Competitive endogenous RNA is an intrinsic component of EMT regulatory circuits and modulates EMT

Yuwei Liu\textsuperscript{1,2}, Mengzhu Xue\textsuperscript{1}, Shaowei Du\textsuperscript{1,3}, Wanwan Feng\textsuperscript{4}, Ke Zhang\textsuperscript{4}, Liwen Zhang\textsuperscript{2,5,6}, Haiyue Liu\textsuperscript{1,2}, Guoyi Jia\textsuperscript{1,3}, Lingshuang Wu\textsuperscript{7}, Xin Hu\textsuperscript{8,9}, Luonan Chen\textsuperscript{5,7,10,11} & Peng Wang\textsuperscript{1,2,4,8,9}

The competitive endogenous RNA (ceRNA) hypothesis suggests an intrinsic mechanism to regulate biological processes. However, whether the dynamic changes of ceRNAs can modulate miRNA activities remains controversial. Here, we examine the dynamics of ceRNAs during TGF-\(\beta\)-induced epithelial-to-mesenchymal transition (EMT). We observe that TGFBI, a transcript highly induced during EMT in A549 cells, acts as the ceRNA for miR-21 to modulate EMT. We further identify FN1 as the ceRNA for miR-200c in the canonical SNAIL-ZEB-miR200 circuit in MCF10A cells. Experimental assays and computational simulations demonstrate that the dynamically induced ceRNAs are directly coupled with the canonical double negative feedback loops and are critical to the induction of EMT. These results help to establish the relevance of ceRNA in cancer EMT and suggest that ceRNA is an intrinsic component of the EMT regulatory circuit and may represent a potential target to disrupt EMT during tumorigenesis.

\textsuperscript{1}Laboratory of Systems Biology, Shanghai Advanced Research Institute, Chinese Academy of Sciences, 200031 Shanghai, China. \textsuperscript{2}University of Chinese Academy of Sciences, 200031 Shanghai, China. \textsuperscript{3}School of Life Sciences, Shanghai University, 200031 Shanghai, China. \textsuperscript{4}Bio-Med Big Data Center, CAS Key Laboratory of Computational Biology, CAS-MPG Partner Institute for Computational Biology, Shanghai Institute of Nutrition and Health, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 200031 Shanghai, China. \textsuperscript{5}Key Laboratory of Systems Biology, Center for Excellence in Molecular Cell Science, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 200031 Shanghai, China. \textsuperscript{6}Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 200031 Shanghai, China. \textsuperscript{7}School of Life Science and Technology, ShanghaiTech University, 201210 Shanghai, China. \textsuperscript{8}Department of Breast Surgery, Key Laboratory of Breast Cancer in Shanghai, Fudan University Shanghai Cancer Center, Fudan University, 200032 Shanghai, China. \textsuperscript{9}Precision Cancer Medicine Center, Fudan University Shanghai Cancer Center, 200032 Shanghai, China. \textsuperscript{10}Center for Excellence in Animal Evolution and Genetics, Chinese Academy of Sciences, 650223 Kunming, China. \textsuperscript{11}Research Center for Brain Science and Brain-Inspired Intelligence, 201210 Shanghai, China. These authors contributed equally: Yuwei Liu, Mengzhu Xue, Shaowei Du. Correspondence and requests for materials should be addressed to X.H. (email: xinhu@fudan.edu.cn) or to L.C. (email: inchen@sibs.ac.cn) or to P.W. (email: wangpeng@picb.ac.cn)
MicroRNAs (miRNAs) are ubiquitous post-transcriptional regulators that impact RNA stability and the rate of translation by pairing to complementary sites (referred to as miRNA response elements [MREs]) within target RNAs\(^1\). The interaction between miRNAs and their RNA targets is characterized by a many-to-many relationship in which a single miRNA can repress multiple-RNA targets and a single RNA can contain MREs of multiple miRNAs. Hence, ceRNA hypothesis has been proposed; this hypothesis suggests that RNAs can regulate each other by competing for a limited pool of miRNAs\(^4,5\). Studies have suggested that ceRNA crosstalk may regulate essential biological processes such as cancer\(^6-10\). However, these studies often lack the absolute quantification of miRNAs and the corresponding ceRNAs\(^11-13\). Consequently, whether the effects of ceRNA exist under physiological conditions has been challenged\(^14\). For example, a recent quantitative hypothesis has been proposed; this hypothesis suggests that RNAs can regulate each other by competing for a limited pool of target RNAs\(^1\).

FOXP1, an evolutionary conserved transcription factor (TF) that plays an important role in regulating embryonic stem cell pluripotency\(^29\), is highly induced and could represent a key EMT regulator in A549 cells (Supplementary Fig. 1A–C). To investigate the role of FOXP1 in EMT, we knocked down FOXP1 in A549 cells undergoing TGF-β-induced EMT using short hairpin RNA. Notably, the knockdown of FOXP1 significantly enhanced the expression of CDH1 and repressed the expression of CDH2 (Fig. 1A, B). Moreover, the loss of FOXP1 substantially reduced the migration and invasion of these cells, diminishing the key characteristic of EMT (Fig. 1C, D). To further demonstrate that FOXP1 is a key inducer of EMT, we overexpressed FOXP1 in A549 cells. Consistent with the results of loss-of-function assays, cells overexpressing FOXP1 gained an mesenchymal-like morphology, lost the expression of CDH1, showed upregulated expression of VIM, and acquired an enhanced ability to migrate and invade (Fig. 1E–H, Supplementary Fig. 1D). Taken together, these data confirmed that FOXP1 is a potent transcriptional inducer of EMT in A549 cells.

FOXP1 and miR-21 forms a double-negative feedback loop. Interestingly, FOXP1 expression reached a plateau at 48 h after TGF-β treatment (Supplementary Fig. 1A–C). Because the canonical EMT-regulatory network is characterized by double-negative feedback loops between SNAIL-miR-34 and ZEB-miR-200c, we speculated that miRNAs might also regulate FOXP1 activity in A549 cells to establish equilibrium. To identify potential miRNA regulators of FOXP1, we used deep sequencing (miRNA-seq) to profile miRNA expression during TGF-β-induced EMT and identified 19 and 126 differentially expressed miRNAs at 24 and 96 h into EMT, respectively (Fig. 2A, Supplementary Fig. 2A). We focused on miRNAs that were differentially expressed at 96 h into EMT because FOXP1 expression maintained an equilibrium from 48 to 96 h into EMT. To identify candidate regulatory miRNAs for FOXP1, we examined the overlap between miRNAs differentially expressed at 96 h into EMT and miRNAs predicted to regulate FOXP1 by targetScan\(^30\). While five miRNAs were identified by both targetScan and the differential expression analysis, four of the five miRNAs (miR-122-5p, miR-129-5p, miR-200b-3p, and miR543) were expressed at low levels (counts per million [CPM] < 10) (Supplementary Fig. 2B). Candidate regulatory miRNAs have been typically identified by changes in relative expression, in which larger changes in the relative expression indicate more significant functions. However, increasing evidence has demonstrated that, for miRNAs, a sufficiently high number of miRNA transcripts in cells is essential for the miRNA to be functional, because a low number of miRNA transcripts (<100/cell) cannot effectively repress their targets owing to the dilution effects of large number of MREs\(^31\). Using published miRNA absolute qPCR and miRNA-seq data, we extrapolated the absolute copy number of the five miRNAs and observed that only miR-21, a well-established oncomiR, was expressed at >100 copies/cell in A549 cells. Thus, we focused on our subsequent analyses on miR-21. Interestingly, the

Results

**FOXP1 is a critical inducer of EMT in A549 cells.** In a previous study, we profiled the transcriptional dynamics of TGF-β-induced EMT in A549 cells\(^22\). While the canonical EMT regulators, such as SNAI1/2 or ZEB1/2, are not abundantly expressed in A549 cells, differential expression analyses have suggested that FOXP1, a transcription factor (TF) that plays an important role in regulating embryonic stem cell pluripotency\(^29\), is highly induced and could represent a key EMT regulator in A549 cells (Supplementary Fig. 1A–C). To investigate the role of FOXP1 in EMT, we knocked down FOXP1 in A549 cells undergoing TGF-β-induced EMT using short hairpin RNA. Notably, the knockdown of FOXP1 significantly enhanced the expression of CDH1 and repressed the expression of CDH2 (Fig. 1A, B). Moreover, the loss of FOXP1 substantially reduced the migration and invasion of these cells, diminishing the key characteristic of EMT (Fig. 1C, D). To further demonstrate that FOXP1 is a key inducer of EMT, we overexpressed FOXP1 in A549 cells. Consistent with the results of loss-of-function assays, cells overexpressing FOXP1 gained a mesenchymal-like morphology, lost the expression of CDH1, showed upregulated expression of VIM, and acquired an enhanced ability to migrate and invade (Fig. 1E–H, Supplementary Fig. 1D). Taken together, these data confirmed that FOXP1 is a potent transcriptional inducer of EMT in A549 cells.

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
FOXP1 is a critical inducer of EMT in A549 cells. a Quantitative reverse transcription polymerase chain reaction (qRT-PCR) results showing the levels of gene expression in A549 cells during TGF-β-induced EMT after silencing FOXP1 expression using specific shRNA. n = 3. b Immunoblotting analysis of the protein abundance of indicated genes in A549 cells undergoing EMT after treatment with an shRNA targeting FOXP1. c A549 cells undergoing TGF-β-induced EMT were treated with an shRNA targeting FOXP1 and subjected to a migration assay (upper panel) and an invasion assay (lower panel). Scale bars: 100 μm. d The migrated or invaded cells were quantified (bar charts). n = 6. e Morphology of A549 cells overexpressing FOXP1 or an empty control vector. f Immunoblotting analysis of the protein abundance of indicated genes in A549 cells overexpressing FOXP1 or an empty control vector. g A549 cells overexpressing FOXP1 or an empty control vector were subjected to a migration assay (upper panel) and an invasion assay (lower panel). Scale bars: 100 μm. h The migrated or invaded cells were quantified (bar charts). n = 6; error bars indicate the means ± s.d. *p < 0.05, **p < 0.01, determined using the two-tailed Student’s t test. Source data are provided as a Source Data file.

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TGFBI regulates EMT as a ceRNA for miR-21. A canonical theme of EMT-regulatory circuits is that the key miRNAs involved in this process, such as members of the miR-200 family, are downregulated during EMT. Surprisingly, miR-21 demonstrated anorthodox upregulation during TGF-β-induced EMT in A549 cells (Fig. 2a). Although the fold change was modest (1.2 at 96 h into EMT), the increase in the absolute number of miR-21 transcripts was substantial because miR-21 comprised 64.91% of all mappable reads in miRNA-seq at 96 h into EMT. This observation raised the interesting question of how FOXP1 functions in the presence of a large number of miR-21 molecules. An attractive mechanism to overcome the inhibition of miR-21 is through a ceRNA effect, which requires the presence of sufficient MREs to sequester the miR-21 molecules. To examine this hypothesis, we first analyzed the dynamic change of miR-21 MREs using the time-course RNA-SEQ data during TGF-β-induced EMT. This analysis showed that the miR-21 MREs exhibited a fourfold expansion during TGF-β-induced EMT (Fig. 3a, Supplementary Fig. 4A, Supplementary Tables 1 and 2). Importantly, TGFBI, a gene broadly associated with EMT and with experimentally validated miR-21 binding sites, represented

canonical EMT miRNA, miR-200c, only expressed at very low levels in A549 cells comparing to miR-21 (normalized read counts 43.33 vs. 1,026,301.79). Because the canonical EMT TFs such as SNAIL and ZEB are also expressed at very low levels in A549 cells, we speculated that the canonical SNAIL/ZEB-miR-200c EMT-regulatory circuit is not functional in A549 cells, and FOXP1 and miR-21 are the master molecules to regulate EMT in A549 cells.

Unlike ZEB1, which possesses multiple binding sites for the miR-21 binding site in the 3′ UTR of FOXP1 by CRISPR/Cas9

Importantly, TGFBI, a gene broadly associated with EMT and with experimentally validated miR-21 binding sites, represented
over 90% of all the increased miR-21 MREs in A549 cells during EMT (Supplementary Table 2). Importantly, recent studies have demonstrated that the absolute quantification of miRNAs and their corresponding ceRNAs is critical to establish ceRNA effects. Hence, we performed absolute qRT-PCR to quantify miR-21 expression was quantified (bar charts). 

**Fig. 2** FOXP1 and miR-21 form a double-negative feedback loop. a Volcano plot showing the differential expression of miRNAs at 96 h into TGF-β-induced EMT in A549 cells. The red dots represent miRNAs with a differential expression FDR < 0.05 and absolute log2-fold change > 1. The horizontal dotted line represents the log2(CPM) corresponding to 100 copies/cell. b Graph showing the sequence alignment of FOXP1 3′UTR with miR-21-5p. c The results of the luciferase reporter assay were quantified (bar charts). d Immunoblotting analysis of the protein abundance of indicated genes in A549 cells during TGF-β-induced EMT after a specific antagomiR was used to silence miR-21 expression. e Same as (d) for the qRT-PCR assay. f qRT-PCR analysis of the estimated number of MREs and miR-21 (Fig. 3b and Supplementary Fig. 4C). Consistent with the dynamic expression patterns of TGFBI and miR-21 during EMT, the 8mer miR-21 binding site, but not the control 3′UTR without the miR-21 binding site, could effectively rescue the expression of FOXP1, because TGFBI expression peaked around 24 h into EMT. Hence, we knocked down TGFBI using siRNA 24 h prior to TGF-β treatment and analyzed the impact of TGFBI-specific siRNA on miR-21 expression. g A549 cells undergoing TGF-β-induced EMT were treated with a siRNA targeting FOXP1, and the impact on miR-21 expression was quantified using qRT-PCR. n = 3; error bars indicate the means ± s.d. *p < 0.01, determined using a two-tailed Student’s t test. Source data are provided as a Source Data file.
Fig. 3 TGFBI is a functional ceRNA for miR-21 to regulate EMT. a Graph showing the number of miR-21 MREs extrapolated from RNA-seq data during TGF-β-induced EMT in A549 cells using targetScan-based predictions. b Graph showing the dynamics of modeled B-mer miR-21 binding site occupancy during TGF-β-induced EMT in A549 cells using targetScan-based predictions. c Graph showing the level of indicated RNAs in A549 cells during TGF-β-induced EMT subjected to the indicated treatments. d Same as (c) for the immunoblotting analysis. e A549 cells undergoing TGF-β-induced EMT were treated as indicated and subjected to a migration assay (left panel) and an invasion assay (right panel). Scale bars: 100 μm. The migrated or invaded cells were quantified (bar charts). n = 6. f Graph showing the level of indicated RNAs in A549 cells during TGF-β-induced EMT subjected to the indicated treatments, using cells whose DICER1 has been knocked out with CRISPR-Cas9. g Same as (f) for the immunoblotting analysis. h Kaplan–Meier survival analyses based on the overexpression of TGFBI in three independent lung cancer data sets. n = 3; error bars indicate the means ± s.d. *p < 0.01, determined using a two-tailed Student’s t test. Source data are provided as a Source Data file.

knockdown of TGFBI significantly reduced the migration and invasion of the cells (Fig. 3e). Consistent with the results of qRT-PCR and immunoblot assays, the reduction of motility could be effectively rescued by overexpressing functional TGFBI 3′ UTR containing the miR-21 binding site (Fig. 3e).

A potential pitfall of siRNA-based assays is the saturation of the RISC machinery, which could affect miRNA function indirectly. Thus, we further analyzed the TGFBI ceRNA activity using two additional approaches. We first utilized CRISPR/Cas9 technology to knockout DICER1, which is essential for the biogenesis of miRNAs (Supplementary Fig. 5A, B). As expected, the knockout of DICER1 gene reduced the mature miR-21 abundance by >90% (Supplementary Fig. 5C, D) comparing to wide type controls. Consistent with the hypothesis that the ceRNA effects of TGFBI require functional miRNAs, depleting TGFBI in DICER1-less A549 cells demonstrated no detectable impacts on the expression of key EMT genes (Fig. 3f, g). Moreover, the introduction of TGFBI 3′ UTR with miR-21 binding sites into A549 cells also generated negligible impacts on key EMT molecules, further confirming that the impact of TGFBI is through the associated ceRNA activities that require mature miRNAs (Fig. 3f, g). Secondly, we mutated the seed region of miR-21 binding sites in the 3′ UTR of TGFBI with CRISPR/Cas9 technology (Supplementary Fig. 6A–D). We then repeated the ceRNA function assays by depleting TGFBI with specific siRNAs. As expected, no significant impacts on the expression of FOXP1, CDH1, CDH2, and miR-21 were detected in A549 cells lacking functional miR-21 binding sites in the 3′ UTR of TGFBI (Supplementary Fig. 6E, F). Interestingly, introducing TGFBI 3′ UTR with functional miR-21 binding sites did generate a small but significant impact on the expression of FOXP1, CDH2, and miR-21 (Supplementary Fig. 6E, F). Taken together, these data
demonstrated that the observed ceRNA effects of TGFBI operate specifically through the associated miR-21 binding site. Finally, TGFBI knockdown only impacted the expression of genes with experimentally validated miR-21 binding sites, further demonstrating the specificity of the ceRNA effects associated with TGFBI (Supplementary Fig. 6G, H).

We next examined whether the key TFs controlling EMT regulate TGFBI expression. Previously, we established that three master TFs, ETS2, HNF4A, and JUNB, synergistically regulate EMT in A549 cells. As expected, the knockdown of ETS2, HNF4A, or JUNB significantly reduced TGFBI expression, confirming that the master TFs for EMT positively regulate TGFBI expression (supplementary Fig. 6I, J). Moreover, the knockdown of FOXP1 significantly enhanced TGFBI expression, which was consistent with the observed downregulation of TGFBI late into EMT (Supplementary Fig. 6I, J). Taken together, these data suggested that TGFBI, the key ceRNA regulating EMT in A549 cells, is directly coupled with the FOXP1-miR-21 double-negative feedback loop and represents an intrinsic component of the EMT-regulatory circuits.

Because EMT is a crucial step of metastasis, it is of great interest to examine whether TGFBI expression is associated with the clinical outcome of cancer patients. We then performed Kaplan–Meier survival analyses on three independent lung cancer data sets. Reassuringly, the overexpression of TGFBI consistently associated with a poor clinical outcome in tested data sets. Reassuringly, the overexpression of TGFBI was associated with a poor clinical outcome in all tested data sets. Reassuringly, the overexpression of TGFBI was associated with a poor clinical outcome in all tested data sets.

Next, we examined whether ceRNA also represents a crucial component in the canonical ZEB-miR200 regulatory model of EMT using MCF10A cells. We first used publicly available time-course microarray data to examine whether the MREs for miR-200c, the miRNA that plays a key role in regulating EMT in MCF10A cells, demonstrate significant dynamics during EMT (Supplementary Fig. 7A, B, Supplementary Tables 3 and 4). Strikingly, we observed a pattern analogous to that observed in A549 cells: the MREs for miR-200c displayed significant upregulation, and a single RNA, FN1, represented over 90% of all increased MREs during EMT in MCF10A cells at 72 h into EMT (Supplementary Fig. 7A, B, Supplementary Table 4). Importantly, the binding sites for miR-200c in FN1 mRNA have been experimentally validated, suggesting that FN1 could display substantial ceRNA effects. Encouraged by the results of MRE dynamics analyses, we quantified the number of miR-200c and FN1 mRNA in MCF10A cells during TGF-β-induced EMT. Expectedly, the absolute qPCR analyses confirmed that while miR-200c outnumbered FN1 by 4.43-fold in unstimulated MCF10A cells, FN1 quickly out-numbered miR-200c upon TGF-β-induced EMT (Fig. 4a).

To confirm that the ceRNA effect of FN1 could regulate EMT, we first performed computational simulations to estimate the changes of miR-200c binding site occupancies analogous to miR-21. Unlike miR-21, which was upregulated during EMT, miR-200c expression was gradually repressed during EMT in MCF10A cells. More importantly, FN1, the putative miR-200c ceRNA,
displayed a monotonic upregulation (Fig. 4a). Consequently, mathematical simulation showed that the 8mer miR-200c site occupancy gradually decreased as cells undergoing EMT, and declined from about 90% at 0 h to around 55% at 72 h into EMT using MREs estimated by pictar (Fig. 4b). Critically, the reduction in miR-200c binding site occupancy was predominantly due to FN1 because the removal of FN1 MREs from the equation restored the miR-200c binding site occupancy to about 85% at 72 h into EMT (Fig. 4b). Reassuringly, a similar pattern was observed using MREs estimated from targetScan predictions (Supplementary Fig. 7C).

We next performed experimental analyses to demonstrate that FN1 indeed could regulate EMT as a ceRNA. We first knocked down FN1 using siRNA at 48 h into EMT and examined its effects 48 h later. The time point to introduce siRNA was carefully selected to isolate the ceRNA effects from transcriptional repression, which manifested its effects at approximately 120 h, and to target the time point with maximal ceRNA potency, which occurred at 96 h as shown by the dynamics of binding site occupancy (Fig. 4b). Consistent with the postulated regulatory role of FN1 as a ceRNA for miR-200c, the knockdown of FN1 significantly enhanced the expression of CDH1 and miR-200c and repressed the expression of ZEB1 and CDH2 (Fig. 4c, d). Importantly, rescue experiments with FN1 3′ UTR containing miR-200c binding sites, but not control 3′ UTR lacking the miR-200c sites, restored the expression of the tested genes; this result confirmed that the observed changes indeed reflected ceRNA effects (Fig. 4c, d). We next performed two additional sets of experiments analogous to TGFBI. In the first approach, we depleted DICER1 from MCF10A with DICER1-specific siRNA (Supplementary Fig. 8), and remeasured the impact of FN1 on the core EMT molecules. Consistent with the essential role of mature miRNAs in mediating ceRNA effects, FN1 knockdown had no detectable impact on ZEB1, CDH1, CDH2, and miR-200c in DICER1-less MCF10A cells (Fig. 4e, f). In a second approach, we mutated the seed region of miR-200c binding site in the 3′ UTR of FN1 with CRISPR/Cas9 technology (Supplementary Fig. 9), and reexamined the ceRNA activity of FN1 in the absence of endogenous functional miR-200c binding sites. Although pictar predicted two miR-200c binding sites in the 3′ UTR of FN1, only one was also predicted by targetScan as “conserved”. A careful examination of the secondary structures suggested that only the conserved site is functional because the poorly conserved site is located in a stem region of the modeled secondary structures (Supplementary Fig. 9A, B). We then knocked out the poorly conserved site with CRISPR/Cas9 technology and observed no detectable impact on the ceRNA activities of FN1 (supplementary Fig. 9C–E), confirming that the poorly conserved miR-200c binding site is not functional. We then mutated the conserved miR-200c binding site in FN1 3′ UTR with CRISPR/Cas9 and measured the impact on the ceRNA activities of FN1 (Fig. 4g, h, and Supplementary Fig. 9F, G). Reassuringly, FN1 knockdown in MCF10A cells without endogenous functional miR-200c binding site in FN1 3′ UTR did not produce significant impact on the key EMT molecules (Fig. 4g, h). In sharp contrast to DICER1-less MCF10A cells, where the addition of exogenous FN1 3′ UTR with functional miR-200c binding site displayed no detectable impact on key EMT molecules due to the lack of mature miRNAs (Fig. 4e, f), the introduction of exogenous FN1 3′ UTR into MCF10A cells without endogenous functional miR-200c binding site in FN1 3′ UTR did generate a small but significant upregulation of ZEB1 and CDH2, further demonstrating that the observed ceRNA activity of FN1 is highly specific and dependent on the presence of mature miRNAs and functional miR-200c binding site in FN1. Finally, FN1 knockdown only impacted the expression levels of genes with experimentally confirmed miR-200c binding sites, further confirming the specificity of the ceRNA effects associated with FN1 (supplementary Fig. 10A, B).

FN1 is a marker for mesenchymal cells, and its upregulation has been universally associated with EMT22. Thus, the key TFs regulating EMT, such as SNAIL and ZEB, were suggested to regulate FN1 expression. Consistent with this hypothesis, the knockdown of SNAI1 or ZEB1 in MCF10A cells significantly reduced FN1 expression (Supplementary Fig. 10C, D). Hence, the key TFs controlling EMT directly upregulate FN1, the ceRNA that dilutes the inhibitory effects of miR-200c prior to the transcriptional repression of miR-200c via ZEB1; this finding further demonstrates that ceRNA is tightly coupled with double-negative feedback loops and is an intrinsic component of the EMT-regulatory circuits.

Unlike A549 cells, in which the key EMT-regulatory miRNA (miR-21) is the most abundant miRNA, miR-200c is only the 15th highest expressing miRNA in MCF10A cells (Supplementary Table 5). Importantly, the higher ranked miRNAs in MCF10A cells are expressed at substantially higher levels than miR-200c. For example, the normalized read count for the highest expressing miR-378a-3p is about 28 times higher than that of miR-200c (5,886,078 vs. 211,178). Assuming a linear relationship between normalized read counts and absolute molecule numbers, we estimated that there are about 38,910 miR-378a-3p molecules in MCF10A cells, which is about 12 times higher than FN1 miRNAs at 96 h into EMT. This discrepancy suggested that if the higher expressing miRNAs also target ZEB1, then the ceRNA effect from FN1 is unlikely to be functional. To address this issue, we compared the miRNAs predicted by targetScan to regulate ZEB1 with the top 14 highest expressing miRNAs in MCF10A cells. Reassuringly, none of the 14 miRNAs targets ZEB1 (Supplementary Table 6). This analysis demonstrated that the FN1-miR-200c axis operates independent of the higher expressing miRNAs in MCF10A cells, and is functional despite the presence of excessive miRNAs.

Mathematical simulation of the EMT-regulatory circuit. To further demonstrate the role of ceRNA in regulating EMT, we investigated whether a mathematical model incorporating the ceRNA effect of FN1 could capture the dynamics of EMT, which are characterized by the slow dynamics (long half-life) of miR-200s and the fast dynamics of ZEB1, CDH1, and CDH228. We compared the simulation results of the canonical model and the new model that included FN1, the ceRNA for miR-200c (Fig. 5a). To demonstrate that the canonical model could not capture EMT dynamics using miR-200c-related parameters that were consistent with the long half-life of miRNAs, we performed simulations using the canonical EMT model but with modified parameters. Specifically, the parameters were modified such that the majority (90%) of miR-200c in the mRNA–miRNA complexes was recycled, which is consistent with experimental observations. Bifurcation analyses demonstrated that the cells did not reach the mesenchymal state because ZEB1 could not overcome the repression of miR-200c (Fig. 5b, c, Supplementary Fig. 11A, B). We subsequently modified the model to incorporate ODEs describing the kinetics of FN1-related reactions (Supplementary Model 1). Expectedly, simulation using the model incorporating FN1 successfully recapitulated the temporal dynamics of key EMT molecules (Supplementary Fig. 11C). Moreover, the bifurcation analysis recapitulated three stable states, suggesting that the incorporation of FN1 successfully captured the dynamic state transitions during EMT (Fig. 5d). Finally, we simulated FN1 knockdown by increasing the degradation rate constant of free
miR-200c degradation rate constant in the EMT model incorporating FN1 increased to 0.5 to 0.9, indicating an increased miR-200c half-life. Specifically, the parameters controlling the recycling rates of miR-200c in the miR-200c-ZEB1 complexes increased from 0.5 to 0.9.

The abundance of ceRNA determines the reversibility of EMT. Although ceRNA is an intrinsic component of EMT-regulatory circuits in the two cell lines examined, the dynamics of ceRNA displayed a striking difference. In benign MCF10A cells, FN1 was upregulated and consistently outnumbered miR-200c (Fig. 4a). However, TGFBI was first upregulated and subsequently downregulated in cancerous A549 cells, resulting in a similar number of TGFBI and miR-21 molecules in the mesenchymal state (Supplementary Fig. 4B). Because the stoichiometry between ZEB and miR-200c play critical roles in controlling the reversibility of EMT, we hypothesized that the stoichiometry between ceRNA and miRNA could play similar roles.

To examine this hypothesis, we first examined whether A549 cells induced by TGF-β to undergo EMT could also undergo a spontaneous MET transition upon the removal of TGF-β. As expected, A549 cells transited from an epithelial phenotype (Fig. 7a) to a mesenchymal phenotype (Fig. 7b) upon TGF-β treatment. However, the removal of TGF-β failed to trigger a spontaneous MET and MCF10A cells maintained the mesenchymal phenotype after TGF-β removal (Fig. 7d), and the effects of siFN1 could be effectively reversed by the addition of FN1 3′UTR (Fig. 7e). Finally, overexpressing miR-200c after TGF-β removal also promoted a transition to the epithelial phenotype, suggesting that both ceRNA and miRNA possess the ability to regulate MET (Fig. 7f). Similar to A549 cells, flow cytometry analyses confirmed that the downregulation of VIM and upregulation of CDH1 occurred simultaneously with morphology transitions in MCF10A cells (Fig. 7g, Supplementary Fig. 12B). Taken together, these data suggested that the stoichiometry between ceRNA and miRNA represents a critical parameter controlling the reversibility of the EMT, which could be a key barrier modulating the efficiency of metastasis.

Discussion

Here, we showed that a single mRNA dynamically induced during EMT could represent the vast majority of induced MREs and regulate EMT through a ceRNA effect. Because highly expressed mRNAs can readily exceed 1000 copies/cell and because some cancer cells express a substantially lower number of miRNAs than normal tissues, these results indicate that, at least
in cancer cells that miRNAs are downregulated, the dynamic expression of a single RNA could effectively function as a ceRNA.

Several lncRNAs that are aberrantly overexpressed in metastatic cancers have been reported to regulate EMT via a ceRNA mechanism. However, these ceRNAs represent a static overexpression that modifies the steady state equilibrium of the mesenchymal state. Recently, Title et al. reported that the miR-200-ZEB1 axis, which is crucial for tumorigenesis, demonstrates extreme signal sensitivity for regulating EMT. In line with their finding, we showed that the dynamically induced ceRNAs are directly coupled with the double-negative feedback loops of EMT, and operate in a dynamic and hypersensitive fashion. These results suggest that ceRNAs may represent candidate regulators in pathways utilizing TF-miRNA feedback loops.

Previous studies have suggested that the balance between ZEB1 and miR-200 controls the reversibility of EMT. However, those analyses were conducted by artificially manipulating the levels of ZEB1 or miR-200; moreover, the mutually inhibitory nature of ZEB1 and miR-200 and the model simulation suggest that the transition into mesenchymal state is largely irreversible. Here, we showed that the stoichiometry between ceRNA and miRNA represents a critical parameter that determines the reversibility of EMT in cancer cell models. Importantly, the cancerous A549 cells express similar numbers of ceRNAs and miRNAs, suggesting that ceRNAs may play a role in the regulation of EMT in these cells.
IRNA and qRT-PCR. Total RNA was isolated from cells using TRIzol reagent (TaKaRa). Reverse transcription was performed using the PrimeScriptTM RT Reagent Kit (TaKaRa) and a qDNA Eraser (Perfect Real Time) according to the manufacturers’ instructions. qRT-PCR was performed using the SYBR Premix Ex Taq (Tli RNase H Plus) system on an ABI Step One Plus machine (Applied Biosystems). The experiments were performed in triplicate, and the values of miRNAs were normalized to that of GAPDH and the values of mRNAs were normalized to that of U6 snRNA. The primer sequences were as follows: U6 forward, CGTCGCTTGCCAGCACA; U6 reverse, AAGGTCCTCAGATTGC; GT; U6-200c forward, GAAGTCCTCAGATTGC; GT; U6-200c reverse, CAGTGCTCATATCAGTCTAG; GAPDH forward, TGGGTACCAAGGTCG; GAPDH reverse, GCTGTCGCTCTCGTGCG; TGFBI forward, TGAGGGTCTGAGGCTGG; TGFBI reverse, CTTCGCCCTCATTCAAGCGT; SNAIL1 forward, TCGGAAGCCTAACTACAGCGA; SNAIL1 reverse, ACATTTGACAGAACATTTCAACTCA; FN1 forward, GGAGTTTCCTGAGGGTTT; FN1 reverse, GGAGCGAGATCCATTGAAT; GAPDH forward, GGAGCGAGATCCATTGAAT; GAPDH reverse, GCTGTCGCTCTCGTGCG.

Transfection of siRNA and plasmids containing 3′UTR of FNL or TGFβ1 were performed using DharmaFECT Duo (Dharmacon) according to the manufacturer’s instructions. Data were analyzed with t tests after confirming that the control and treatment groups exhibit similar variance. The following are the siRNA sequences: TGFβ1-Homo, sense, UUGUGGCUAUUGGCUAAGU; TGFβ1-Anti, sense, UUGUGAUCGUGUCGUCAAGTTT; FOXP1-Homo-1008, sense, GGAUCAUUGGAGAUAU; FOXP1-Anti, sense, GACAGUGAAUUGGUAAGU; SNAIL1-Homo, sense, CAGAGUCAUAAAGGACAGCAGT; SNAIL1-Anti, sense, UUGUCAUGUGAGUGGUACU; Dicer1-Homo-14215s, sense, AGUGUGUCCUGAGCUGAG; Dicer1-Anti, sense, AUAAAUACUUCGACAGGACTT; ZEB1-Homo, sense, GCAGCUGCUGGUUCUCGUUTT, antisense, AACGAGAACCA; DICER1-Homo-1008, sense, GCCCAAUAUUGCAAGAAAT; DICER1-Anti, sense, UUGUCAUGUGUAAUGGCUCTT; DICER1-Homo-4380, sense, GAAGUGGUGCAGCAAGG; DICER1-Anti, sense, GUGUCAUGUGUGGUAAUGGCU. For rescue experiments, the 3′UTR for TGFβ1 (hg19, chr5:135,399,223-135,399,462) and FN1 (hg19, chr2:216,225,719-216,226,087) were synthesized by Sangon Biotech (Shanghai) Co., Ltd. and cloned in pcDNA3.1 plasmid.
Luciferase reporter assay. The cells were seeded onto six-well plates at a density of 1 × 10^5 cells/well and the luciferase signal was detected after 24 h using a luciferase assay system (Promega, E2510) according to the manufacturer’s instructions.

Flow cytometry. The cells were fixed with 4% paraformaldehyde (15 min at room temperature). For VIM staining, the cells were permeabilized by slowly adding ice-cold 100% methanol to pre-chilled cells under gentle vortexing to a final concentration of 40%. To block nonspecific binding, the cells were suspended in incubation buffer (10% goat serum in PBS, Gibco) and incubated on ice for 30 min. Staining was performed using CD1H (Life Technologies, A15757, 1:100) or VIM (Abcam, ab203428, 1:100) antibodies conjugated to fluorescent dye at room temperature for 1 h. The cells were analyzed using a Beckman Coulter CytoFLEX S system, and the data analysis was performed using CytExpert 1.2.11.0 software.

Estimating number of MREs using gene-expression data. We estimated the relative number of MREs by multiplying the transcripts per million (TPM) of each transcriptional isoform containing corresponding miRNA binding site with the number of conserved miRNA binding sites in their 3′UTR as predicted by targetScan or pictar23,34. Specifically, for miR-21 we used the time course gene-expression data of TGFβ1-induced EMT in A549 cells (GSE69667)23. For miR-200c, we used the time course gene expression data of MCF10A cells over-expressing SNAIL (GSE25929)24. Because the MCF10A time course data is measured by microarray, we first estimated the gene expression TPM in unstimulated MCF10A cells (GSE48213)25, and extrapolated the gene expression TPM during EMT by multiplying the base TPM with gene expression fold changes estimated from microarray time course data in SNAI1 overexpressing MCF10A cells. Finally, assuming absolute qPCR provides a more accurate measurement of the number of molecules in cells, we scaled number of MREs derived with RNAseq TPM by the ratio between qPCR measured number of TGFβ1 or FN1 with their corresponding TPM, and used the scaled MREs for downstream analysis.

MicroRNA sequencing and data analysis. The sequencing libraries were constructed according to the protocol for the Illumina small RNA Sample preparation kit. Sequencing was performed on the Illumina HiSeq 2000 sequencer. Library construction and sequencing were performed at the Genergy Biotech Co., Ltd. (Shanghai). miRNA expression was analyzed by miDeep2.0.0.7-26 and differentially expressed microRNAs were identified using an FDR cutoff value of 0.05 and absolute log2-fold change greater than 1.

Mathematical modeling of miRNA binding site occupancy. We utilized the model developed by Jens and Rajewsky27 to simulate the changes of miRNA binding site occupancy during EMT. We first estimated the number of molecules using the conserved miRNA binding sites predicted by targetScan 7.2 and the miRNA binding sites predicted by pictar (https://pictar.mdc-berlin.de/). The number of miRNA molecules were determined by absolute qPCR. The software developed by Jens et al. was downloaded from http://dorina.mdc-berlin.de/public/rajewsky/miRNA_compositions/ and the “simplified model” was used to estimate the miRNA binding site occupancies.

Mathematical modeling of EMT-regulatory circuits. We utilized the Cascading Bistable Switches (CBS) model proposed by Zhang et al.28 for the mathematical simulation. We generated the following modifications to incorporate FNI into the CBS model. First, we assumed that the majority (90%) of miR-200c could be recycled from miR-200c-ZEB1 complexes, resulting in a miR-200c half-life that was consistent with experimental observations. Specifically, we changed the recycling ratio 1 (1–5) in the ZEB1/miR-200 module from 0.5 to 0.9. Second, we added ODEs describing the kinetics of FNI mRNA and estimated the related parameters using experimental gene expression data. Specifically, we modeled one conserved binding site of miR-200c in FN1 based on targetScan prediction and employed Hill functions to model the transcriptional regulation of FN1 by SNAI1 and ZEB1. One-parameter bifurcation analyses and 300-h time course analyses were performed using Oscil8. The parameters that differed from those of the CBS model of Zhang et al. are provided in Supplementary Table 7.

Data availability
The miRNA-seq data reported in this study have been deposited in GEO under accession number GSE87338. All other data are available from the authors upon reasonable request.

Code availability
The ODEs describing the EMT model with FN1 are provided in Supplemental Note.

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