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TWIST1-induced microRNA-424 reversibly drives mesenchymal programming while inhibiting tumor initiation

David J. Drasin¹, Anna L. Guarnieri², Deepika Neelakantan¹, Jihye Kim³, Joshua H. Cabrera², Chu-An Wang², Vadym Zaberezhnyy⁴, Pierluigi Gasparini⁵, Luciano Cascione⁵, Kay Huebner⁵, Aik-Choon Tan³, and Heide L. Ford^{1,2,4}

¹Program in Molecular Biology, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

²Department of Pharmacology, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

³Department of Medicine, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

⁴Department of Biochemistry, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

⁵Department of Molecular Virology, Immunology & Molecular Genetics, Ohio State University, Columbus, OH, USA

Abstract

Epithelial-to-mesenchymal transition (EMT) is a dynamic process that relies on cellular plasticity. Recently, the process of an oncogenic EMT, followed by a reverse mesenchymal-to-epithelial transition (MET), has been implicated as critical in the metastatic colonization of carcinomas. Unlike governance of epithelial programming, regulation of mesenchymal programming is not well understood in EMT. Here, we describe and characterize the first microRNA that enhances exclusively mesenchymal programming. We demonstrate that microRNA-424 is upregulated early during a TWIST1 or SNAI1-induced EMT, and that it causes cells to express mesenchymal genes without affecting epithelial genes, resulting in a mixed/intermediate EMT. Furthermore, microRNA-424 increases motility, decreases adhesion and induces a growth arrest, changes associated with a complete EMT, that can be reversed when microRNA-424 expression is lowered, concomitant with an MET-like process. Breast cancer patient microRNA-424 levels positively associate with TWIST1/2 and EMT-like gene signatures, and miR-424 is increased in primary tumors versus matched normal breast. However, microRNA-424 is downregulated in patient metastases versus matched primary tumors. Correspondingly, microRNA-424 decreases tumor initiation and is post-transcriptionally downregulated in macrometastases in mice, suggesting the need for biphasic expression of miR-424 to transit the EMT-MET axis. Next-generation RNA sequencing revealed microRNA-424 regulates numerous EMT and cancer stemness-associated genes, including TGFBR3, whose downregulation promotes mesenchymal

Corresponding author: Heide L. Ford, 12800 E. 19th Ave, MS 8303, Aurora, CO 80045, USA, Phone: (303) 724-3509, Fax: (303) 724-3663, Heide.Ford@ucdenver.edu.

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phenotypes, but not tumor-initiating phenotypes. Instead, we demonstrate that increased MAPK/ERK signaling is critical for miR-424-mediated decreases in tumor-initiating phenotypes. These findings suggest microRNA-424 plays distinct roles in tumor progression, potentially facilitating earlier, but repressing later, stages of metastasis by regulating an EMT-MET axis.

Keywords

miR-424; EMT; MET

Introduction

Over the last decade, there has been a growing emphasis on understanding the metastatic cascade of carcinomas by examining the epithelial-to-mesenchymal transition (EMT) (1). An oncogenic EMT, similar to a developmental EMT, causes epithelial cells to lose tight cell-cell contacts and gain a more motile and invasive phenotype, traits postulated to facilitate dissemination from a primary tumor (2). Carcinoma-associated experimental studies and patient tissue analyses have further demonstrated that acquisition of a more mesenchymal phenotype is linked with other pro-metastatic traits such as tumor-initiating cell (TIC) characteristics (e.g. self-renewal, multi-potency) and resistance to conventional therapeutics (3–5). Many of these informative studies have demonstrated that EMT-inducing transcription factors such as SIX1, TWIST1/2 and SNAIL/2 can mediate these pro-metastatic and TIC phenotypes (1).

MicroRNAs (miR) have relatively recently been identified as a class of factors that also regulate oncogenic EMTs. Such mechanisms include, but are not limited to, ability of miRs to target the 3' untranslated region (UTR) of *CDH1* (E-cadherin) (1) or to target the 3' UTR of the *CDH1* repressors *ZEB1/2* (6). Furthermore, miRs have been reported to alter signaling pathways implicated in EMT, such as the EGFR pathway (1). To date, no miRs have been identified that solely upregulate the mesenchymal arm of an oncogenic EMT without repressing epithelial programming.

Identification of potential mesenchymal-specific regulators has likely been hindered by the ability of EMT-inducing factors to influence one another (7). The ubiquitous loss of functional E-cadherin during an EMT has further confounded analysis of mesenchymal programming in isolation from epithelial programming, as E-cadherin loss can be sufficient to drive a full EMT (8). Studies manipulating the mesenchymal half of the EMT program are increasingly relevant, as human carcinoma cells are documented to co-express epithelial and mesenchymal markers (9–11), thus having not fully lost their epithelial identity. Such an intermediate EMT state, as opposed to a fully mesenchymal state, may more easily undergo a reverse mesenchymal-to-epithelial transition (MET), which has been postulated to facilitate metastatic colonization at secondary sites (2), and is compatible with the epithelial nature of carcinoma metastases and the similarity of a metastatic lesion to its originating primary tumor.

Studies now experimentally support that an MET, following an EMT, facilitates metastatic colonization. Such studies focused on the EMT-inducers TWIST1 and PRRX1, which

promoted invasive phenotypes *in vitro* and *in vivo*, but needed to be downregulated, concurrent with an MET, for metastatic outgrowth to occur (12,13). Contrary to previous studies that positively link EMT and TIC attributes (3,4), an MET link to stemness appears just as relevant to metastatic outgrowth, as metastases are epithelial in nature. Oncogenic EMT inducers may thus be regulating a more plastic state, rather than a full EMT, to enable reversion to an epithelial phenotype along with self-renewal and proliferative capability at the secondary site. To better understand mediators of this plastic state, we set out to identify novel regulators of the mesenchymal arm of EMT and to ask whether such regulators impinge on TIC phenotypes, thereby influencing various stages of the metastatic cascade. In doing so, we identify miR-424, acting through TGFBR3, as the first miR regulator of mesenchymal programming that simultaneously maintains epithelial morphology and molecular characteristics. While miR-424 enhances early pro-metastatic functions, our data demonstrate that this miR inhibits TIC phenotypes, mediated by ERK signaling, and ultimately becomes downregulated in experimental and clinical macrometastases.

Materials and Methods

Cell culture

HMLE derivatives and SUM149PT lines were generous gifts from Dr. Robert Weinberg (Massachusetts Institute of Technology; 2009) and Dr. Stephen Ethier (Medical University of South Carolina; 2012), respectively. MCF12A cells were obtained from The University of Colorado Comprehensive Cancer Center (UCCC) Tissue Culture Shared Resource and cultured according to ATCC recommendations. Cell lines were authenticated on 4/3/13 by short tandem repeat profiling or by methods previously described (14). TWIST1 and SNAI1 induction were performed as previously described (4). MEK inhibitors, 5 μ M PD98059 (Sigma) or 5 μ M AZD6244 (Otava Chemicals), were used for 24 hours. Doxycycline (Sigma) inductions were done at 0.5 μ g/mL in complete SUM149PT media for the indicated amount of time for each assay. Inductions were always performed on cells that had never previously been exposed to doxycycline.

BrdU/PI analysis

Attached cells were serum-starved, then given complete medium supplemented with 10 μ M BrdU (Sigma) for 2 hours. Cells were fixed with ice cold 70% ethanol, permeabilized with 0.5% Tween-20/PBS and denatured with 2N HCl. Cells were incubated with anti-BrdU antibody (Roche; 1:100), FITC-anti-mouse IgG (Sigma; 1:20), and propidium iodide/RNaseA. Cells were analyzed as in *Flow cytometry analyses*.

MicroRNA microarray and next generation RNA sequencing

10 μ g total RNA was isolated using the miRNeasy Mini Kit (Qiagen) and was submitted in triplicate to LC Sciences for microRNA microarray services and data analysis using miRBase v15.0. Results are deposited under GSE58560.

1 μ g total RNA isolated using the miRNeasy Mini Kit (Qiagen) was submitted in triplicate to the University of Colorado AMC Genomics and Microarray Core for construction and sequencing of the RNA-seq library on a HiSeq-2000 (Illumina). On average, we obtained

approximately 54 million (range=44–60 million) single-end 50 bp sequencing reads per sample. RNA-seq analysis was performed as previously described (15,16). Results are deposited under GSE54505.

Cell migration and adhesion assays

Motility was determined by gap closure assay, where a culture insert (Ibidi) created a 500 μm gap between $4\text{--}6 \times 10^4$ cells/chamber. Inserts were removed the next day, and distance migrated was measured using DP2-BSW software (v2.2; Olympus). Adhesion assays were performed as previously described (17).

Immunoblot analysis

Whole cell extracts were collected in RIPA buffer as previously described (17) and run on 7, 8 or 10% SDS-polyacrylamide gels. Antibodies used for immunoblots (IB): α -catenin, E-cadherin, fibronectin, N-cadherin, plakoglobin (BD Biosciences), actin, β -tubulin, vimentin (Sigma), total ERK1/2, p-ERK1/2 (Cell Signaling), TGFBR3 (Santa Cruz), ST3GAL5 (Abgent) and TMPRSS4 (Proteintech).

Flow cytometry analyses

ALDEFLUOR assays (Stemcell Technologies) to detect ALDH1 activity were performed according to manufacturer's recommendation. Diethylaminobenzaldehyde (DEAB) was used as an ALDH1 inhibitor to set ALDH1 gates. Surface TGFBR3 levels were determined with a polyclonal goat primary antibody (R&D Systems) and AlexaFluor-594 secondary antibody (Life Technologies). All samples were analyzed on a Gallios Flow Cytometer (Beckman Coulter) at the UCCC Flow Cytometry Shared Resource.

Immunocytochemistry

Immunocytochemistry (ICC) was performed as previously described (17). Antibodies used: N-cadherin (BD Biosciences; 1:50), E-cadherin (Cell Signaling; 1:200), AlexaFluor-488 (Life Technologies; 1:800), AlexaFluor-594 (Life Technologies; 1:800). Coverslips were mounted with Vectashield (Vector Laboratories) and DAPI (1 $\mu\text{g}/\text{mL}$).

Mammosphere formation

Primary and secondary mammosphere formation were performed as previously described (18). Mammosphere media: MEM basal media (500 mL), 1X B27, hydrocortisone (1 $\mu\text{g}/\text{mL}$), insulin (5 $\mu\text{g}/\text{mL}$), β -mercaptoethanol (5 $\mu\text{g}/\text{mL}$) and EGF (10 ng/mL).

3' UTR reporter

Reporter constructs and 5 nM miR-424 or negative control mimic (Thermo Scientific) were co-transfected into cells with X-tremeGENE siRNA Transfection Reagent (Roche). Lysates were collected and analyzed as previously described (18). See Supplementary Materials and Methods for construct design.

Statistical analyses

Prism software (v5.0; GraphPad) was used to conduct appropriate statistical procedures, as noted in figure legends. P-value <0.05 or FDR <0.25 were considered significant unless noted otherwise. The frequency of stem cells in the tumor-initiation experiment was estimated with 95% confidence using the ELDA analysis tool (19).

Mouse models

Tumor-initiation experiments and experimental metastasis assays are detailed in Supplementary Materials and Methods. All animal studies were performed according to protocols reviewed and approved by the Institutional Animal Care and Use Committee at the University of Colorado AMC.

Results

TWIST1 and SNAI1 induce mesenchymal markers prior to repressing epithelial markers during an EMT

To examine the temporal regulation of mesenchymal and epithelial genes during an EMT, we used inducible models of EMT previously established in the non-tumorigenic, human mammary epithelial HMLE cell line (4). In these models, TWIST1 or SNAI1 are fused to the estrogen receptor ligand-binding domain, allowing for post-translational activation by 4-hydroxytamoxifen (4-OHT). After 12 days of 4-OHT treatment, we observed the expected morphologic and molecular changes (Fig. 1A,C; Supplementary Fig. S1A,C) characteristic of an EMT (4). Interestingly, 4 days of 4-OHT treatment was not sufficient to alter the epithelial morphology (Fig. 1A; Supplementary Fig. S1A – middle panels) or to decrease levels of the epithelial markers E-cadherin or miR-200c, but was sufficient time for TWIST1 and SNAI1 to induce expression of the mesenchymal markers fibronectin, N-cadherin and vimentin (Fig. 1B; Supplementary Fig. S1B). These findings suggested the existence of a mesenchymal regulator(s) that operates upstream of E-cadherin repression during an EMT.

TWIST1 and SNAI1 upregulate miR-424 early during an EMT

To identify a potential mechanism by which mesenchymal markers are upregulated early during an EMT, we screened RNA from different time points during a TWIST1-induced EMT using a miR microarray. As members of the miR-8 family (e.g. miR-200a/b/c) have been well-documented to promote an epithelial phenotype (20), we reasoned that another miR may serve as a mesenchymal-promoting counterpart. 41 and 77 miRs were differentially expressed after 4 (early EMT) and 12 (late EMT) days of TWIST1 induction, respectively (Fig. 1D; Supplementary Tables S1,S2). Notable microarray changes included members of the miR-8 family, miR-146a, miR-31 and the previously identified TWIST1-regulated miR-10b, each of which have been implicated in the metastatic process (20–23). Because the miR-8 family was not downregulated until day 12 (Fig. 1D), yet mesenchymal markers were upregulated earlier in the EMT process, additional mechanisms downstream of TWIST1 that mediate the mesenchymal phenotype likely exist.

We used quantitative PCR (qPCR) with independent TWIST1 inductions to validate the changes of miR-200a/b/c, as well as miR-429, miR-10a and miR-152 (data not shown).

Additional validation of the screen led to the confirmation that miR-424 was upregulated in HMLE cells early after TWIST1/SNAI1 induction (Fig. 1E; Supplementary Fig. S1D), which we further pursued, as miR-424 had not previously been directly implicated as an exclusive regulator of mesenchymal programming. Primary miR-424 (pri-miR-424), the first precursor during miR-424 biogenesis, was also upregulated by TWIST1 and SNAI1 induction (Fig. 1F; Supplementary Fig. S1E). Together, these data demonstrate that TWIST1 and SNAI1 can regulate miR-424 expression.

MiR-424 regulates the mesenchymal arm of an EMT

We next asked whether miR-424 could mediate a change in expression of mesenchymal markers by creating three human breast cell lines that stably overexpressed miR-424. The rationale for cell line choice was: HMLE – cell line used for the original miR screen; MCF12A – non-transformed human mammary cell line to verify HMLE findings; SUM149PT – human breast cancer cell line with low endogenous miR-424 useful for *in vivo* study of exogenous miR-424. In SUM149PT cells, miR-424 (Fig. 2A) induced a dramatic upregulation of N-cadherin with more modest increases in vimentin and fibronectin (Fig. 2B). In contrast, miR-424 had little to no effect on the levels of the epithelial markers E-cadherin, α -catenin, plakoglobin and miR-200c, nor did miR-424 alter cellular morphology (Fig. 2B; Supplementary Fig. S2A,B). Similar results were observed in miR-424-expressing MCF12A and HMLE cells (Supplementary Fig. S2C–F, S2G–J, respectively). Notably, ICC revealed miR-424 expression caused a mixed/intermediate phenotype, where 52% of SUM149PT cells simultaneously expressed N-cadherin and E-cadherin at cell membranes, as compared to 10% of empty vector (EV) control cells (Figures 2C,D); ICC also demonstrated localization of E-cadherin was unaffected by miR-424. Thus, miR-424 is sufficient to regulate the mesenchymal arm of an EMT in multiple cell lines, independent of epithelial arm repression, mimicking an early TWIST1 or SNAI1-induced EMT.

Gains in mesenchymal EMT markers, particularly N-cadherin, have been associated with increased motility (24). We thus tested if miR-424 affects cellular migration, focusing on the SUM149PTs and MCF12As in order to characterize the tumorigenic cell line chosen for *in vivo* studies and an additional non-transformed cell line that had the more robust intermediate EMT profile (Supplementary Fig. S2), respectively. Using gap closure assays, we found that miR-424 increased migration of SUM149PT cells (Fig. 2E). Additionally, miR-424 shifted cells into the G₁ phase, akin to an EMT-associated growth arrest (Fig. 2F). Lastly, we examined cell-matrix adhesion, finding that miR-424 decreased cell adhesion to fibronectin-coated plates (Fig. 2G). Similar effects of miR-424 on motility, growth and adhesion were seen in MCF12A cells (Supplementary Fig. S3A–C). Together, these data demonstrate that miR-424 induces EMT-associated functions while promoting an intermediate EMT.

Inducible miR-424 reversibly recapitulates EMT-associated phenotypes

As recent evidence has demonstrated the importance of an MET following an oncogenic EMT, we asked if the intermediate EMT state was reversible by developing a doxycycline-inducible miR-424 system (i-miR-424) in SUM149PT cells. We characterized clones from the EV control (i-EV 1, i-EV 2) and miR-424 (i-miR-424 1, i-miR-424 2) expressing cells.

3' UTR luciferase reporters with a perfect miR-424 binding site demonstrated that doxycycline led to repression of luciferase in the i-miR-424 clones, which was alleviated by mutating the reporter's miR-binding site (Fig. 2H). Removal of doxycycline also alleviated luciferase repression, demonstrating that i-miR-424 activity is functionally reversible (Supplementary Fig. S4A).

Importantly, induction of miR-424 led to an increase in N-cadherin and fibronectin levels without affecting levels of E-cadherin (Fig. 2I), and did so in a reversible manner (Fig. 2J). This proof of principle experiment demonstrates that reduction of miR-424, after it has already driven an intermediate EMT, is sufficient to reverse mesenchymal phenotypes similar to an MET.

We also validated that i-miR-424 recapitulated functions observed with constitutive miR-424 expression. Doxycycline treatment shifted i-miR-424 cells into the G₁-phase in a reversible manner (Supplementary Fig. S4B – white, gray bars, respectively). Additionally, while doxycycline treatment led to an increase in migration in the control i-EV 2 clone, doxycycline increased migration to a significantly greater extent in the i-miR-424 1 clone (Supplementary Fig. S4C).

To address the temporal role of miR-424 in EMT-MET and metastasis, we decided to characterize our inducible model *in vivo*. By using doxycycline to manipulate miR-424 levels before or after injections (i.e. high to low miR-424, low to high miR-424, always low miR-424, always high miR-424), we aimed to determine which metastatic phenotypes miR-424 regulates and whether manipulation of miR-424 to allow completion of an EMT-MET axis was required. However, neither orthotopic nor tail vein injections resulted in metastatic burden after 70 and 90 days, respectively, with either the i-EV or i-miR-424 clones (data not shown). We were also unable to detect circulating tumor cells (CTCs) after orthotopic injection. As SUM149PT cells have been recently documented to consist of varying subpopulations (25), interclonal cooperativity may be necessary for SUM149PT cells to metastasize, or the clones selected may lack the innate ability to metastasize. Regardless, *in vitro* reversibility of the intermediate EMT and its associated functions, by manipulating miR-424 levels, is consistent with an EMT, followed by an MET, potentially facilitating the various steps of metastasis.

MiR-424 is post-transcriptionally downregulated in overt metastatic lesions

While we could not evaluate inducible miR-424 effects *in vivo*, the role of an intermediate EMT *in vivo* remained unknown. This question is important, as tumor cells have been found to exist in an intermediate state both in primary tumors and in circulation (11). Thus, we characterized our constitutive miR-424 system in an experimental metastasis mouse model. EV control and miR-424 overexpressing SUM149PT cells were tagged with firefly luciferase to allow detection of metastatic burden by *in vivo* imaging system (IVIS), and were verified to express exogenous primary and mature miR-424 prior to injection (Fig. 3A). Cells were subsequently injected into the left ventricle of nude female mouse hearts. Following injection, each mouse was imaged to ensure systemic luciferase signal indicative of dissemination. Over 15 weeks, a trend towards decreased metastasis incidence was observed in the miR-424 group, though this difference was not significant (Fig. 3B).

To verify that ectopic expression of miR-424 was maintained in metastases that occurred in the miR-424 group, metastatic tissues were collected using *ex vivo* luciferase imaging to confirm the presence of a metastatic lesion and to discard as much normal tissue as possible, prior to RNA extraction. Remarkably, levels of mature miR-424 were relatively similar in metastases from mice injected with either EV or miR-424 SUM149PT cells (Fig. 3C). This similarity contrasted with mature miR-424 expression levels in cells pre-injection, where there was an approximately 15-fold difference in mature miR-424 levels (Fig. 3A – left). However, we found that ectopic pri-miR-424 was still expressed at relatively high levels within the miR-424 metastases (Fig. 3D). This expression of pri-miR-424 was similar to pre-injection cells (Fig. 3A – right), demonstrating that miR-424 was post-transcriptionally regulated *in vivo* in the miR-424 metastases. Global miR processing did not appear affected, as multiple other mature miRs were not downregulated in the same metastatic tissue (Supplementary Fig. S5).

A recent study showed that while ectopic TWIST1 increased tumor cell dissemination, subsequent downregulation of TWIST1 enhanced metastatic colonization (13). These data suggest a requirement for biphasic TWIST1 expression at different stages of the metastatic cascade, or for biphasic expression of TWIST1-induced genes that regulate various aspects of the metastatic process. Thus, we examined how miR-424 levels are regulated upon long-term TWIST1 and SNAI1 expression, especially in light of miR-424 post-transcriptional downregulation in metastases. We found that constitutive TWIST1 or SNAI1 expression in HMLE cells resulted in downregulation of mature miR-424 (Fig. 3E, S6A), as opposed to the upregulation observed early after TWIST1 or SNAI1 induction (Fig. 1E). This downregulation is controlled post-transcriptionally, as pri-miR-424 is not repressed by either constitutive TWIST1 or SNAI1 (Fig. 3E, S6A). Together, these data are consistent with tumor cells downregulating mature miR-424 to reverse the intermediate EMT to allow outgrowth at a secondary site.

MiR-424 decreases tumor-initiating characteristics

Increasing evidence suggests that the EMT state does not always increase TIC characteristics (12,26,27), therefore we asked if miR-424 influences TIC characteristics, as this may help explain why miR-424 trends towards decreasing metastasis incidence. We first determined ALDH1 activity in response to miR-424, as ALDH1-active populations are enriched for TICs (28). Overexpression of miR-424 in SUM149PT and MCF12A cells decreased ALDH1 activity (Fig. 4A,B; Supplementary Fig. S3D), while alternative TIC-enriching markers (i.e. CD24, CD44 and EPCAM (ESA)) were not consistently changed. Additionally, inducible miR-424 also decreased ALDH1 activity, which was reversible by doxycycline removal (Supplementary Fig. S4D – white, gray bars, respectively). The lack of CD24/CD44 changes was unexpected given the ALDH1 changes and the fact that both sets of markers enrich for a self-renewal phenotype. However, CD24⁻/CD24⁺ populations are known to have relatively little overlap with ALDH1⁺ populations (28,29), thus miR-424 seems to specifically affect ALDH1⁺ cells.

We then examined mammosphere formation as a functional TIC assay (30). Consistent with the effects on ALDH1, miR-424 decreased numbers of secondary mammospheres formed

(Fig. 4C). Thus, miR-424 is sufficient to impart EMT-related functions on cells, while simultaneously repressing TIC characteristics, traits previously thought to positively associate with one another (1).

MiR-424 decreases orthotopic tumor initiation

Our *in vitro* TIC data prompted us to address whether miR-424 can impact tumor initiation by performing a limiting-dilution analysis *in vivo*. Between 20 and 300 SUM149PT cells overexpressing miR-424 or control EV were injected into the mammary fat pads of female NOD/SCID mice, which were then palpated weekly for tumor formation. While no differences in tumor-initiating capability were observed when larger numbers of cells were injected, injection of lower numbers of cells (20 cells) led to a significant reduction in TIC frequency due to miR-424, as assessed (19) up to three months (Fig. 4D), supporting miR-424 as an inhibitor of tumor initiation.

Interestingly, an oncogenic EMT has been shown to increase tumor initiation in RAS-transformed HMLE (HMLER) cells constitutively expressing TWIST1 or SNAI1 (4). Because we initially observed TWIST1 and SNAI1 increasing miR-424 levels, which is sufficient to decrease TIC properties, we attempted to reconcile the conflicting TIC data by examining miR-424 levels in HMLER cells, finding HMLER cells post-transcriptionally downregulated miR-424 (Supplementary Fig. S6B). This additional example of miR-424 post-transcriptional downregulation is consistent with decreased miR-424 as a mechanism to allow tumor initiation or metastatic outgrowth, possibly by removing signals that inhibit tumor initiation and cell proliferation.

MiR-424 represses a breast cancer stem cell gene signature

To investigate how miR-424 controls TIC and EMT-like phenotypes, we utilized Gene Set Enrichment Analysis (GSEA) after performing next-generation RNA sequencing (RNA-seq) on MCF12A cells overexpressing miR-424. The non-tumorigenic MCF12A cells were used to reduce potentially masking effects of broader genomic alterations in the transformed SUM149PT cells. GSEA revealed that miR-424 represses several genes that are normally downregulated in an EMT signature (7), revealing hypothetical targets of miR-424 involved in mesenchymal programming (Supplementary Fig. S7A). MiR-424 also repressed genes typically upregulated in a breast cancer stem cell gene signature (3), giving insight into how miR-424 represses TIC phenotypes (Supplementary Fig. S7B). TargetScan (31) analysis of the 3' UTRs of these downregulated genes revealed that a significant number (20 of 60, $p < 10^{-5}$; 8 of 23, $p = 0.002$, respectively) of the most negatively associated core genes in the two signatures had predicted miR-424 binding sites (Supplementary Fig. S7, asterisks). Further GSEA analysis, using genome-wide predicted miR-424 targets (TargetScan) as a gene signature, revealed that our RNA-seq results were enriched with repressed predicted target genes ($p < 0.001$), demonstrating utility in coupling functional data and *in silico* predictions. These data suggest that miR-424 may drive mesenchymal EMT programming and inhibit TIC phenotypes by targeting multiple genes simultaneously.

TGFBR3 is a novel target of miR-424

To narrow down a mechanism downstream of miR-424, we validated several RNA-seq hits. IB analyses of SUM149PT lysates confirmed that miR-424 downregulates TGFBR3, likely observed as a number of bands due to known extensive post-translational modifications (32), ST3GAL5 and TMPRSS4 (Fig. 5A). TGFBR3 was identified directly from RNA-seq differences, while ST3GAL5 and TMPRSS4 came through GSEA of the breast cancer stem cell and EMT signatures, respectively. Each gene also contains a predicted miR-424 binding site (TargetScan) and has not previously been identified as a target of miR-424.

Because TGF β signaling has been implicated in both EMT and TIC phenotypes (32–35), we focused on TGFBR3 downstream of miR-424. QPCR confirmed that miR-424 decreases *TGFBR3* mRNA in SUM149PT, HMLE and MCF12A cells (Fig. 5B; Supplementary Fig. S7C,D), while a 3' UTR reporter assay demonstrated that miR-424 can repress the *TGFBR3* 3' UTR (Fig. 5C). Mutating the putative miR-424 seed-binding site in the *TGFBR3* 3' UTR reporter construct significantly alleviated miR-424 repression of the reporter, though to a small degree (Fig. 5C).

TGFBR3 mediates the intermediate EMT phenotype driven by miR-424

To determine if TGFBR3 inhibition, mimicking miR-424 expression, is sufficient to recapitulate an intermediate EMT, we used two shRNAs to knock down (KD) TGFBR3 in parental SUM149PT cells (Fig. 5D). TGFBR3 KD increased vimentin and fibronectin levels, by qPCR and IB, and N-cadherin, by qPCR, without affecting E-cadherin levels (Fig. 5D,E). We also determined if TGFBR3 loss is necessary for a miR-424-induced intermediate EMT by restoring the coding-sequence of *TGFBR3*, which cannot be repressed by miR-424 as it lacks a 3' UTR, to miR-424 overexpressing cells (Supplementary Fig. S7E). TGFBR3 restoration reversed the mesenchymal marker levels originally increased by miR-424 while not affecting E-cadherin levels (Fig. 5F). Together, decreased TGFBR3 is necessary in mediating miR-424-dependent EMT changes and sufficient to regulate, at least in part, mesenchymal EMT programming.

ERK signaling mediates miR-424 repression of TIC phenotypes

Because TGFBR3 is responsible for the majority of mesenchymal changes induced by miR-424, we asked if TGFBR3 also plays a role in repressing TIC phenotypes. We found that TGFBR3 KD, phenocopying miR-424 expression, did not lower ALDH1 activity (Fig. 6A), nor was TGFBR3 restoration able to rescue decreased ALDH1 activity downstream of miR-424 (Fig. 6B).

Subsequently, we shifted our focus to ERK signaling, as we observed a marked increase in this non-canonical TGF β signaling arm downstream of miR-424 (Fig. 5F), and as ERK signaling is known to negatively regulate self-renewal of embryonic stem cells (36), though in other contexts ERK has been found to positively regulate self-renewal (37). Interestingly, while TGFBR3 KD increased phospho-ERK1/2 (Fig. 5E), revealing TGFBR3 can inhibit ERK, restoring TGFBR3 did not reverse miR-424's ability to increase phospho-ERK1/2 levels (Fig. 5F), indicating miR-424 activates the ERK pathway through multiple mechanisms.

To examine ERK signaling functionally, we used two small-molecule MEK inhibitors, AZD6244 (38) and PD98059 (39), to prevent MEK from phosphorylating ERK1/2 (Fig. 6C). ERK signaling inhibition reversed miR-424's ability to decrease ALDH1 activity (Fig. 6D). Extending the PD98059 MEK inhibitor to functional mammosphere formation assays, we observed a partial reversal of mammosphere repression due to miR-424 (Fig. 6E). Interestingly, while MEK inhibition downstream of miR-424 increased the number of mammospheres, they were smaller in size (Fig. 6F), suggesting divergent effects of the ERK pathway on self-renewal and proliferation in mammosphere-initiating cells and the progeny that comprise the bulk of the mammosphere, respectively. We thus decided against using MEK inhibitors to examine tumor initiation, as effects on TIC properties would likely be masked by the anti-proliferative properties. In light of TGFBR3 KD not altering ALDH1 activity, yet being sufficient to increase ERK signaling, these data together suggest that ERK signaling is necessary, but not sufficient, for repressing *in vitro* tumor-initiating phenotypes downstream of miR-424.

MiR-424 associates with TWIST1 and is biphasically expressed in human breast cancers

To determine the clinical relevance of miR-424, we assessed the expression of miR-424 and EMT-inducing transcription factors in human breast cancer data sets containing patient-matched miR and mRNA expression data. In two data sets (40,41), a positive relationship existed between miR-424 and *TWIST1* mRNA (Fig. 7A), in addition to *TWIST2* mRNA (Fig. 7B), which has similar EMT and TIC-associated properties as *TWIST1* (42).

Recently, CTCs from triple-negative breast cancers, which largely overlap with basal tumors, and from HER2-enriched breast cancers have been shown to consist largely of mesenchymal-like and intermediate EMT-like cells (11). We thus examined The Cancer Genome Atlas (TCGA) data (43) to determine whether miR-424 levels were associated with a particular breast cancer subtype. Basal tumors had significantly higher miR-424 levels than luminal A and B tumors, while HER2-enriched tumors expressed significantly more miR-424 than luminal A tumors (Fig. 7C). These subtype differences are consistent with miR-424 inducing an intermediate EMT phenotype.

We then examined patient mRNA profiles from high and low miR-424 groups (40). GSEA identified a significant enrichment of three different EMT-associated gene signatures (7,8,44) in patient tumors that expressed high miR-424 (Fig. 7D). These data corroborate our experimental findings that miR-424 induces an intermediate EMT and suggest that miR-424 may regulate an EMT-like program in human breast cancers.

Lastly, to assess miR-424 levels during cancer progression, we investigated our own independent cohort of triple-negative breast cancer patients (45) where high expression of miR-424 was expected. We found that miR-424 was upregulated in primary tumors compared to matched normal tissue (Fig. 7F), and in these primary tumors, miR-424 inversely correlated with TGFBR3 (Fig. 7E). Further, miR-424 was downregulated in metastases compared to matched primary tumors (Fig. 7G), similar to what occurred in our experimental metastases. Thus, examination of multiple patient cohorts revealed that miR-424 was upregulated in primary tumors in association with EMT drivers and EMT gene signatures, but was downregulated in matched metastatic lesions. These data are consistent

with our hypothesis that miR-424 expression may promote early stages of metastasis associated with an EMT, but that downregulated miR-424 facilitates metastatic outgrowth.

Discussion

The oncogenic EMT has been implicated as a critical mediator of metastasis. Previous controversy in the field may have been due to the challenge of identifying human tumor cells that are histologically mesenchymal and definitively originate from a carcinoma (46). However, recent studies using experimental models and human tumors indicate that disseminating cells may exist in a more plastic state where cells transition between epithelial and mesenchymal phenotypes, or exist in an intermediate state between the two (11,13). We thus examined an inducible EMT model to characterize a dynamic, intermediate EMT state. In doing so, we demonstrated that EMT-inducing factors TWIST1 and SNAIL1 can increase expression of mesenchymal genes prior to repression of epithelial genes. Furthermore, we found that miR-424, downstream of TWIST1 and SNAIL1, is sufficient to induce multiple mesenchymal EMT characteristics, without altering epithelial attributes, in a reversible manner. To our knowledge, this is the first demonstration of a miR that can control specifically the mesenchymal arm of an EMT independent of epithelial changes.

While miR-424 induced functions associated with a full oncogenic EMT, miR-424 decreased TIC characteristics *in vitro* and *in vivo*, in contrast to other EMT-inducing factors (4,42). The negative growth effects of miR-424, observed by others (47) and us, could be partially responsible for the TIC repression; however, over the last month of the experiment, no new miR-424 tumors were formed, whereas new control tumors formed each week. Additionally, while miR-424 overexpression led to smaller mammosphere formation, possibly due to growth effects, there were importantly fewer mammospheres, possibly indicative of TIC inhibition. These data, coupled with ALDH1 repression, suggest that miR-424 may decrease tumor initiation through TIC effects and not just through growth inhibition.

Interestingly, while miR-424 did not significantly affect overt metastasis incidence, we observed post-transcriptional regulation of miR-424 in metastatic outgrowths. HMLE and HMLER cells constitutively expressing TWIST1 and SNAIL1 over time demonstrated post-transcriptional reduction of miR-424. While post-transcriptional regulation of miR-424 has also been observed in endothelial cells (48), the mechanism remains unknown. Post-transcriptional regulation of miR-424 in the constitutive TWIST1 and SNAIL1 HMLER cells, which have increased TIC frequency (4), may have allowed those cells to overcome the inhibitory tumor-initiation effects of miR-424, while maintaining other pro-tumorigenic phenotypes mediated by TWIST1 or SNAIL1.

Examination of how miR-424 mediates an intermediate EMT led to the identification of TGFBR3. Previous studies demonstrated that TGFBR3 decreases during breast cancer progression (49), as well as during an EMT in prostate cancer prior to E-cadherin downregulation (33); similarly during an EMT, miR-424 increases prior to E-cadherin downregulation. Importantly, TGFBR3 KD, while increasing vimentin and fibronectin protein levels, did not increase N-cadherin protein. These data suggest that while TGFBR3

functions downstream of miR-424 for induction of the mesenchymal phenotype, it does not act alone, and may act in combination with other genes identified by GSEA.

To repress TIC attributes, we found that miR-424 requires ERK signaling. A similar negative TIC role for ERK signaling has been shown in normal and tumorigenic settings (36,50), but ERK signaling can also increase TIC properties (37), implicating important contextual differences. Additionally, since ERK signaling is necessary, but not sufficient, for repression of TIC phenotypes, we speculate that numerous effectors of miR-424 are responsible for mediating TIC phenotypes, particularly as miR-424 represses part of a breast cancer stem cell signature.

Clinically, we found multiple associations with miR-424 that parallel our experimental data. Analysis of primary breast tumors from four independent cohorts demonstrated a positive link between miR-424 and EMT-associated molecular changes. Our own cohort revealed increased miR-424 in primary tumors compared to matched normal tissue, with an inverse correlation between TGFBR3 and miR-424 in the same tumors. Critically, miR-424 was decreased in metastases compared to matched primary tumors. These human data are consistent with our experimental findings that miR-424 induced a mesenchymal-like phenotype, but that mature miR-424 was downregulated in metastases *in vivo*, supporting the possibility that decreased miR-424 at a distant site may facilitate metastatic outgrowth (Supplementary Fig. S8).

Recently, miR-424 was shown to induce a full oncogenic EMT (51), though we have not observed alterations in the epithelial EMT arm in four different models. Comparing techniques may help reconcile differing results, as ectopic miR-424 expression was achieved through very different approaches. Nonetheless, our findings contribute to other studies dissociating mesenchymal and TIC phenotypes (12,26,27). These studies demonstrate that an EMT may only be relevant to the metastatic cascade in the context of an EMT-MET spectrum. Critically, the intermediate EMT state has been observed in human tumor cells (9–11). Our findings that miR-424 drives an intermediate EMT, where the mesenchymal N-cadherin protein is co-expressed with the epithelial E-cadherin protein, suggests that miR-424 may poise cells for movement along the epithelial-mesenchymal spectrum as a means to mediate plasticity that facilitates an MET and metastatic outgrowth.

As more experimental studies demonstrate roles for both EMT and MET in metastatic progression, we must re-evaluate whether clinically targeting EMT-inducing factors will be uniformly efficacious. The position of a tumor cell along the EMT-MET axis, as well as physical location in a patient, may dictate different responses to novel therapeutics. Thus, obtaining a better understanding of the role of plasticity within the metastatic cascade will help identify which patients will benefit from potential EMT-related therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TWIST1 induces mesenchymal markers prior to repression of epithelial genes, concomitant with miR-424 upregulation

(A) Phase contrast images of HMLE Empty Vector and HMLE TWIST1-inducible cells after 4 and 12 days of TWIST1 induction. Bar-100 μ m. Epithelial and mesenchymal markers, by qPCR, after (B) 4 and (C) 12 days of TWIST1 induction, two-tailed unpaired t-test. (D) MiR microarray results ($p < 0.1$) after 4 and 12 days of TWIST1 induction compared to HMLE control cells. (E) Mature and (F) primary miR-424 levels, by qPCR, after 4 and 12 days of TWIST1 induction, two-way ANOVA, Bonferroni multiple comparison post-test.

SEM shown. Representative images/graphs of n 3. #p<0.1, *p<0.05, **p<0.01, ***p<0.001.

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Figure 2. MiR-424 drives a reversible intermediate EMT without repressing epithelial characteristics

(A) Level of stable constitutive miR-424, by qPCR, SEM shown. (B) IB of mesenchymal (N-cadherin, fibronectin, vimentin) and epithelial (E-cadherin, α -catenin, plakoglobin) markers in stable miR-424 cells. Representative loading control (β -tubulin) shown. Asterisk, incomplete stripping. (C) Quantification of E-cadherin and N-cadherin ICC localization, n = 155 nuclei. (D) Representative ICC of E-cadherin (red) and N-cadherin (green). DAPI, blue. Arrows, co-expression of E-cadherin & N-cadherin. Stable miR-424 expressing SUM149PT cells displaying: (E) distance migrated over 8 hours; (F) cell cycle analysis, by

BrdU/PI staining; (G) adhesion to fibronectin-coated wells. Doxycycline and reversed SUM149PT inducible clones were treated with 0.5 µg/mL doxycycline for 7–10 days, while reversed conditions were cultured for an additional 7–10 days in the absence of doxycycline: (H) 3' UTR luciferase reporter with perfect or mutant miR-424 binding sites; EMT markers by (I) IB of whole cell lysates and (J) by qPCR. Two-tailed unpaired t-test for all except (H) two-way ANOVA, Bonferroni multiple comparison post-test. SEM shown, compiled experiments of n = 3. NS-not significant, *p<0.05, **p<0.01, ***p<0.001.

Figure 3. MiR-424 is post-transcriptionally downregulated in an experimental metastasis mouse model

(A) Pre-intracardiac injection levels of mature and primary miR-424, by qPCR, of luciferase-tagged SUM149PT cells overexpressing constitutive miR-424. (B) Kaplan-Meier graph of metastasis-free survival in nude mice after intracardiac injection with cells in (A). Levels of (C) mature and (D) primary miR-424, by qPCR, in metastases from mice injected with EV or miR-424 cells profiled in (A), SD shown. (E) Levels of mature and primary miR-424, by qPCR, in HMLE cells constitutively expressing TWIST1 past 20 days, SEM shown. Two-tailed unpaired t-test, **p<0.01, ***p<0.001, N.S. not significant.

^AStem cell frequency: EV – 1/13, miR-424 – 1/41 (p=0.012 by ELDA)

Figure 4. MiR-424 represses tumor-initiating properties

(A) Representative ALDH1 activity, as measured by ALDEFLUOR assay, with and without DEAB (ALDH1 inhibitor) treatment in constitutive miR-424 versus Empty Vector (EV) control SUM149PT cells. (B) Quantification of ALDH1 activity without DEAB in the same cell lines as (A). (C) Secondary mammosphere formation of SUM149PT cells with constitutive miR-424 or EV. SEM shown, n = 3. *p<0.05, ***p<0.001, two-tailed unpaired t-test. (D) Tumor initiation results from palpating NOD/SCID mice injected with SUM149PT-424 cells in limiting dilution.

Figure 5. TGFBR3 KD is necessary and sufficient to recapitulate much of an intermediate EMT SUM149PT cells expressing constitutive miR-424 assayed for (A) TGFBR3, ST3GAL5 and TMPRSS4 by IB and (B) *TGFBR3* mRNA by qPCR. (C) Luciferase reporter using 3' UTR of *TGFBR3* co-transfected with 5 nM miR-424 mimics. Mutant – mutated miR-424 seed binding site in reporter construct. Two-way ANOVA with Bonferroni post-test. (D) QPCR, with two-tailed unpaired t-test, and (E) IB for EMT markers in SUM149PT cells with stable TGFBR3 KD. (F) IB for EMT markers and phospho-ERK1/2 in SUM149PT cells

overexpressing miR-424 and a TGFBR3 rescue construct. Representative actin show for (F). SEM shown, n = 3; *p<0.05, **p<0.01, ***p<0.001.

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Figure 6. ERK signaling is necessary for miR-424-mediated repression of tumor-initiating properties

(A) ALDH1 activity of SUM149PT cells with stable KD of TGFBR3. (B) ALDH1 activity in SUM149PT cells expressing miR-424 with restoration of TGFBR3. (C) Total and phospho-ERK1/2 levels of SUM149PT cells expressing miR-424 with vehicle or MEK inhibitor (5 μ M) treatment after 24 hours. (D) ALDH1 activity of cells in (C). (E) Secondary mammosphere formation results and (F) phase contrast images of SUM149PT cells expressing miR-424 and treated with PD98059. SEM shown, n = 3. Bar=100 μ m. Two-way

ANOVA with Bonferroni post-test for all except (A) one-way ANOVA with Bonferroni post-test. # $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 7. Human data support a positive role for miR-424 in EMT, but a negative role in metastasis

Contingency table analysis of above/below median miR-424 levels versus above/below median levels of (A) TWIST1 and (B) TWIST2. (C) MiR-424 levels in PAM50 subtypes from the TCGA dataset, quartiles shown, n=291. ANOVA test with multiple comparisons corrected by Tukey HSD. (D) GSEA using mRNA from breast cancer patients with high or low miR-424 expression in the Buffa data set (40) compared to upregulated genes from 3 EMT signatures by Taube et al. (7), Onder et al. (8) and Blick et al. (44). (E) Expression of miR-424 and TGFBR3 in individual primary breast tumors; Pearson correlation analysis.

Expression of miR-424 in (F) normal breast versus matched primary tumor (n=46) and (G) primary tumor versus matched metastasis (n=18), in a human triple-negative breast cancer data set (45), two-tailed paired t-test. #p<0.0001 versus LumA, \$p<0.05 versus LumB, %p<0.01 versus LumA, *p<0.05.

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