Title: 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 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 demonstrate that loss of REST results in up-regulation 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 demonstrate a critical role for the REST-LIN28A axis in tumor aggression and suggest a causative relationship between REST loss and tumorigenicity in vivo.

Introduction
We recently described a highly aggressive subset of human breast tumors in which loss of REST is associated with decreased disease-free survival(1). During an unbiased screen 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 demonstrated 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 ~2,000 target genes(4, 6) and recruiting chromatin modifying enzymes including histone deacetylases (HDACs) and histone methyltransferases (HMTs)(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. Over-expression 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 up-regulated in these cancers. In this report, we demonstrate that REST directly regulates LIN28A expression in breast cancer cell lines, and that REST loss up-regulates 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 over-expressed 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 Supplemental Material.

Cell culture & generation of stable knockdown cell lines

All cell lines were purchased and authenticated from ATCC and grown as previously described(1). MDA-MB-231 low-serum growth was performed as previously described(17). REST knockdown was previously described(1). Stable knockdown of LIN28A (LIN28A\text{low}) was achieved via lentiviral delivery of an anti-LIN28A shRNA (TRCN0000102579) in pLKO.1 vector (Open Biosystems). Plasmid 1864 (Addgene) was used as a control (LIN28A\text{norm}); lentiviral particles were generated and used as previously described(18).

Westerns and Chromatin immunoprecipitation

Antibodies used for western: REST (Upstate 05-579), LIN28A (Abcam ab46020), β-actin (MP Biomedicals). ChIP as previously described(10); 2µg of antibody (REST H-290, Santa Cruz Biotech; G9a 07-551, Upstate; rabbit IgG, Sigma-Aldrich) was added to 300µg total protein. All washes performed with TSE with 500mM NaCl.
Quantitative RT-PCR

Total RNA was harvested from cultured cells (in biological triplicate) or xenograft tumors using TRIzol (Invitrogen) according to the manufacturer’s protocol, re-suspended in sodium citrate (1mM, 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 was performed using SYBR Premix Ex Taq (Takara Bio Inc.).

Luciferase reporter assay

~2kb 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. 48 hours post-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 ab46020) and Alexa Fluor goat anti-rabbit 594, (Invitrogen); Vectashield Mounting Medium with DAPI (Vector Laboratories). Microscopic imaging was performed with a digital camera (Spot II; Diagnostic Instruments) on a Nikon E600 Eclipse epifluorescent microscope with 20x 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 performed using Adobe Photoshop according to AACR guidelines.

Clonogenic assay and soft agar colony formation

Clonogenic assays performed as previously described(19).
Xenograft Experiments

All procedures were performed with the approval of the University of Wisconsin-Madison School of Medicine and Public Health Institutional Animal Care and Use Committee and according to national guidelines and policies. Adult intact female athymic nude-\textit{Foxn1}^nu mice (Harlan Laboratories) were used and MCF7 cells were treated as previously described(20).

Results

\textit{REST} knockdown enhances tumorigenic phenotypes in vitro

Recently we demonstrated that \textit{REST} is lost in 20% of human breast cancers, and that these \textit{REST}less tumors are highly aggressive(1). Here, we demonstrate that \textit{REST} loss drives tumor aggression. We used lentiviral delivery of shRNAs to generate stable control (\textit{REST}^\text{norm}) or \textit{REST} knockdown cell lines (\textit{REST}^\text{low} cells; the term “\textit{REST}less” refers only to human tumors). We knocked down \textit{REST} in a non-transformed breast cell line (MCF10A), and in estrogen receptor positive (ER\textsubscript{α}+) and triple negative (ER-/PR-/Her2-) breast cancer cell lines (MCF7 and MDA-MB-231, respectively); \textit{REST} knockdown was confirmed by western blot (Fig 1A-B and previously shown(1)).

We first tested the tumorigenicity of \textit{REST}^\text{low} and \textit{REST}^\text{norm} breast cells using a clonogenic assay(19), which estimates the ability of individual cells to form colonies. In all three cell lines tested, \textit{REST} knockdown significantly increased plating efficiency (Fig 1C-E).

Using an ER\textsubscript{α}+ (MCF7) and an ER negative (ER-; MCF10A) cell line, we repeated the clonogenicity assay using a soft agar protocol. In both cell lines, \textit{REST} knockdown significantly increased (>3-fold) the number of colonies observed (Fig 1F-G).

\textit{REST} knockdown increases tumor growth in mice

We performed xenograft experiments to test the \textit{in vivo} tumorigenicity of \textit{REST}^\text{norm} and \textit{REST}^\text{low} MCF7 cells. Work that will be described elsewhere suggested that \textit{REST}^\text{low} MCF7 cells would show enhanced growth \textit{in}}
in the absence of estrogen supplementation; \textit{REST}^{\text{norm}} or \textit{REST}^{\text{low}} MCF7 cells were injected subcutaneously into the flanks or mammary fat pads of intact female athymic Foxn1\textsuperscript{nu} mice, and tumor growth was monitored. 200 days post-injection, 30\% (8/28) of \textit{REST}^{\text{low}} mammary fat pad injection sites developed tumors (1980 mm\textsuperscript{3} tumor volume), compared with 0\% (0/28, 0 mm\textsuperscript{3} tumor volume) of \textit{REST}^{\text{norm}} injections (Fig 2A-B). The tumor take rate was also significantly greater for \textit{REST}^{\text{low}} versus \textit{REST}^{\text{norm}} cells injected subcutaneously into the flanks of the mice, with 34.4\% (11/32) of \textit{REST}^{\text{low}} injection sites giving rise to tumors, compared to 12.5\% (4/32) of \textit{REST}^{\text{norm}} sites (Fig 2C). The total tumor burden for flank tumors was significantly greater for \textit{REST}^{\text{low}} than \textit{REST}^{\text{norm}} tumors, at 4314 mm\textsuperscript{3} and 1017 mm\textsuperscript{3}, respectively (Fig 2D). \textit{REST} knockdown therefore results in a significant increase in tumorigenicity of MCF7 cells at both orthotopic and non-orthotopic sites.

Histopathological examination of \textit{REST}^{\text{low}} tumors showed that they were highly anaplastic, with enlarged nuclei, prominent nucleoli, many convoluted nuclear envelopes and a high mitotic rate (Fig 2E). \textit{REST}^{\text{low}} tumors contain ~1.5-fold more cells undergoing mitosis than \textit{REST}^{\text{norm}} tumors; however, due to the small number of \textit{REST}^{\text{norm}} tumors available for analysis (n=3), this difference did not reach statistical significance (p=0.1). 62.5\% (5/8) of \textit{REST}^{\text{low}} flank tumors examined show localized invasion into adjacent muscle (Fig 2F), and one showed lympho-vascular invasion (Fig 2G).

\textit{REST} regulates LIN28A expression in breast cancer cells

We hypothesized that one or more of the genes that become simultaneously de-repressed upon \textit{REST} knockdown\cite{1} must contribute to \textit{REST}less breast tumor aggression. One such gene was LIN28A; given that LIN28A was up-regulated in \textit{REST}less cells \textit{in vitro} and \textit{in vivo}, was a \textit{REST} target\cite{4, 12}, and was previously implicated in breast cancer progression\cite{15, 16}, we sought to determine whether LIN28A was an effector of the \textit{REST}less phenotype. We confirmed previously published microarray data showing that LIN28A mRNA was elevated in the breast cancer cell line T47D upon \textit{REST} knockdown\cite{1} by quantitative RT-PCR (Fig 3A); this increase in mRNA correlated with increased LIN28A protein (Fig 3B). LIN28A mRNA and protein are similarly up-regulated upon \textit{REST} knockdown in MCF7 cells (Fig 3C-D). \textit{REST} has been shown to regulate LIN28 in embryonic stem and neural progenitor cells\cite{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 performed chromatin immunoprecipitation 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 performed 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 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 demonstrate the specificity of REST binding and co-repressor recruitment to the LIN28 RE1 site we performed a ChIP using an anti-REST antibody or anti-G9a (a REST co-repressor(10)) antibody, and sham IgG; the ChIP DNA was analyzed via qRT-PCR using primers spaced every 2kb from the LIN28A transcriptional start site (TSS) to 12kb upstream, and primers 8kb downstream of the TSS. REST and G9a enrichment is found exclusively at the LIN28A RE1 site and not elsewhere in the vicinity of the LIN28A gene (Fig 3F).

To determine whether REST directly represses transcription by binding to the LIN28A RE1 site, we transfected a luciferase reporter containing ~2kb of the LIN28A promoter region including (+RE1) or excluding (-RE1) the REST binding site into REST\textsuperscript{norm} and REST\textsuperscript{low} MCF7 and HEK cells. As predicted, luciferase activity was decreased by greater than 50% in the presence (+RE1) compared to the absence (-RE1) of the RE1 site in REST\textsuperscript{norm} cells; this repression was lost in REST\textsuperscript{low} cells (Fig 3H-I). This repression therefore requires both REST and the REST binding site. These data indicate that REST directly represses transcription from the LIN28A promoter region by binding to the RE1 site.

To test whether the regulation of LIN28A by REST observed \textit{in vitro} was also present \textit{in vivo}, we measured LIN28A RNA levels in REST\textsuperscript{norm} and REST\textsuperscript{low} MCF7 tumor tissue. Both LIN28A mRNA, as measured by two 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 REST\textsuperscript{low} versus REST\textsuperscript{norm} MCF7 tumors (Fig 3J). None of the housekeeping genes analyzed show significant differences in expression between REST\textsuperscript{norm} and REST\textsuperscript{low} tumors.
**REST**<sub>low</sub> in vitro phenotypes are **LIN28A-dependent**

To evaluate the contribution of LIN28A to the **REST**<sub>low</sub> phenotype, **REST**<sub>norm</sub> and **REST**<sub>low</sub> MCF7 cells were transduced with a lentiviral construct expressing an anti-LIN28A shRNA (**LIN28A**<sub>low</sub>) or control shRNA (**LIN28A**<sub>norm</sub>), and LIN28A knockdown was verified (Fig 4A); the anti-LIN28A shRNA used does not knock down LIN28B (data not shown). We then performed 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 **REST**<sub>low</sub> and **REST**<sub>norm</sub> cells; however, LIN28A knockdown significantly retarded the growth of **REST**<sub>low</sub> but not **REST**<sub>norm</sub> cells (Fig 4C), suggesting that the **REST**<sub>low</sub> cells have acquired a dependence upon LIN28A that is not present in **REST**<sub>norm</sub> cells. Because MDA-MB-231 cells proliferate at near-maximal rate in the presence of 10% FBS, we then performed 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, **REST**<sub>low</sub> cells show increased proliferation relative to **REST**<sub>norm</sub> 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 over-expression is a functional effector of the **REST**<sub>low</sub> phenotypes, LIN28A was stably over-expressed in MCF7 cells (Fig 4E). LIN28A over-expression caused a ~2x increase in plating efficiency and colonies grown in soft agar (Fig 4F-G). We therefore conclude that in MCF7 cells, LIN28A over-expression is sufficient to induce the cell culture phenotype of **REST**<sub>low</sub> cells.

**LIN28A contributes to RESTless tumor formation**
To determine whether the up-regulation of LIN28A observed in REST\textsuperscript{low} cells contributes to the tumorigenicity observed \textit{in vivo}, we compared the tumorigenicity of REST\textsuperscript{low} cells with and without LIN28A expression. REST\textsuperscript{lowLIN28Anorm} or REST\textsuperscript{lowLIN28Alow} MCF7 cells were injected subcutaneously into the mammary fat pads of athymic nude mice as for Figure 1. After 100 days, 50% (6/12) of REST\textsuperscript{lowLIN28Anorm} mammary fat pad injections had given rise to tumors, compared with only 8.3% (1/12) of fat pads injected with REST\textsuperscript{lowLIN28Alow} cells (Fig 5A). The tumor burden in the mammary fat pads was also significantly decreased when LIN28A was knocked down, with a total tumor volume of 345mm\textsuperscript{3} for REST\textsuperscript{lowLIN28Anorm} compared to only 56mm\textsuperscript{3} for REST\textsuperscript{lowLIN28Alow} tumors (Fig 5B).

\textit{LIN28A expression is increased in human RESTless breast tumors}

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

\textbf{Discussion}

We demonstrate 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 \textit{in vitro}. We find that REST is a direct transcriptional repressor of the tumor promoter LIN28A in breast epithelial cells, and we present \textit{in vitro} and \textit{in vivo} data demonstrating 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 et al.(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 publically-available microarray data from ~1,000 breast tumor samples, our group was unable to detect any significant decrease in REST mRNA in tumor samples compared to healthy tissue, and in fact found that REST mRNA was significantly increased in tumors relative to normal tissue in multiple independent datasets(1). We further saw no significant difference in REST mRNA level across tumor stage or grade. The reason for this discrepancy is unclear, may be due to the small sample size used by Reddy et al.

Lv and colleagues(28) used immunohistochemical staining for REST in normal and breast tumor tissues, and found that REST was detectable in all normal and benign samples tested (n=10 and 22, respectively), but was absent from 30.9% (21/68) of tumor samples. Consistent with their findings, when we examined REST expression in primary tumors via immunohistochemistry, we found that REST staining was absent in ~20% (37/182) of tumor samples. We further found that patients with RESTless tumors had a significantly poorer outcome compared to patients whose tumors stained positive for REST protein(1).

Here, we demonstrate that REST loss is not only a marker, but a driver of tumor aggression. Using REST knockdown breast cell lines, we examined the effects of REST loss in in vitro tumorigenicity assays. We found that loss of REST increases clonogenicity in MCF7, MCF10A and MDA-MB-231 cell lines (Fig 1C-E). We confirmed the transformed phenotype of RESTlow cells in a soft agar colony formation assay, which measures anchorage-independent growth; because REST loss is observed in ER+ and ER negative human tumors, we tested the effect of REST loss on both MCF7 (ER+) and MCF10A (ER-) cells in this assay. In both cell lines, REST knockdown significantly increased the number of colonies formed in soft agar, a hallmark of transformation (Fig 1F-G); this is consistent with previous data showing that REST knockdown promotes the growth of MCF7 cells in soft agar(28).
We next confirmed the transformative capacity of REST loss \textit{in vivo}; this is the first study to examine the effects of REST loss on tumorigenicity in a xenograft model. Xenograft experiments using MCF7 cells are often performed in estrogen-supplemented mice, as estrogen supplementation of the host mice increases the tumor take rate of MCF7 cells\cite{29}. Consistent with previous reports, REST\textsuperscript{norm} MCF7 cells generate tumors at low frequency in either mammary fat pads or flanks in the absence of estrogen supplementation; however, REST\textsuperscript{low} MCF7 cells gave rise to tumors in both orthotopic and non-orthotopic sites in the absence of estrogen supplementation, further demonstrating the enhanced tumorigenicity of REST\textsuperscript{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\cite{1}; consistent with this, REST\textsuperscript{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\textsuperscript{low} tumors also show lympho-vascular infiltration (Fig 2G), consistent with their human counterparts showing increased lymph node metastasis.

\textit{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 \textit{in vitro} and is elevated in RESTless tumors, we hypothesized that LIN28A contributes to the aggressive phenotype of RESTless breast cancer, and find support for our hypothesis both \textit{in vitro} and \textit{in vivo}. REST knockdown cells show enhanced soft agar colony formation \textit{in vitro}, and this phenotype is dependent upon aberrant LIN28A expression (Fig 4B). Ectopic LIN28A expression in MCF7 cells increases growth in clonogenicity assays, thus recapitulating the REST\textsuperscript{low} phenotype (Fig 4E-F). In MDA-MB-231 cells, REST knockdown has no effect on cellular growth rate, but concomitant knockdown of LIN28A severely retards the growth of REST\textsuperscript{low}, but not REST\textsuperscript{norm}, cells (Fig 4C), suggesting a synthetic lethal interaction between REST and LIN28A. Exploring this interaction further exposed a LIN28A-dependent growth phenotype in low-serum conditions (Fig 4D).
We further find that LIN28A expression contributes to the tumorigenicity of REST knockdown cells: the ability of REST\textsuperscript{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\cite{16}, the higher levels of lymph node metastasis in RESTless breast cancer\cite{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\cite{16}; here, we describe the up-regulation of LIN28A upon loss of the transcriptional repressor REST.

LIN28A has a REST binding site ~2kb upstream of the promoter and we ChIP REST at this site in MCF7 cells (Fig 3C-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\cite{4}. Reporter assays confirmed the necessity of the RE1 site for REST-mediated regulation of LIN28A in mammary epithelial cells (Fig 3H-I). REST has previously been shown to regulate LIN28A expression in embryonic stem and neural progenitor cells\cite{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\cite{13,14,31-33}, which in turn reduce the expression of multiple breast cancer oncogenes. However, we did not observe a change in the expression of the let-7 family members let-7a, let-7b, let-7e, let-7g, let-7i and miR-98 upon REST knockdown in MCF7 cells by either miRNA array or qPCR; we saw no change in let-7 expression (as measured by qPCR) in MDA-MB-231 or T47D cells upon REST knockdown; nor did we observe a change in let-7 expression in REST\textsuperscript{low} versus REST\textsuperscript{norm} xenograft tumors (via qPCR), in spite of measuring a robust increase in LIN28A 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 \emph{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)(35) and regulates the translation and stability of mRNAs including \emph{Oct4}(35-37) and several cell cycle regulators(38). Our observation that LIN28A mediates tumor aggression in a \emph{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 over-expressed 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 REST\textsuperscript{low} cells are LIN28A-dependent, and that ectopic LIN28A expression is sufficient to recapitulate the REST\textsuperscript{low} phenotypes observed \textit{in vitro}. Thus, although our data show that LIN28A is necessary for our REST\textsuperscript{low} \textit{in vivo} phenotypes and is sufficient for a subset of REST\textsuperscript{low} \textit{in vitro} phenotypes, how LIN28B integrates into this mechanism is not yet clear.

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

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References

Figure Legends

Figure 1. REST knockdown enhances tumorigenicity of cells in culture.

A-B, Western blot showing REST knockdown in MCF7 and MDA-MB-231 cells.

C-E, Plating efficiency of REST^{low} and REST^{norm} MCF7, MCF10A and MDA-MB-231 cells in a clonogenic assay (p≤0.050).

F-G, Soft agar colony formation assay comparing anchorage-independent growth of REST^{low} versus REST^{norm} MCF7 and MCF10A cells (p≤0.050).

Figure 2. REST knockdown increases the aggressiveness of MCF7 tumor growth in nude mouse xenografts.

A, 10^{6} control (REST^{norm}) or REST knockdown (REST^{low}) MCF7 cells were injected subcutaneously into the mammary fat pads of female athymic nude mice. Tumor incidence was monitored weekly, and is significantly higher for REST^{low} versus REST^{norm} cells (p=0.0022).

B, Tumor burden in the mammary fat pads is significantly larger in REST^{low} versus REST^{norm} tumors (p<0.0001).

C, 10^{6} control (REST^{norm}) or REST knockdown (REST^{low}) MCF7 cells were injected subcutaneously into the flanks of female athymic nude mice. Tumor incidence upon injection into flank is significantly higher for REST^{low} versus REST^{norm} MCF7 cells (p=0.0382).

D, Tumor burden in flanks is significantly larger in REST^{low} versus REST^{norm} tumors (p=0.0016).

E-G, Bright field photomicrographs of H&E stained sections of REST^{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^{low} tumor and adjacent mammary fat pad; arrows indicate lympho-vascular invasion.

Figure 3. REST is a direct transcriptional repressor of LIN28A in breast cancer cells.
A, LIN28A mRNA in REST\textsuperscript{low} and REST\textsuperscript{norm} T47D cells was analyzed by qPCR and normalized to actin (p=0.05).

B, Western blot for LIN28A and β-actin (loading control) in REST\textsuperscript{low} and REST\textsuperscript{norm} T47D cells.

C, LIN28A mRNA in REST\textsuperscript{low} and REST\textsuperscript{norm} MCF7 cells was analyzed by qPCR and normalized to actin (p=0.0053).

D, Immunofluorescent staining for LIN28A in REST\textsuperscript{norm} and REST\textsuperscript{low} MCF7 cells.

E, Chromatin immunoprecipitations (ChIP) 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\textsuperscript{norm} and REST\textsuperscript{low} MCF7 (F) and HEK (G) cells. Luciferase expression was normalized to renilla.

J, LIN28A immature and processed mRNA in REST\textsuperscript{low} versus REST\textsuperscript{norm} tumors measured by qPCR (p≤0.04). Housekeeping genes showed no significant difference.

Figure 4. LIN28A is necessary and sufficient for REST\textsuperscript{low} phenotypes \textit{in vitro}.

A, Immunoblot for LIN28A or β-actin (loading control) in control (LIN28A\textsuperscript{norm}) or LIN28A knockdown (LIN28A\textsuperscript{low}) MCF7 cells.

B, Soft agar colony formation of REST\textsuperscript{norm} and REST\textsuperscript{low} MCF7 cells with and without anti-LIN28 shRNA (LIN28\textsuperscript{low}) (p=0.0383).

C-D, Growth of REST\textsuperscript{norm} and REST\textsuperscript{low} MDA-MB-231 cells with and without anti-LIN28 shRNA (LIN28\textsuperscript{low}) in high (C, 10%) or low (D, 2.5%) serum (72h, p≤0.05).
E-G, Lentiviral delivery of a LIN28A expression construct was used to generate LIN28 over-expressing MCF7 cells; expression was verified by western blot (E). Clonogenicity (F) and soft agar colony formation (G) were measured (p≤0.05).

Figure 5. LIN28A contributes to the tumorigenicity of REST\textsuperscript{low} MCF7 cells in mice. 10\textsuperscript{6} REST\textsuperscript{low}/LIN28A\textsuperscript{norm} or REST\textsuperscript{low}/LIN28A\textsuperscript{low} MCF7 cells were injected subcutaneously into the mammary fat pads of athymic nude mice, and tumor incidence and growth were monitored weekly.

A-B Tumor incidence (A, p=0.020) and burden (B, p=0.0002) are decreased upon LIN28A knockdown.

Figure 6. LIN28A mRNA is increased in human REST\textit{less} tumors.

A-B, Box and whisker plot of fold median LIN28A mRNA expression in REST\textit{less} and REST-containing (“REST\textit{fl}”) breast tumors from datasets GSE2034 (A, p<0.0001) and GSE2990 (B, p=0.0050). Lines of the box represent 75\textsuperscript{th}, 50\textsuperscript{th} and 25\textsuperscript{th} percentiles; whiskers represent 90\textsuperscript{th} and 10\textsuperscript{th} percentile of LIN28A expression in each group.

Supplemental Figure 1 A-B. Analysis of datasets GSE2990 and GSE2034 were performed to define tumors as REST\textit{less} or REST\textit{fl} using the gene signature derived in Wagoner \textit{et al.} \textsuperscript{(1)}. Tumors were clustered using Euclidean distance and REST\textit{less} tumors are highlighted by a red bar.

Supplemental Table 1. The list of tumors in GSE2990 and GSE2034 are listed and labeled as REST\textit{less} or REST\textit{fl}.
Figure 4

A

LIN28<sup>low</sup>  LIN28<sup>high</sup>
LIN28
Actin

B

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<tr>
<th>shREST</th>
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<td>shLIN28</td>
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C

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<td>LIN28&lt;sup&gt;low&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>+ +</td>
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<td>Relative Growth (% control)</td>
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D

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<td>Relative Growth (% control)</td>
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MCF7

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G

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<td>Colonies</td>
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* indicates significance.
Figure 5

A

Percent of injection sites tumor-free, Mammary fat pads

Days post-injection

B

Tumor volume (mm$^3$), Mammary fat pads

Days post-injection
Figure 6

A

GSE2034

p < 0.0001

B

GSE2990

p = 0.0050

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


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