miR-206 Inhibits Stemness and Metastasis of Breast Cancer by Targeting MKL1/IL11 Pathway

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Abstract

Purpose: Effective targeting of cancer stem cells is necessary and important for eradicating cancer and reducing metastasis-related mortality. Understanding of cancer stemness-related signaling pathways at the molecular level will help control cancer and stop metastasis in the clinic.

Experimental Design: By analyzing miRNA profiles and functions in cancer development, we aimed to identify regulators of breast tumor stemness and metastasis in human xenograft models in vivo and examined their effects on self-renewal and invasion of breast cancer cells in vitro. To discover the direct targets and essential signaling pathways responsible for miRNA functions in breast cancer progression, we performed microarray analysis and target gene prediction in combination with functional studies on candidate genes (overexpression rescues and pheno-copying knockdowns).

Introduction

Cancer stem cells (CSCs) are a subset of cancer cells that are capable of self-renewing and mediating tumor generation and metastasis (1–3). It is necessary and essential to discover and target the stemness regulators of breast cancer, the leading cancer in women, to reduce cancer-related mortality. Although both genetic and epigenetic alterations regulate CSC functions, we focused on identifying miRNA regulators of breast cancer tumorigenesis and metastasis. miRNAs are short, noncoding RNAs that have emerged as important posttranscriptional regulators of genes in various cellular processes such as cell growth, motility, invasion, and stress response (4, 5). The major mechanisms by which miRNAs regulate target genes include induction of mRNA degradation and repression of mRNA translation by binding to partial complementary sequences in the 3′ untranslated regions (3′UTR; refs. 6, 7). Discovery of miRNA-regulated molecular pathways in controlling breast cancer would lead to better understanding of cancer biology as well as future development of novel targeting strategies for the treatment of breast cancer.

Using a forward functional screen on a list of miRNAs differentially expressed in less metastatic tumors, we discovered miR-206 as a suppressor of breast tumor initiation and metastasis. Although miR-206 has been reported in promoting myogenic differentiation (8), its role and signaling in breast cancer stemness were unclear. We sought to identify the role and signaling pathway of miR-206 in stemness-related self-renewal and metastasis-related invasion. Self-renewal is a process of cell division in which at least one daughter cell is identical to the parent cell thereby maintaining the pool of stem cells (2). The gold standard assay of CSCs is the tumor regenerating assay in vitro, which is complemented by colony formation or sphere assays in vitro. Mesenchymal features have been implicated in cellular invasion, characterized by cancer cells losing cell-to-cell contacts and migrating away from the cohesive tumor tissue along with altered expression levels of adhesion molecules and cellular skeletons (9, 10).
translational relevance
Metastasis accounts for 90% of breast cancer mortality and demands better strategies for its understanding and treatment. Cancer stem cells have been reported to mediate metastasis and resist conventional therapies, thus being considered the root of cancer and the seeds of metastasis. To stop cancer regeneration and metastasis requires effective targeting of cancer stem cells using innovative approaches against both stemness and invasion. We discovered that miR-206 inhibits both self-renewal and invasion through suppressing expression and/or activities of the cytoskeleton gene TWFI, mesenchymal transcription factors MKL1/SRF, and subsequent cytokine IL11 in breast cancer cells. The newly identified miR-206/MKL1/IL11 signaling pathway in regulation of breast cancer stemness provides new therapeutic targets and facilitates miRNA/siRNA-based drug development, thereby potentially impacting cancer treatment and preventing/blocking metastasis.

Furthermore, epithelial-to-mesenchymal transition (EMT) enables epithelial cells to adopt a mesenchymal-like morphology along with the formation of actin-rich protrusive and invasive structures, such as invadopodia (11). The question remains whether actin-related cytoskeleton proteins and mesenchymal transcription factors are involved in the miR-206 signaling pathway and EMT regulation.

To fully elucidate the downstream signaling pathway of miR-206, we utilized a global profiling approach to identify the genes inhibited by miR-206. In combination with target gene prediction software and functional rescuing studies, twinfilin (TWFI or PTK9) was the top candidate target of the list downregulated by miR-206. The connection of TWFI function with cell motility and chemotherapy sensitivity was revealed in a systemic RNA interference screening study (12). However, it was unknown whether TWFI is an essential target of miR-206 and what downstream signaling of TWFI would regulate stemness. TWFI is a conserved actin-binding protein with two actin depolymerizing factor homology (ADF/H) domains and belongs to a family of proteins containing the ADF/H domain (13), including coflinl, N-WASP, Arp2/3, and cortactin (14–18). Our previous work demonstrated that TWFI promotes F-actin (stress fiber) formation as a mesenchymal marker (19, 20). We hypothesized that TWFI may relay the messages from the actin structures to downstream transcription factors that regulate mesenchymal phenotypes.

Notably, recent breast cancer genomics studies identified susceptibility-related SNPs located within megakaryoblastic leukemia (translocation) 1 (MKL1) introns (21), implicating a potential relevance of the well-known muscle cell regulator MKL1 in breast cancer development. The transcriptional activity of MKL1, also known as myocardin-related transcription factor (MRTF-A), is regulated by the G-actin monomer concentration and dependent upon binding to and activating serum response factor (SRF; refs. 22, 23). However, the molecular mechanisms by which MKL1 regulates breast cancer progression are unclear. Our work examined whether miR-206 and/or TWFI signaling pathways were involved in the activation of the mesenchymal lineage transcription factors MKL1/SRF and whether MKL1 and SRF regulate the EMT and invasive phenotype of breast cancer cells. By profiling the self-renewal related cytokines regulated by miR-206, we discovered IL11 as an important effector of miR-206 and further linked it to the MKL1/SRF pathway.

Materials and Methods
Animal studies (PDX models and cell line xenografts)
Patient-derived human-in-mouse breast tumor xenograft (PDX) models with spontaneous metastases were generated using patient tumors as described (3). Experiments were performed under the approval of the Institutional Biosafety Committee, Institutional Review Board, and the Administrative Panel on Laboratory Animal Care of Case Western Reserve University and the University of Chicago.

Cell culture and transfections
MDA-MB-231, MCF-7, HS578t, and Hek293T were obtained from ATCC or NCI cancer cell line database. Authentication was done by microarray analyses (MDA-MB-231) and phenotypic analyses (MCF-7, HS578t, and Hek293T). Cells were maintained in DMEM with 10% FBS and 1% penicillin–streptomycin. Cells were transfected with miRNAs (Dharmacon; negative control #4) or pooled siRNAs (Dharmacon) at 100 nmol/L using Dharmafect (Dharmacon) according to manufacturer’s instructions. Six-hour transfections were repeated twice over consecutive days. TWFI (as described in ref. 19) IL11, SRF cDNA vectors (Origene) were transfected using Fugene HD (Promega) according to manufacturer’s instructions and repeated twice over consecutive days.

Vectors and cloning
A lentiviral gateway vector pFU-attr-PGK-L2G (19) was used to construct miR-206 expression vector with a precursor entry clone (provided by Dr. Jun Lu at Yale University, New Haven, CT) using LR clonase II (Invitrogen). Human TWFI cDNA was subcloned into pDEST40 (Invitrogen; ref. 19). Human G6PD, SRF, and IL11 expression vectors were purchased from Origene. Luciferase vectors containing the 3′UTR of TWFI, G6PD, and Notch were obtained from Switchgear Genomics.

RNA extraction and RT-PCR
Cell pellets from six-well plates were dissolved in TRlzol and total RNA was extracted using isopropyl alcohol (Invitrogen). Approximately 1 μg RNA per sample was reverse transcribed using qScript SuperMix (Quanta Biosciences). RT-PCR was performed using TaqMan primers (Invitrogen) and the relative expressions of the target genes were quantified using the comparative CT method. Mean gene expression levels were normalized to the mean expression levels of the GAPDH reference gene.

Gene expression microarray analyses
Samples were profiled as described previously using oligo microarrays (ref. 19; Agilent Technologies). All microarray data are available in the University of North Carolina (UNC) Microarray Database and have been deposited in the Gene Expression Omnibus (GEO) under the accession number GEO:GSE59751. The log2-transformed expression levels are presented in the Supplementary Table with paired t test analyses between miR-206 and other controls in three experiments.
Western blot analysis

Cell were harvested and lysed in RIPA buffer (Amresco N653). A total of 20 to 50 μg of protein samples were loaded and run on mini-PROTEAN 4% to 20% gradient TGX Bio-Rad gels. Transferred membranes were blocked in 5% BSA, and probed for antibodies to TWF1 (Genetex GTX11439), MKL1 (Atlas Antibodies), or FAK (Cell Signaling Technology, 3285) overnight at 4°C. Appropriate fluorophore-conjugated secondary antibodies were incubated for 1 hour at room temperature for TWF1 and MKL1, and the signals were visualized using LI-COR scanner (LI-COR). Horseradish peroxidase (HRP)-conjugated secondary antibody was used for FAK blotting and incubated for 1 hour at room temperature and signals were visualized on exposed films.

Cell count analysis

A total of 50,000 transfected cells were plated in 24-well plates and harvested every 24 hours more than 3 days. Cells were stained with Trypan blue and then counted on hemocytometer. At each time point three wells of cells were counted.

Quantitative invasion assays

Cells were transfected as described earlier except for miR-206 rescue assays where target gene cDNAs were transfected first followed by miR-206 transfection. 100K of cells in triplets were seeded in serum-free DMEM onto matrigel (BD Biosciences) precoated transwell inserts (BD Biosciences) with DMEM + 10% PBS in the bottom wells and incubated for 24 hours at 37°C. Cells were then stained with Calcein AM (BD Biosciences). Cells on the top of inserts were swabbed. Cells on the bottom side of inserts were dissociated in a cell dissociation buffer (Trevisgen) shaking for 1 hour at 37°C and the fluorescent signal was measured using a plate reader (Perkin-Elmer).

Cell-cycle analysis

Cells were transfected twice as stated before and harvested using trypsin. They were washed and resuspended at a concentration of 1 × 10^6 cells/mL in PBS, then resuspended in 0.3 mL of PBS. To fix the cells, 70% cold ethanol was added dropwise while vortexing gently followed by incubation in ice for at least an hour. Cells were washed twice and resuspended in 0.25 mL of PBS. Five microliters of RNase A was added (stock concentration 20 mg/mL) and incubated by an hour at 37°C. 10% FBS in the bottom wells and incubated for 24 hours at 37°C. Cells were then stained with Annexin V-PE (BD Biosciences) and propidium iodide solution (Sigma) was added. Cells were transfected twice as stated before and harvested using trypsin. They were washed and resuspended at a concentration of 1 × 10^6 cells/mL in PBS, then resuspended in 0.3 mL of PBS. To fix the cells, 70% cold ethanol was added dropwise while vortexing gently followed by incubation in ice for at least an hour. Cells were washed twice and resuspended in 0.25 mL of PBS. Five microliters of RNase A was added (stock concentration 20 mg/mL) and incubated by an hour at 37°C. Appropriate fluorophore-conjugated secondary antibodies were incubated for 1 hour at room temperature for TWF1 and MKL1, and the signals were visualized using LI-COR scanner (LI-COR). Horseradish peroxidase (HRP)-conjugated secondary antibody was used for FAK blotting and incubated for 1 hour at room temperature and signals were visualized on exposed films.

Soft agar colony formation assay

For this experiment, agarose was used instead of agar. A six-well plate was coated at a 1:1 ratio of 1% agarose and 2× media, solidify for 30 minutes. The top portion was prepared with 0.6% agarose and 2× media with cells plated at a 4,000 cells/mL density. Pictures were taken after 10 days using DeltaVision microscopy.

Annexin V staining

MCF-7 cells were transfected once for 48 hours and collected for analyses. During collection, cells were washed with PBS and resuspended in Annexin V binding buffer (BD Biosciences) and 5 μL of Annexin V-PE (BD Biosciences) as per the manufacturer’s instructions. Cells were also analyzed using the BD LSR II flow cytometer.

Mammosphere assay

A 24-well plate was coated with poly hema overnight to avoid adherence. Cells were plated at a density of 500 cells per well in serum-free PRIME-XV mammary tumor sphere medium (Irvine Scientific) with 2 U/mL of heparin (Sigma Aldrich) and 0.5 μg/mL hydrocortisone ( Stem Cell Technologies). Pictures of mammospheres were taken at 6 days after seeding.

IHCl staining

After deparaffinization and rehydration, the tissue sections were incubated in antigen retrieval buffer and heated in steamer at more than 97°C for 20 minutes. Anti-TWF1 (PTK9; Genex, GT111439, rabbit IgG) was applied on tissue sections overnight at 4°C at 1:800 dilution in a humidity chamber. Following TBS wash, the antibody binding was detected with Envision+ system and DAB+ chromogen (DAKO, K4010). Tissue sections were briefly immersed in hematoxylin for counterstaining, washed with water and covered with coverslips.

Statistical analysis

For all assays and analyses in vitro, if not specified, Student t test was used to evaluate the significant difference or P values and SD of mean values were depicted as error bars in figures. For animal studies in vivo, Shapiro–Wilk test was applied to check the normality of the raw data, and MANOVA, ANOVA, or Student t test was applied in exploring the P value and the significant differences between vector and 206 groups. Linear mixed model was fitted to generate the tumor growth graphs with tumor volumes (bioluminescent signals) as response variables, time, and each mouse ID as the random effects. The data applied on linear mixed models was derived by taking log transformation of the original tumor volumes. Dot plot was used to represent a comparison of the data distribution in metastatic burden. All the tests and models are performed in R software.

Methods on F-actin quantification, immunofluorescence staining and microscopy, gelatin invadopodia assay, sub-G1 population analysis, luciferase assay, and bioluminescence imaging are provided in Supplementary Information.

Results

miR-206 inhibits breast tumorigenesis and metastasis

To screen miRNA regulators of breast cancer progression, we previously established patient-derived human-in-mouse breast tumor xenograft (PDX) models in immunodeficient NOD/SCID mice and labeled with Luc2-eGFP (L2G) or Luc2-tdTomato (L2T; ref. 3). Triple-negative breast tumor PDXs develop spontaneous lung metastases with altered miRNA expression profiles and differentiation scores (3, 24). Higher miR-206 levels were detected in the relatively nonmetastatic tumor cells compared with the metastatic primary tumor cells sorted from L2G and L2T-labeled PDXs (Fig. 1A). To investigate the functional significance of miR-206 in breast cancer progression, we modulated miR-206 precursor expression in breast cancer cell lines and PDXs. We first cloned the miR-206 precursor into a gateway lentiviral vector coexpressing the fusion reporter gene L2G, and then generated MDA-MB-231 stable clones overexpressing miR-206 or vector control (backbone). These clones were used to examine the role...
Figure 1.
miR-206 suppresses breast cancer initiation and metastasis in vivo. A, miR-206 expression levels (fold change) in sorted primary breast tumor cells, metastatic (mainly CD44^+), and non-metastatic (mainly CD44^-) from triple negative patient-derived xenografts (PDX), measured by Taqman RT-PCR. B, log_{10} plots of bioluminescent signals (total flux, p/s) of mammary tumors versus day, after mammary fat pad injections of L2G vector (blue) or miR-206 overexpressing (red) MDA-MB-231 cells within a time course of 72 days. P values are calculated between linear curves using R software MANOVA. C, Representative images of mammary tumors on day 1 and day 72, post fat pad injections of L2G vector (left: clone #1 and #17) or miR-206 overexpressing (right: clone #4 and #9) MDA-MB-231 cells. D, Representative images of dissected lungs from the mice bearing vector controls (left: clone #1 and #17) and miR-206 expressing tumor cells (right: clone #4 and #9) on day 72. E, Antagonist of miR-206 (anti-206) increased lung metastasis burden of breast tumor PDX, whereas the primary tumor growth was not significantly changed.
of miR-206 in both spontaneous metastasis models as well as blood stream–inoculated metastasis models. Compared with the vector controls (clones #1 and #17), orthotopic injection of the miR-206 stable-expression clones (#4 and #9) into the mammary fat pad of NOD-SCID mice resulted in a failure of tumor initiation and progression, as measured by bioluminescence imaging (BLI) of the growth curves over a time course of 72 days (Fig. 1B–C). Compared with the empty vector controls, the lungs dissected from the mice bearing miR-206 clones also showed negative bioluminescent tumor signals (Fig. 1D). We then inhibited miR-206 function in primary tumor cells isolated from PDXs by transducing its antagonist anti-miR-206. Although breast tumor growth did not show significant changes, lung metastasis burden (ratio) based on primary tumor signals significantly increased (Fig. 1E), suggesting an inhibitory role of miR-206 in breast cancer progression.

To further evaluate the regulatory effects of miR-206 in lung metastasis, we conducted colonization assays with tail vein injections of the miR-206 stable clones (#4 and #9 clones) or control cells (#1 and #17 clones) into NOD-SCID mice. The miR-206 cells regressed whereas the control cells colonized the lungs with increased bioluminescent tumor metastases (Fig. 2A and B). Two-photon imaging of the dissected lungs on day 72 post tail vein injection showed remarkable GFP+ metastases of control cells but an absence of miR-206 overexpression cells in the lungs (Fig. 2C). These studies demonstrate the importance of miR-206 in breast cancer initiation and metastasis in vivo.

miR-206 suppresses self-renewal, invasion, and EMT

We continued to examine the effects of transiently transfected miR-206 mimics on cell cycle, self-renewal, invasion, migration, growth, and EMT of triple negative breast cancer cells (MDA-MB-231, BT-20, and HS578T) as well as estrogen receptor-positive MCF-7 cells.

We first investigated the role of miR-206 in self-renewal related cell-cycle regulation. Shown by DNA content analyses with PI staining, miR-206-transfected MDA-MB-231 cells increased G1-phase population along with decreased S and G2-M phases within 48 hours, and further strengthened the cell-cycle arrest at 72 hours posttransfection (Fig. 3A and B). Furthermore, upregulation of miR-206 in MCF-7 cells caused not only G1 arrest but also additional cell death within 48 hours posttransfection, measured by Annexin-V staining (Supplementary Fig. S1A–S1C). By seeding individual tumor cells into solid culture of soft agar or suspension culture with stem cell medium, we demonstrated that miR-206 overexpression abolished the colony formation of MDA-MB-231 cells (Fig. 3C) and dramatically reduced mammosphere formation efficiency (counts) of MCF-7 cells (Fig. 3D). The phenotypic regulation of miR-206 in cell cycle, colony formation, and mammosphere generation in vitro is consistent with its role in inhibiting breast tumor initiation in vivo.

We then examined the effects of miR-206 in metastasis-related cell motility such as cellular invasion and migration using the matrigel-coated transwells (20). Enforced expression of miR-206 significantly reduced invasion of MDA-MB-231 cells, measured by high throughput plate-reader signals of calcein-AM-stained cells that invaded through matrigel, the membrane, and moved to the bottom side of the transwell within 24 hours post plating (Fig. 3E). Similarly, miR-206 inhibited the cellular invasion of both BT-20 and HS578T breast cancer cells (Supplementary Fig. S2A-B). Elevated levels of miR-206 also suppressed the migration of MDA-MB-231 cells in a 6-hour vertical transwell assay (Fig. 3F).

To determine whether the decreased invasion and migration were caused by altered cell growth and proliferation within 24 hours, we performed multiple cell growth analyses, both calcein-AM-based metabolic cell viability staining and cell counts–based growth curve analyses. We first compared the overall cell growth (metabolic) and proliferation (cell count) signals measured by calcein-AM staining, which is used in the invasive cell signal quantitation in parallel to trypan blue–based cell counting. With calcein-AM staining, we did not observe a significant effect of miR-206 on the overall cell growth in both MDA-MB-231 and HS578T cells within 24 hours (time point equal to the 24-hour invasion assay or longer than the 6-hour migration assay; Supplementary Fig. S2C–S2D), indicating that miR-206-mediated inhibition of invasion and migration was due to intrinsic cell motility changes. The cell count–based growth curves revealed that miR-206 started to alter MDA-MB-231 cell proliferation at 72 to 96 hours (Supplementary Fig. S2E), which is consistent with the cell-cycle analyses (Fig. 3B), whereas in HS578T cells miR-206 slowed down the proliferation rate at early time points without reducing the calcein AM staining signals (Supplementary Fig. S2D and S2F). Cell-cycle analysis using propidium iodide staining also revealed that at the 24-hour time point, miR-206 caused minimal alterations of MDA-MB-231 cell cycle (G1 and S phase) without detectable sub-G1 proapoptotic cell death (Supplementary Fig. S2G).

To determine whether miR-206 regulates invasion with an effect on EMT, we chose to evaluate two mesenchymal markers, F-actin and vinculin (19, 24). Notably, elevated miR-206 expression significantly reduced the formation of F-actin stress fibers (green) and vinculin-stained focal adhesions (red dots at the end of green fibers; Fig. 3G). We also observed decreased focal adhesion kinase (FAK) levels in miR-206–transfected breast tumor cells (Fig. 3H). These results further suggest that miR-206 suppresses EMT by inhibiting actin polymerization and focal adhesion formation.

TFW1 is an important target of miR-206

To characterize pathways regulated by miR-206, we analyzed the global transcriptome using microarrays of MDA-MB-231 cells transfected with miR-206, scramble, or mock controls. TWF1 was shown on the top of the most downregulated genes by miR-206 (Supplementary Table S1). To focus on the genes directly targeted by miR-206, we used the GeneSet2miR prediction analysis (25), which resulted in 43 potential direct target genes predicted by a minimum of 4 out of 11 established algorithms, as listed in the heat map, including TWF1, NOTCH2, G6PD, and others (Fig. 4A, P value <0.0001). TWF1 regulates both cell morphology and motility through sequestering ADP-actin monomers and capping filament barbed ends (26, 27), NOTCH2 regulates stem cell population, differentiation, and apoptotic programming (28–30), and Glucose-6-phosphate dehydrogenase (G6PD) relates to glucose metabolism and protection against reactive oxygen species (31). Most importantly, all three of these genes have been shown to regulate cancer progression (19, 29, 32).

To determine whether these genes are real targets of miR-206, we first measured their expression levels upon miR-206 modulation in MDA-MB-231 cells using RT-PCR. Forced expression of miR-206 caused a significant decrease in mRNA levels for NOTCH2, G6PD, and TWF1 compared to the scrambled control (Scr; Fig. 4B). We then set out to determine whether these genes...
are direct targets of miR-206. By cotransfecting HEK293T cells with miR-206 and the luciferase vector with the 3'UTR of candidate genes cloned downstream of the luciferase gene, we found that miR-206 mediated an inhibitory interaction with the 3'UTR regions of all three candidate genes, thereby reducing the expression levels and activity of the upstream luciferase reporter (Fig. 4C). Western blot analysis further showed a decrease in protein levels of TWF1 in MDA-MB-231 cells transfected with miR-206 (Fig. 4D).

To determine the importance of its target genes in miR-206-mediated functions such as invasion, we performed rescue assays where candidate genes were overexpressed in cells with elevated miR-206 levels. If a gene is an essential downstream player in the miR-206 signaling, overexpression of the gene would reverse miR-206-mediated phenotype. Among three tested candidates, we only observed that TWF1 overexpression was able to prevent or reverse the decreased invasiveness in MDA-MB-231 cells caused by miR-206 (Fig. 4E). Other direct targets, such as G6PD, failed to
Figure 3.

miR-206 induces G1 arrest and inhibits stemness. **Student t-test, P values <0.001.**

A, miR-206 increased G1-phase and decreased S and G2-M populations of MDA-MB-231 cells at 48 hours posttransfection.

B, miR-206 increased G1-phase and decreased S populations of MDA-MB-231 cells at 72 hours posttransfection.

C, miR-206 abolished MDA-MB-231 single cell-mediated colony formation in soft agar.

D, miR-206 decreased MCF-7 mammosphere formation in serum-free stem cell medium.

E, Transwell invasion of MDA-MB-231 cells transfected with either miR-206 or scramble control.

F, Vertical transwell migration assays of MDA-MB-231 cells transfected with either miR-206 or scramble control.

G, F, Left: representative images (×1,000) of MDA-MB-231 cells transfected with either miR-206 or scramble control; F-actin staining (Phalloidin-green), focal adhesions (Vinculin-red), and DNA (DAPI-blue). Right histogram: quantification of focal adhesions. **Student t-test, P values <0.001.**

H, Decreased FAK protein levels in miR-206-transfected cell lysates compared with that of the scramble control, measured by immunoblotting.

miR-206 Inhibits Stemness and Metastasis

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TWF1 is an important target of miR-206. A, Heatmap of 43 candidate targets downregulated in miR-206-transfected MDA-MB-231 cells (n = 3) compared to scramble (n = 3) and mock-transfected controls (n = 3). B, RT-PCR of G6PD, NOTCH, TWF1 gene expression in MDA-MB-231 cells transfected with either miR-206 or scramble control. **P < 0.01. C, Western blot analysis of MDA-MB-231 cells transfected with either miR-206 or scramble control. β-Actin (red) was used as a loading control. D, Transwell invasion assays of MDA-MB-231 cells transfected with either scramble (Scr) or miR-206 (206) followed by either TWF1 siRNA, or scramble control. **P < 0.01. E, Representative images of Gelatin Invadopodia Assay 24 hours after MDA-MB-231 cell culture. The black dots in scramble is due to degradation of matrix by localized protrusions of invadopodia and are absent in miR-206 and TWF1 KD. F, IHC staining indicates decreased TWF1 (green) in the residual miR-206–transduced breast tumor cells (arrow pointed) compared with the vector control tumor in the lung sections of recipient mice, dissected on day 72 post tail vein injections (as shown in Fig. 2B and C).
sparsely distributed single tumor cells had remarkably lower levels of TWF1 (Fig. 4H).

Knockdown of TWF1 also mimicked miR-206 upregulation in decreasing cell motility (migration) of MCF-7 cells (Supplementary Fig. S2H). However, the downstream signaling of TWF1 was not fully understood, and we decided to seek out mesenchymal regulators related to miR-206/TWF1 functions.

miR-206 and TWF1 regulate MKL1/SRF activities

It has been previously suggested that the mesenchymal lineage transcription factor MKL1 is regulated by G-actin monomers in the cytoplasm, which promotes its nuclear export and inhibits its nuclear import (22, 23). This movement of MKL1 outside of the nucleus prevents the activation of the MKL1–SRF complex in the nucleus that targets many genes encoding cytoskeletal components, including actin (22, 23, 35). We hypothesized that miR-206 and TWF1 regulate the mesenchymal phenotype through actin dynamics and MKL1/SRF signaling.

We first investigated the impact of miR-206 on MKL1 mRNA stability and expression levels. Both overexpression of miR-206 and knockdown of TWF1 decreased the mRNA levels of MKL1 (Fig. 5A and Supplementary Fig. S3A). siRNA-mediated knockdown of either MKL1 or SRF decreased the invasiveness of both MDA-MB-231 and HS578T cells, phenocopying the inhibitory effect of miR-206 overexpression (Fig. 5B and C and Supplementary Fig. S2B). Furthermore, overexpression of SRF was also sufficient to rescue or restore the invasiveness inhibited by miR-206 in MDA-MB-231 cells (Fig. 5D).

We then examined the effects of modulated miR-206 and TWF1 levels on actin dynamics, such as the F/G actin ratio in MDA-MB-231 cells. The ratio of F actin to G actin was reduced by miR-206 overexpression (Fig. 5B and C and Supplementary Fig. S3B). To determine whether miR-206-induced alteration of F/G actin ratio and MKL1/SRF activity regulates invasion, we performed functional rescue assays by using cytochalasin D (CD) to promote MKL1 translocation and SRF activity. As reported, MKL1 is a cytoplasmic protein trapped by G-actin, and its release from G-actin results in its translocation from the cytoplasm to the nucleus where it binds to SRF as a coactivator (36). CD has a high affinity to G-actin, so it releases MKL1 from its G-actin trap and thereby

Figure 5.

miR-206 decreases invasion through regulating MKL1/SRF and IL11. A, RT-PCR of MKL1 expression in MDA-MB-231 cells transfected with either scramble, miR-206, or TWF1 siRNA. *, P < 0.05; **, P < 0.01. B, Transwell invasion assays of MDA-MB-231 cells transfected with scramble or MKL1 siRNA. ***, P < 0.001. C, Transwell invasion assays of MDA-MB-231 cells transfected with scramble or SRF siRNA. *, P < 0.05. D, Transwell invasion assays of MDA-MB-231 cells transfected with miR-206 or scramble control followed by a second transfection with either SRF cDNA or pcDNA control vectors. +, P < 0.05; ***, P < 0.01. E, Transwell invasion assays of MDA-MB-231 cells transfected with either scramble or miR-206, followed by treatment with either DMSO control or CD. +, P < 0.05. F, RT-PCR of IL11 expression in MDA-MB-231 cells transfected with miR-206 or scramble control. ***, P < 0.001. G, IL11 cytokine assay in MDA-MB-231 cells transfected with miR-206, TWF1 siRNA, or scramble control. +, P < 0.05; **, P < 0.01. H, qPCR of IL11 expression in MDA-MB-231 cells transfected with either TWF1, MKL1, or SRF siRNA or scramble control. ****, P < 0.00001. I, qPCR of IL11 expression in MDA-MB-231 cells treated with CD from 30 minutes to 6 hours. +, P < 0.05; **, P < 0.0001. J, qPCR of IL11 expression levels of MDA-MB-231 cells transfected with either miR-206 or scramble control followed by either TWF1 cDNA or SRF cDNA or pcDNA control. ****, P < 0.00001.
triggers MKL1 translocation and SRF activation (36). Relative to the DMSO vehicle control, CD itself promoted cellular invasion and reversed the inhibitory effects of miR-206 on invasion of MDA-MB-231 cells (Fig. 5E). That is consistent with the functional rescuing effects of SRF in reversing the miR-206 phenotype in invasiveness of breast cancer cells (Fig. 5D).

miR-206 and TWF1 regulate IL11 expression

IL6 family members, such as leukemia inhibitory factor (LIF) and IL11 are known to regulate STAT3 activity and self-renewal of embryonic stem cells (37, 38). We previously reported that IL11 promotes EMT and chemoresistance in breast cancer (19). We examined the relevance of these cytokines in the miR-206-mediated signaling pathway. We found that compared to the scramble control, miR-206 mediated a reduction of IL11 at both mRNA and protein levels in MDA-MB-231 cells (Fig. 5F and G). Furthermore, TWF1 siRNA also reduced the IL11 mRNA and/or protein levels in multiple breast cancer cells (Fig. 5G and H and Supplementary Fig. S3C).

We also determined the effects of MKL1–SRF transcription complex on IL11 expression. Similar to the inhibitory effects of miR-206 and siTWF1, knockdown of MKL1 or SRF decreased IL11 expression in MDA-MB-231 and HS578T cells (Fig. 5H and Supplementary Fig. S3C). We then examined the effects of altered activity of MKL1 and SRF on IL11 expression. Within 30 minutes to 6 hours, the treatment with the MKL1 activator CD resulted in a rapid time-dependent increase of the IL11 mRNA levels in both MDA-MB-231 and HS578T cells as measured by real-time PCR (Fig. 5I and Supplementary Fig. S3D), suggesting a quick, likely direct, transcriptional control of IL11 by activated MKL1-SRF. More importantly, functional rescuing studies further demonstrated that both TWF1 and SRF are able to restore the IL11 expression suppressed by miR-206 (Fig. 5I), suggesting that TWF1 and MKL1/SRF are essential downstream players of miR-206 in regulating IL11.

Because there was a report that estrogen receptor alpha (ERα) regulates miR-206 expression in ER-positive breast cancer (39), we sought to determine whether miR-206 induces a similar signaling pathway in ER-positive cells. RT-PCR results also demonstrated that in MCF7 cells miR-206 overexpression led to a comparable reduction of TWF1, MKL1, and IL11 expression levels and signaling from TWF1 to MKL1 and IL11 as in other breast cancer cells (Supplementary Fig. S3E). These observations suggest that miR-206 regulation of breast cancer cell phenotypes through targeting TWF1 pathway is independent of ER status.

The next task was to determine the importance of IL11 in miR-206 signaling pathway and its function in breast cancer.

IL11 promotes invasion and self-renewal of breast cancer cells

We decide to perform functional rescue studies to evaluate the role of IL11 in miR-206 phenotype regulation. Using transwell invasion assays, we observed that IL11 overexpression reversed the inhibitory effect of miR-206 on invasion of MDA-MB-231 cells (Fig. 6A). Furthermore, IL11 siRNAs transfected in MDA-MB-231 and HS578T cells pheno-copied miR-206 in inhibiting cell invasion (Fig. 6B and Supplementary Fig. S2B). These findings show IL11 is an important downstream target of miR-206 and TWF1 in regulating breast cancer invasion and metastasis.

We then continued to examine whether IL11 regulates cell cycle and self-renewal of breast cancer cells. Within 72 hours, IL11 knockdown induced a significant increase of MDA-MB-231 cells at G1 phase arrest along with decreased cell populations in S and G2–M phases, reminiscent of the cell-cycle regulatory effects of miR-206 (Fig. 6C). Consistently, knockdown of IL11 or MKL1 also mimicked miR-206 and remarkably reduced the mammary sphere formation efficiency (both number and size) in MCF-7 mammary tumor cells (Fig. 6D), implicating that the MKL1/IL11 signaling pathway is essential for miR-206 function in regulating breast tumor self-renewal and tumorigenesis. We also observed that both miR-206 upregulation and IL11 knockdown reduced the CD44 expression that is a marker of CSCs in breast cancer (Supplementary Fig. S4A and B).

Overall our data report a novel signaling pathway mediated by miR-206 that targets TWF1, MKL1, and SRF, and subsequently IL11 expression, thereby inhibiting cell cycle and self-renewal in tumorigenesis as well as cell motility (EMT, migration, invadopodia formation, and invasion) in breast cancer metastasis (Fig. 6E). High levels of miR-206 reduce TWF1 expression that not only causes high free G-actin to trap MKL1 in the cytoplasm and reduce its transcriptional activity along with SRF, but also reduces its expression levels. Reduced MKL1/SRF activity leads to lower levels of IL11 mRNA and protein, which are required for both breast cancer initiation and metastasis.

Discussion

Although miR-206 has been shown to play a role in tumor regulation (8, 40–42), our studies uncovered a novel signaling cascade mediated by miR-206, TWF1, MKL1/SRF, and IL11 in breast cancer stemness and invasion, relevant to breast tumor regeneration and metastasis. Many of the components are potential new targets for breast cancer therapeutics, including both miR-206 and the siRNAs of its target genes. TWF1 has been reported to regulate cell cytoskeleton and motility through direct interactions with actin filaments (27, 43). Although coflin, profilin, and other regulators of actin dynamics are well known to regulate cancer cells, the role and signaling pathways of TWF1 in cellular invasion and stemness were underappreciated. Our studies here comprehensively link TWF1 to both upstream miRNA regulators (miR-206) and downstream protein players, including transcription factor complex (MKL1/SRF) and cytokines (IL11), demonstrating that TWF is central to miRNA functions in breast cancer development and progression.

Although MKL1 and SRF are relatively known for their role in transcriptionally regulating cardiovascular growth and muscle cell differentiation (44), our studies link the mesenchymal muscle lineage transcription factors with breast cancer progression. Our data highlight the importance of the G-actin/F-actin dynamics and related MKL1/SRF activity in regulating stemness, invasion, and miRNA/TWF1/IL11 signaling. Along with the other two coactivators, myocardin and MRTF-B, MKL1 (or called MRTF-A) is one of the three major coactivators of the transcription factor SRF (21, 22). Compared to the knockout mice of other SRF coactivators, MKL1−/− mice possess a unique phenotype of deficient mammary myoepithelial cells and premature involution during pregnancy and lactation (44, 45). Nevertheless, our work suggests that MKL1 may be the major regulator of SRF in the dissected miR-206 signaling pathway in breast cancer cells. These are consistent with the evidence reporting a breast cancer risk susceptibility variation within the MKL1 gene (21) as well as its regulatory role in cytoskeletal dynamics and cell motility in a Rho kinase-dependent manner (44, 46). The direct or indirect
regulation nature of IL11 by SRF remains to be elucidated. The immediate response of IL11 mRNA upregulation within 30 minutes upon MKL1 activation may suggest a direct regulation. Many SRF target genes control cytoskeletal components including actin, and the SRF/Co/Co cells have defects in adhesion and migration (35, 47, 48).

As an IL6 family member, IL11 is a cytokine that enhances platelet production (49), and its recombinant protein (oprelvekin) has been used as an approved drug to treat cancer patients undergoing chemotherapy and to prevent thrombocytopenia (50). Our report here, however, confirms an oncogenic role of IL11 in promoting stemness and cancer invasion, consistent with our previous studies demonstrating the association of IL11 with poor clinical outcome including relapse-free survival in breast cancer patients (19). This also raises questions about the current clinical applications of IL11 in cancer patients and calls for a careful reevaluation of its benefits and potential cancer-promoting effects in the clinical setting.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: R. Samaeekia, V. Adorno-Cruz, Y.-F. Chang, N. Ha, G.Y. Moskalenko, H. Liu
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Figure 6.
IL11 promotes invasion, cell cycle and self-renewal. A, Transwell invasion assays of MDA-MB-231 cells transfected with either scramble or miR-206 followed by either IL11 cDNA or pcDNA control vectors. *, P < 0.05; **, P < 0.01. B, Transwell invasion assays of MDA-MB-231 cells transfected with either IL11 siRNA or scramble control. **, P < 0.01. C, siIL11 significantly increased G1-phase and decreased S and G2-M populations of MDA-MB-231 cells at 72 hours posttransfection. **, Student t test P values <0.001; ****, Student t test P values <0.00001. D, siIL11 decreased MCF-7 mammosphere formation in serum-free stem cell medium. ****, Student t test P values <0.00001. E, Diagram of miR-206/TWF1/MKL1-SRF/IL-11 signaling in breast cancer cells. Mechanism proposed for miR-206 regulation in triple-negative breast tumors. Higher levels of miR-206 reduces TWF1 and somewhat reduced MKL1 expression. Reduction of TWF1 leads to more free G-actin, which binds to left-over MKL1 preventing its translocation into the nucleus and its subsequent activation of SRF. A reduction of SRF activity leads to lower levels of IL11 mRNA and protein expression.
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