Induction of the RNA Regulator LIN28A Is Required for the Growth and Pathogenesis of RESTless Breast Tumors

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Abstract

The transcription factor RE1 silencing transcription factor (REST) is lost in approximately 20% of breast cancers. Although it is known that these RESTless tumors are highly aggressive and include all tumor subtypes, the underlying tumorigenic mechanisms remain unknown. In this study, we show that loss of REST results in upregulation of LIN28A, a known promoter of tumor development, in breast cancer cell lines and human breast tumors. We found that LIN28A was a direct transcriptional target of REST in cancer cells and that loss of REST resulted in increased LIN28A expression and enhanced tumor growth both in vitro and in vivo, effects that were dependent on heightened LIN28A expression. Tumors lacking REST expression were locally invasive, consistent with the increased lymph node involvement observed in human RESTless tumors. Clinically, human RESTless breast tumors also displayed significantly enhanced LIN28A expression when compared with non-RESTless tumors. Our findings therefore show a critical role for the REST-LIN28A axis in tumor aggression and suggest a causative relationship between REST loss and tumorigenicity in vivo. Cancer Res; 72(13); 3207–16. ©2012 AACR.

Introduction

We recently described a highly aggressive subset of human breast tumors in which loss of RE1 silencing transcription factor (REST) is associated with decreased disease-free survival (1). During an unbiased screening for tumor suppressors, REST was identified as a factor whose loss confers anchorage-independent growth upon human mammary epithelial cells (2). We identified a group of patients with RESTless breast cancers and showed that these patients have a poor prognosis; however, the causative role of REST loss and the downstream mechanism responsible for the aggression of these tumors has not been described.

REST is a transcriptional repressor that functions by binding to the repressor element 1 (RE1) found in the regulatory regions (3–5) of its approximately 2,000 target genes (4, 6) and recruiting chromatin-modifying enzymes including histone deacetylases (HDAC) and histone methyltransferases (HMT; refs. 7–11).

REST regulates the expression of the RNA-binding protein LIN28A in embryonic stem and neural progenitor cells (12).

LIN28A, which is highly expressed during normal development, maintains the self-proliferative capacity of progenitor cells (12–14). During cellular differentiation, LIN28A expression is decreased, resulting in the repression of genes involved in self-renewal and subsequent lineage commitment.

Aberrant expression of LIN28A in differentiated cells promotes transformation. Overexpression of murine Lin28a drives soft agar colony formation and tumor growth in nude mice (15). LIN28A expression also promotes the metastasis of MDA-MB-231 cells in a mouse model of breast cancer (16) and is associated with a variety of aggressive human cancers (15). However, little is known about how LIN28A is upregulated in these cancers. In this report, we show that REST directly regulates LIN28A expression in breast cancer cell lines and that REST loss upregulates LIN28A expression, which drives breast tumor growth and local invasion in vivo and increased clonogenicity and soft agar colony formation in vitro. We find that LIN28A is overexpressed in RESTless human breast tumors, supporting a clinically relevant role for the REST-LIN28A axis in breast cancer.

Materials and Methods

Detailed materials and methods are available in Supplementary Material.

Cell culture and generation of stable knockdown cell lines

All cell lines were purchased and authenticated from American Type Culture Collection and grown as previously described (1). MDA-MB-231 low-serum growth was conducted as previously described (17). REST knockdown was conducted as previously described (1). Stable knockdown of LIN28A (LIN28Alow) was achieved via lentiviral delivery of an anti-

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LIN28A shRNA (TRCN0000102579) in pLKO.1 vector (Open Biosystems). Plasmid 1864 (Addgene) was used as a control (LIN28A wildtype), lentiviral particles were generated and used as previously described (18).

**Western blotting and chromatin immunoprecipitation**

Antibodies used for Western blotting were REST (Upstate, 05-579), LIN28A (Abcam, ab46020), and β-actin (MP Biomedicals).

Chromatin immunoprecipitation (ChIP) was carried out as previously described (10); 2 µg of antibody (REST H-290, Santa Cruz Biotechnology; G9a 07-551, Upstate; rabbit IgG, Sigma-Aldrich) was added to 300 µg total protein. All washes were conducted with TSE with 500 mmol/L NaCl (10).

**Quantitative real-time PCR**

Total RNA was harvested from cultured cells (in biologic triplicate) or xenograft tumors using TRIzol (Invitrogen) according to the manufacturer’s protocol, resuspended in sodium citrate (1 mmol/L, pH 6.4) containing RNASecure (Ambion), quantitated via NanoDrop (Thermo Scientific), and reverse-transcribed using SuperScript III (Invitrogen) according to manufacturer’s instructions. Real-time PCR (RT-PCR) was carried out using SYBR Premix Ex Taq (Takara Bio Inc.).

**Luciferase reporter assay**

Approximately 2 kb of the LIN28A promoter region including (+RE1) or excluding (−RE1) the REST-binding (RE1) site was amplified from HEK-293T genomic DNA and cloned into pGL3-Basic (Promega). Cells were transfected with pGL3-Promoter, pGL3-Basic, pGL3-Basic + RE1, or pGL3-Basic − RE1, and RL-TK Renilla (Promega) using calcium phosphate.

Forty-eight hours after transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer’s instructions and luciferase signal was normalized to Renilla.

**Immunofluorescence**

Paraffin-embedded cells were deparaffinized, rehydrated, and stained according to antibody manufacturer’s protocol (Abcam). Antibodies used were LIN28A (Abcam ab6020) and Alexa Fluor goat anti-rabbit 594 (Invitrogen); Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Microscopic imaging was conducted with a digital camera (Spot II; Diagnostic Instruments) on a Nikon E600 Eclipse epifluorescent microscope with ×20 plan apochromatic objective and a standard TRITC filter cube. Images were acquired at an initial 36-bit tone scale and saved as 16-bit files. Minimal image manipulation was conducted using Adobe Photoshop according to the AACR guidelines.

**Clonogenic assay and soft agar colony formation**

Clonogenic assays were conducted as previously described (19).

**Xenograft experiments**

All procedures were conducted with the approval of the University of Wisconsin-Madison (Madison, WI) School of Medicine and Public Health Institutional Animal Care and Use Committee and according to national guidelines and policies. Adult intact female athymic nude-Foxn1nu mice (Harlan Laboratories) were used and MCF7 cells were treated as previously described (20).

Results

REST knockdown enhances tumorigenic phenotypes in vitro

Recently, we showed that REST is lost in 20% of human breast cancers, and that these RESTless tumors are highly aggressive (1). Here, we show that REST loss drives tumor aggression. We used lentiviral delivery of short hairpin RNAs (shRNA) to generate stable control (RESTnorm) or REST knockdown cell lines (RESTlow cells; the term RESTless refers only to human tumors). We knocked down REST in a nontransformed breast cell line (MCF10A), and in estrogen receptor-positive (ER+) and triple-negative (ER−/PR−/Her2−) breast cancer cell lines (MCF7 and MDA-MB-231, respectively). REST knockdown was confirmed by Western blotting (Fig. 1A and B and previously shown in ref. 1).

We first tested the tumorigenicity of RESTlow and RESTnorm breast cells using a clonogenic assay (19), which estimates the ability of individual cells to form colonies. In all 3 cell lines tested, REST knockdown significantly increased plating efficiency (Fig. 1C–E).

Using an ER−/c (MCF7) and an ER−/b (MCF10A) cell line, we repeated the clonogenicity assay using a soft agar protocol. In both cell lines, REST knockdown significantly increased (3-fold) the number of colonies observed (Fig. 1F and G).

REST knockdown increases tumor growth in mice

We carried out xenograft experiments to test the in vivo tumorigenicity of RESTnorm and RESTlow MCF7 cells. Work that will be described elsewhere suggests that RESTlow MCF7 cells would show enhanced growth in vivo in the absence of estrogen supplementation. RESTim and RESTlow MCF7 cells were injected s.c. into the flanks or mammary fat pads of intact female athymic Foxn1nu mice, and tumor growth was monitored. Two hundred days after injection, 30% (8 of 28) of RESTlow mammary fat pad injection sites developed tumors (1,980 mm3 = tumor volume), compared with 0% (0 of 28, 0 mm3 = tumor volume) of RESTnorm injections (Fig. 2A and B). The tumor take rate was also significantly greater for RESTlow versus RESTnorm cells injected s.c. into the flanks of the mice, with 34.4% (11 of 32) of RESTlow injection sites giving rise to tumors, compared with 12.5% (4 of 32) of RESTnorm sites (Fig. 2C). The total tumor burden for flank tumors was significantly greater for RESTlow than RESTnorm tumors, at 4,314 mm3 and 1,017 mm3, respectively (Fig. 2D). REST knockdown therefore results in a significant increase in tumorigenicity of MCF7 cells at both orthotopic and nonorthotopic sites.

Histopathologic examination of RESTlow tumors showed that they were highly anaplastic, with enlarged nuclei, prominent nucleoli, many convoluted nuclear envelopes, and a high mitotic rate (Fig. 2E). RESTlow tumors contain approximately
1.5-fold more cells undergoing mitosis than REST\textsuperscript{norm} tumors; however, because of the small number of REST\textsuperscript{norm} tumors available for analysis ($n = 3$), this difference did not reach statistical significance ($P = 0.1$). About 62.5% (5 of 8) of REST\textsuperscript{low} flank tumors examined show localized invasion into adjacent muscle (Fig. 2F) and one showed lymphovascular invasion (Fig. 2G).

REST regulates LIN28A expression in breast cancer cells

We hypothesized that one or more of the genes that become simultaneously derepressed upon REST knockdown (1) must contribute to RESTless breast tumor aggression. One such gene was LIN28A, given that LIN28A was upregulated in RESTless cells \textit{in vitro} and \textit{in vivo}, was a REST target (4, 12), and was previously implicated in breast cancer progression (15, 16), we sought to determine whether LIN28A was an effector of the RESTless phenotype. We confirmed previously published microarray data showing that LIN28A mRNA was elevated in the breast cancer cell line T47D upon REST knockdown (1) by quantitative RT-PCR (Fig. 3A). This increase in mRNA correlated with increased LIN28A protein (Fig. 3B). LIN28A mRNA and protein are similarly upregulated upon REST knockdown in MCF7 cells (Fig. 3C and D). REST has been shown to regulate LIN28 in embryonic stem and neural progenitor cells (12), and the LIN28A promoter region contains an evolutionarily conserved REST-binding site. To determine whether LIN28A is a direct target of transcriptional regulation by REST in our tumor model (MCF7 cells),
we conducted ChIP with an anti-REST antibody and interrogated for the LIN28A RE1 sequence (Fig. 3E); a region of DNA that does not contain an RE1 site serves as a negative control (NC). To confirm that REST binding to the putative RE1 site was ablated by REST knockdown, this ChIP was conducted in both REST\textsuperscript{norm} and REST\textsuperscript{low} MCF7 cells. We find robust enrichment of REST at the LIN28A RE1 site in REST\textsuperscript{norm} MCF7 cells. Indeed, REST enrichment at the LIN28A RE1 is twice that at the brain-derived neurotrophic factor (BDNF) RE1, a well-characterized REST-binding site (21–23). As expected, upon REST knockdown, REST-binding enrichment is lost at both the BDNF and LIN28 RE1 sites. To

Figure 2. REST knockdown increases the aggressiveness of MCF7 tumor growth in nude mouse xenografts. A, a total of 10^6 control (REST\textsuperscript{norm}) or REST knockdown (REST\textsuperscript{low}) MCF7 cells were injected s.c. into the mammary fat pads of female athymic nude mice. Tumor incidence was monitored weekly and is significantly higher for REST\textsuperscript{low} versus REST\textsuperscript{norm} cells (P = 0.0022). B, tumor burden in the mammary fat pads is significantly larger in REST\textsuperscript{norm} versus REST\textsuperscript{low} tumors (P < 0.0001). C, a total of 10^5 control (REST\textsuperscript{norm}) or REST knockdown (REST\textsuperscript{low}) MCF7 cells were injected s.c. into the flanks of female athymic nude mice. Tumor incidence upon injection into flanks is significantly higher for REST\textsuperscript{low} versus REST\textsuperscript{norm} MCF7 cells (P = 0.0382). D, tumor burden in flanks is significantly larger in REST\textsuperscript{low} versus REST\textsuperscript{norm} tumors (P = 0.0016). E–G, bright field photomicrographs of hematoxylin and eosin-stained sections of REST\textsuperscript{low} tumors. Representative images show the histopathology (E) and local invasion (F) observed in these tumors. Arrows in F indicate muscle fibers incorporated into the tumor. G, REST\textsuperscript{low} tumor and adjacent mammary fat pad; arrows indicate lymphovascular invasion.
Figure 3. REST is a direct transcriptional repressor of LIN28A in breast cancer cells. A, LIN28A mRNA in REST<sup>norm</sup> and REST<sup>low</sup> T47D cells was analyzed by quantitative PCR and normalized to actin (\( P = 0.05 \)). B, Western blot analyses for LIN28A and \( \beta \)-actin (loading control) in REST<sup>low</sup> and REST<sup>norm</sup> T47D cells. C, LIN28A mRNA in REST<sup>norm</sup> and REST<sup>low</sup> MCF7 cells was analyzed by quantitative PCR and normalized to actin (\( P = 0.0053 \)). D, immunofluorescent staining for LIN28A in REST<sup>norm</sup> and REST<sup>low</sup> MCF7 cells. EChIP in MCF7 cells with anti-REST antibody and IgG (sham). DNA analyzed by qRT-PCR with primers in the LIN28 and BDNF (positive control) promoter regions; negative control (NC) lacks RE1 site. F, ChIP in MCF7 cells using anti-REST or anti-G9a antibody and IgG (sham). DNA was analyzed via qRT-PCR using primers up- and downstream of TSS. G–I, schematic representation of the LIN28 promoter region showing placement of primers used to clone the LIN28 promoter region including (+ RE1) or excluding (− RE1) the REST-binding site into a pGL3 luciferase reporter plasmid, which was transfected into REST<sup>norm</sup> and REST<sup>low</sup> MCF7 (F) and HEK (G) cells. Luciferase expression was normalized to Renilla. J, LIN28A immature and processed mRNA in REST<sup>low</sup> versus REST<sup>norm</sup> tumors measured by quantitative PCR (\( P < 0.04 \)). Housekeeping genes showed no significant difference.
To test whether the regulation of LIN28A by REST observed in vitro was also present in vivo, we measured LIN28A RNA levels in RESTnorm and RESTlow MCF7 tumor tissue. Both LIN28A mRNA, as measured by 2 different primer pairs (pair 1 and pair 2) and LIN28A immature RNA (pre-mRNA), measured using primers in the second intron, are significantly increased in RESTlow versus RESTnorm MCF7 tumors (Fig. 3J). None of the housekeeping genes analyzed shows significant differences in expression between RESTnorm and RESTlow tumors.

**Figure 4.** LIN28A is necessary and sufficient for RESTlow phenotypes in vitro. A, immunoblotting for LIN28A or β-actin (loading control) in control (LIN28Anorm) or LIN28A knockdown (LIN28Alow) MCF7 cells. B, soft agar colony formation of RESTnorm and RESTlow MCF7 cells with and without anti-LIN28 shRNA (LIN28Alow; P = 0.0383). C and D, growth of RESTnorm and RESTlow MDA-MB-231 cells with and without anti-LIN28 shRNA (LIN28Alow) in high (C, 10%) or low (D, 2.5%) serum (72 hours; *, P ≤ 0.05). E–G, lentiviral delivery of a LIN28A expression construct was used to generate LIN28A-overexpressing MCF7 cells; expression was verified by Western blot analysis (E). Clonogenicity (F) and soft agar colony formation (G) were measured (*, P ≤ 0.05).

**RESTlow in vitro phenotypes are LIN28A-dependent**

To evaluate the contribution of LIN28A to the RESTlow phenotype, RESTnorm and RESTlow MCF7 cells were transduced with a lentiviral construct expressing an anti-LIN28A shRNA (LIN28Alow) or control shRNA (LIN28Anorm), and LIN28A knockdown was verified (Fig. 4A). The anti-LIN28A shRNA used does not knockdown LIN28B (data not shown). We then conducted a soft agar colony formation assay and found that the increase in colony formation observed upon REST knockdown is ablated by concurrent knockdown of LIN28A (Fig. 4B).
We then examined the effects of REST and LIN28A on the growth rate of estrogen receptor-negative MDA-MB-231 cells. When cells were grown in high serum (10% FBS), there was no significant difference in growth rate between RESTlow and RESTnorm cells; however, LIN28A knockdown significantly retarded the growth of RESTlow but not RESTnorm cells (Fig. 4C), suggesting that the RESTlow cells have acquired a dependence upon LIN28A that is not present in RESTnorm cells. Because MDA-MB-231 cells proliferate at near-maximal rate in the presence of 10% FBS, we then conducted the growth assay using low-serum (2.5%) conditions previously shown to unmask growth phenotypes in MDA-MB-231 cells (17). Under low-serum conditions, RESTlow cells show increased proliferation relative to RESTnorm cells but lose their growth advantage upon LIN28A knockdown (Fig. 4D).

**LIN28A is sufficient to recapitulate REST knockdown phenotypes in MCF7 cells**

To determine whether LIN28A overexpression is a functional effect of the RESTlow phenotypes, LIN28A was stably overexpressed in MCF7 cells (Fig. 4E). LIN28A overexpression caused approximately a 2× increase in plating efficiency and colonies grown in soft agar (Fig. 4F and G). We therefore conclude that in MCF7 cells, LIN28A overexpression is sufficient to induce the cell culture phenotype of RESTlow cells.

**LIN28A contributes to RESTless tumor formation**

To determine whether the upregulation of LIN28A observed in RESTlow cells contributes to the tumorigenicity observed in vivo, we compared the tumorigenicity of RESTlow cells with and without LIN28A expression. RESTlow/LIN28Anorm or RESTlow/LIN28Alow MCF7 cells were injected s.c. into the mammary fat pads of athymic nude mice, and tumor incidence and growth were monitored weekly. A and B, tumor incidence (A; P = 0.020) and burden (B; P = 0.0002) are decreased upon LIN28A knockdown.

**LIN28A expression is increased in human RESTless breast tumors**

Given the dependence of tumor growth on LIN28A in RESTlow cells in xenograft assays, we assessed LIN28A levels in human RESTless tumors. We analyzed publicly available cDNA microarray data from 2 breast tumor cohorts, GSE2034 (24, 25) and GSE2990 (26) and classified ER+ tumors as RESTless or REST-expressing (REST+) using the 24-gene signature method as previously described (ref. 1; Supplementary Fig. S1A and S1B). In these 2 independent breast tumor cohorts, LIN28A expression was significantly higher in RESTless versus REST+ tumors (Fig. 6A and B). The list of tumors in each data set defined as RESTless or REST+ is provided in Supplementary Table S1. Our results strongly argue that LIN28A overexpression contributes to the previously described aggression of RESTless tumors (1).

**Discussion**

We show that REST is a tumor suppressor; the loss of which promotes tumor growth in xenografts and increases clonogenicity, soft agar colony formation, and growth rate in vitro. We find that REST is a direct transcriptional repressor of the tumor promoter LIN28A in breast epithelial cells, and we present in vitro and in vivo data showing that LIN28A expression is required for the increased tumorigenicity of cells lacking REST. Finally, we show that LIN28A expression is heightened in human RESTless breast tumors, highlighting the clinical relevance of the REST-LIN28A axis.

**Identification of REST as a tumor suppressor in breast cancer**

REST was first identified as a tumor suppressor in a screen for genes whose loss confers anchorage independence upon human mammary epithelial cells (2). In 2009, Reddy and colleagues (27) examined 19 normal or cancerous breast biopsy samples and claimed a statistically significant, inverse correlation between tumor grade and REST expression. However, in examining publicly available microarray data from approximately 1,000 breast tumor samples, our group was unable to...
with previous reports, RESTnorm MCF7 cells generate tumors at increases the tumor take rate of MCF7 cells (29). Consistent using MCF7 cells are often conducted in estrogen-supplemen-
on tumorigenicity in a xenograft model. Xenograft experiments fi
assay. In both cell lines, REST knockdown signi
the growth of MCF7 cells in soft agar (28).

With previous data showing that REST knockdown promotes increased the number of colonies formed in soft agar, a
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tion of REST loss increases clono-
lines, we examined the effects of REST loss in
in vitro tumor-
-GENICITY assays. We found that loss of REST increases clono-

We previously found that RESTlow MCF7 (ER
and ER
) and MCF10A (ER
) cells in this

We further found that patients with RESTless tumors showed a signi

defined as the absence of estrogen supplementation. However, REST
low MCF7 cells gave rise to tumors in both orthotopic and nonorthotopic sites in the absence of estrogen supplementation, further showing the enhanced tumorigenicity of REST
low cells. This finding suggests that REST loss could confer a growth advantage under conditions of low estrogen availability. REST loss could be one mechanism by which tumors escape the growth restriction normally conferred by low estrogen availability.

We previously found that RESTless tumors show increased lymph node metastasis relative to their REST-containing counterparts (1). Consistent with this, REST
low MCF7 tumors in mice show local invasion into adjacent muscle tissue, as shown by the presence of muscle fibers within the tumors (Fig. 2F). REST
low tumors also show lymphovascular infiltration (Fig. 2G), consistent with their human counterparts showing increased lymph node metastasis.

**LIN28 in RESTless breast cancer**

Given the established role of LIN28 in cancers, including breast cancer, and our findings that LIN28A is regulated by REST in vitro and is elevated in RESTless tumors, we hypoth-
ized that LIN28A contributes to the aggressive phenotype of RESTless breast cancer and find support for our hypothesis both in vitro and in vivo. REST knockdown cells show enhanced soft agar colony formation in vitro, and this phenotype is dependent upon aberrant LIN28A expression (Fig. 4B). Ectopic LIN28A expression in MCF7 cells increases growth in clono-

The lack of effect on cellular growth rate, but concomitant knockdown of LIN28A severely retards the growth of REST
low, but not REST
low cells (Fig. 4C), suggesting a synthetic lethal inter-

We further find that LIN28A expression contributes to the tumor-suppressive activity of REST knockdown cells. The ability of REST
low cells to form tumors in mice is significantly diminished by knockdown of LIN28A (Fig. 5). Importantly, we found that human RESTless breast tumors also have higher
levels of LIN28A mRNA (Fig. 6), supporting a clinically relevant role for the REST–LIN28A axis. Given the critical role of LIN28A in mouse models of breast cancer metastasis (16), the higher levels of lymph node metastasis in RESTless breast cancer (1) and the aberrant expression of LIN28A in other aggressive cancers, we propose that LIN28A contributes to the aggressive nature of RESTless breast cancer.

**Regulation and mechanism of action of LIN28A**

Recently, it has been shown that LIN28A is a tumor promoter. However, little is known about how it becomes expressed in tumors. Myc has been shown to induce LIN28A expression by direct binding to an E-box proximal to the LIN28A promoter (16). Here, we describe the upregulation of LIN28A upon loss of the transcriptional repressor REST.

LIN28A has a REST-binding site approximately 2 kb upstream of the promoter and it chromatin immunoprecipitated REST at this site in MCF7 cells (Fig. 3C and D), consistent with the findings of a genome-wide ChIPseq screen that found robust binding of REST to the LIN28A RE1 in Jurkat T cells (4). Reporter assays confirmed the necessity of the RE1 site for REST-mediated regulation of LIN28A in mammary epithelial cells (Fig. 3H and I). REST has previously been shown to regulate LIN28A expression in embryonic stem and neural progenitor cells (12, 30), but this is the first report to show regulation of LIN28A by REST in epithelial cancer cells.

The best-characterized mechanism by which LIN28A promotes tumor progression and metastasis is by blocking the maturation of the let-7 family of tumor-suppressing miRNAs (13, 14, 31–33), which in turn reduce the expression of multiple breast cancer oncoproteins. However, we did not observe a change in the expression of the let-7 family members let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, and miR-98 upon REST knockdown in MCF7 cells by either miRNA array or quantitative PCR. We saw no change in let-7 expression (as measured by quantitative PCR) in MDA-MB-231 or T47D cells upon REST knockdown, nor did we observe a change in let-7 expression in RESTlow versus RESTnorm xenograft tumors (via quantitative PCR), despite measuring a robust increase in let-7 expression upon REST knockdown in all cell lines and tumors (data not shown). These findings suggest a let-7 processing-independent role for LIN28A in RESTless tumor growth. It is well established that LIN28A is not limited to regulating the maturation of miRNAs. During gliogenesis, LIN28A alters cell fate independently of let-7 (34) and the first reports of the molecular mechanism of LIN28A focused on its function as an mRNA-binding protein. LIN28A binds to RNA helicase A (RHA; ref. 35) and regulates the translation and stability of mRNAs including Oct4 (35–37) and several cell-cycle regulators (38). Our observation that LIN28A mediates tumor progression in a let-7-independent manner in our model suggests the need for further investigation of the mechanistic role of LIN28A in cancers.

LIN28B, which is encoded by a separate gene but is functionally similar to LIN28A, is also overexpressed in advanced human malignancies and transforms cells in a manner similar to LIN28A (15). LIN28B is a REST target in other systems (4). The shRNA used to knock down LIN28A expression in our studies does not target LIN28B (data not shown) and our data show that the focus formation, soft agar colony formation, and xenograft tumor growth of RESTlow cells are LIN28A-dependent, and that ectopic LIN28A expression is sufficient to recapitulate the RESTlow phenotypes observed in vitro. Thus, although our data show that LIN28A is necessary for our RESTlow in vivo phenotypes and is sufficient for a subset of RESTlow in vitro phenotypes, how LIN28B integrates into this mechanism is not yet clear.

The work presented here shows that REST is a breast cancer tumor suppressor that works, at least in part, by suppressing aberrant expression of LIN28A.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** K.T.W. Gunsalus, M.P. Wagoner, K. Meyer, W.B. Potter, A. Friedl, A. Roopra

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**Study supervision:** A. Roopra

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