 393P_vector cells. Scale bar: 200 μm. Values are mean ± SD. n = 3, unless otherwise indicated. P values, 2-tailed Student’s t test and Dunnett’s test for 2-group and more-than-2-group comparisons, respectively. Results were replicated (n ≥ 2 experiments).
assay, 10,000 cells were seeded into 96-well plates, and intracellular cAMP levels were detected using cAMP-Glo Max Assay (Promega) according to the manufacturer’s instructions.

**qPCR analysis.** Total RNA was isolated from cells using TRIzol and subjected to reverse transcription using the qScript CDNA super-Mix (Quanta Biosciences). mRNA levels were determined using SYBR Green Real-Time PCR Master Mixes (Thermo Fisher Scientific) and subjected to reverse transcription using the qScript cDNA super-mega) according to the manufacturer’s instructions. RNA was subsequently purified using the RNeasy RNA Synthesis Kit (New England BioLabs) according to the manufacturer’s instructions. For the AGO2 RIP assay, the 344SQ cells transfected with ITGA1 siRNA or control siRNA or 393P cells transfected with WT or mutated ITGA1 3′-UTR or empty vector were harvested and subjected to RIP assay using an anti-AGO2 antibody.

**Bisulfite sequencing.** Genomic DNA was extracted from 393P, 344SQ, 393P vector, and 393P_ZEB1 cells with the QIAamp DNA Mini Kit (Qiagen) and treated with bisulfite using the EpTect Bisulfite Kit (Qiagen). The ITGA1 promoter sequence was amplified from bisulfite-converted genomic DNA, and the PCR products were subcloned into T vector (Promega). Single clones were selected and subjected to direct Sanger sequencing.

**Animal husbandry.** All mouse studies were approved by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center. Immunocompetent 129/Sv mice syngeneic to the KP cells were bred in house and were injected subcutaneously in the right flank with tumor cells (n = 8-10 mice per group) and necropsied after 5 weeks or injected intravenously with tumor cells (n = 5–8 mice per group) and necropsied after 7-10 days. Primary tumors were weighed, and lung metastases on the pleural surfaces were counted. Mice received standard care and were euthanized according to the standards set forth by the Institutional Animal Care and Use Committee.

**Statistics.** The results are representative of replicated experiments and are the means ± SD from triplicate samples or randomly chosen cells within a field unless otherwise indicated. Statistical evaluations were carried out with Prism 6 (GraphPad Software Inc.). Unpaired 2-tailed Student’s t tests were used to compare the means.
of 2 groups. ANOVA with Dunnett’s tests was used to compare multiple treatments with a control.

The database of biological targets of miRs downloaded from TargetScan (www.targetscan.org) was used to predict biological targets of miRs (46). For miR target enrichment analysis, we performed data bioinformatics analyses using the R and Bioconductor package (https://www.r-project.org/; https://www.bioconductor.org/), a publicly available statistical computing tool that is widely used for high-throughput “omics” data analysis. To determine whether ZEB1-regulated genes are enriched in specific miR targets, we first used an R package, DESeq2, to identify genes that were differentially expressed between groups (393P_vector versus 393P_ZEB1). The Benjamini-Hochberg method (47) was then applied to adjust for multiple hypothesis testing and to estimate false discovery rate. Genes with a false discovery rate (q value) less than or equal to 0.001 were considered to be differentially expressed between 393P_vector and 393P_ZEB1. We then used the hypergeometric test to test for enrichment of differentially expressed genes in predicted miR targets that were identified using a prediction algorithm (www.targetscan.org).

To determine whether ZEB1, ITGAI, and ADCY9 levels were correlated in human cancers, we used RNA-Seq data from The Cancer Genome Atlas that included acute myeloid leukemia (LAML, n = 173); adrenocortical carcinoma (ACC, n = 79); bladder urothelial carcinoma (BLCA, n = 408); brain lower-grade glioma (LGG, n = 516); breast invasive carcinoma (BRCA, n = 1095); cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC, n = 304); cholangiocarcinoma (CHOL, n = 36); colorectal adenocarcinoma (CRC, combining COAD and READ projects, n = 623); esophageal carcinoma (ESCA, n = 184); glioblastoma multiforme (GBM, n = 161); head and neck squamous cell carcinoma (HNSC, n = 520); kidney chromophobe (KICH, n = 66); kidney renal clear cell carcinoma (KIRK, n = 533); kidney renal papillary cell carcinoma (KIRP, n = 290); liver hepatocellular carcinoma (LIHC, n = 371); lung adenocarcinoma (LUAD, n = 515); lung squamous cell carcinoma (LUSC, n = 501); lymphoid neoplasm diffuse large B cell lymphoma (DLBCL, n = 48); mesothelioma (MESO, n = 87); ovarian serous cystadenocarcinoma (OV, n = 262); pancreatic adenocarcinoma (PAAD, n = 178); pheochromocytoma and paraganglioma (PCPG, n = 179); prostate adenocarcinoma (PRAD, n = 497); sarcoma (SARC, n = 259); skin cutaneous melanoma (SKCM, n = 469); stomach adenocarcinoma (STAD, n = 415); testicular germ cell tumors (TGCT, n = 150); thymoma (THYM, n = 120); thyroid carcinoma (THCA, n = 503); uterine carcinosarcoma (UCS, n = 57); and uterine corpus endometrial carcinoma (UCEC, n = 545). P less than 0.05 was considered statistically significant.

To analyze a compendium of human lung adenocarcinoma cohorts (48) for the presence or absence of the ADCY9-derived expression signature, we used a t score described elsewhere (29).

Author contributions
JNK and XT conceived and designed the study. XT, PB, and Xiuping Liu developed the methodology. JMK, XT, Xin Liu, PB, MS, CB, JF, and IiW acquired data (provided animals, acquired and managed patients, provided facilities, etc.). XT, PB, CJ, LD, XZ, JY, JW, and KLS analyzed and interpreted the data (e.g., statistical analysis, bio-statistics, computational analysis). JMK and XT wrote, reviewed, and/or revised the manuscript. JMK, XT, PB, Xin Liu, DLG, CJ, AJ, MS, CB, JF, LD, JW, Xiuping Liu, and CGL provided administrative, technical, or material support (i.e., reporting or organizing data, constructing databases). JMK supervised the study.

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Address correspondence to: Jonathan M. Kurie, The University of Texas MD Anderson Cancer Center, Box 432, Department of Thoracic/Head and Neck Medical Oncology, 1515 Holcombe Boulevard, Houston, Texas 77030, USA. Phone: 713.745.6747; Email: jkurie@mdanderson.org.


