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miR-200a inhibits migration of triple-negative breast cancer cells through direct repression of the *EPHA2* oncogene

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Abstract

Triple-negative breast cancer (TNBC) is characterized by aggressiveness and affects 10–20% of breast cancer patients. Since TNBC lacks expression of ERα, PR and HER2, existing targeted treatments are not effective and the survival is poor. In this study, we demonstrate that the tumor suppressor microRNA miR-200a directly regulates the oncogene EPH receptor A2 (*EPHA2*) and modulates TNBC migration. We show that *EPHA2* expression is correlated with poor survival specifically in basal-like breast cancer and that its expression is repressed by miR-200a through direct interaction with the 3′UTR of *EPHA2*. This regulation subsequently affects the downstream activation of AMP-activated protein kinase (AMPK) and results in decreased cell migration of TNBC. We establish that miR-200a directs cell migration in a dual manner; in addition to regulating the well-characterized E-cadherin pathway it also regulates a EPHA2 pathway. The miR-200a-EPHA2 axis is a novel mechanism highlighting the possibility of utilizing miR-200a delivery to target TNBC metastases.

Introduction

Triple-negative breast cancer (TNBC) is a highly invasive subtype with a high incidence of relapse (1). It affects 10–20% of breast cancer patients with a disproportionate incidence among younger women (2–4). TNBC is characterized by a lack of ERα, PR and HER2 expression. Since available targeted treatments of breast cancer are directed towards the ERα and HER2 receptors, they are not efficient against TNBC. In addition, TNBC cells are also relatively resistant to chemotherapy and radiation. As a result, patients diagnosed with this type of breast cancer exhibit a poor overall survival (OS) (5). Therefore, alternative therapeutic approaches are urgently needed.

A promising approach to targeting cancer pathways is through microRNA (miRNA) replacement therapy (6). miRNAs are small non-coding RNAs that have a capacity to act as tumor suppressors and are frequently lost in several types of cancer (7). Because miRNAs usually target multiple genes and pathways simultaneously, an important advantage with miRNA-replacement therapy is a lower potential for resistance. Human clinical trials of miRNA delivery have been successfully performed for hepatitis and cancer patients with no adverse effects observed (8,9). The miR-200 family is emerging as critical tumor suppressor miRNAs and replacement of miR-200 family members has been implicated as a possible therapeutic approach against some human cancers (10). Thus, it is important to understand their mechanism of action.

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Abbreviations

AMPK	AMP-activated protein kinase
DMEM	Dulbecco's modified Eagle's medium
EPHA2	EPH Receptor A2
EMT	Epithelial-to-mesenchymal transition
miRNA	microRNA
OS	overall survival
ORF	open reading frame
RISC	RNA-induced silencing complex
TNBC	triple-negative breast cancer
3′UTR	3′ untranslated region

Low expression of the miR-200 family is observed in breast cancer stem cells (11) and in TNBC (12), and is associated with enhanced stem cell self-renewal (11), epithelial-to-mesenchymal transition (EMT) (13,14) tumor progression (15) and an aggressive tumor phenotype (16). The human miR-200 family consists of five members; with miR-200a, miR-200b and miR-429 in one cluster on chromosome 1 and miR-141 and miR-200c in a second cluster on chromosome 12. miR-200a, b and c all oppose EMT by targeting the E-cadherin suppressors *ZEB1, ZEB2* and *SUZ12,* resulting in increased levels of E-cadherin (17,18). Given that reduced E-cadherin expression is a characteristic for the TNBC subgroup classification (19) and these miRNAs are low in TNBC cells, miR-200 replacement therapy is an intriguing possibility for future TNBC treatment.

By studying the differentiation of non-tumorigenic murine mammary epithelial HC11 cells (20), we found that mRNA and miRNA expression profiles of the undifferentiated HC11 cells overlap with profiles of TNBC clinical samples and cell lines (21), Further, we found that miR-200a was the most upregulated miRNA during mammary cell differentiation, exhibiting a 160-fold increase in differentiated compared to undifferentiated HC11 cells. Analysis of mRNA and miRNA expression profiles indicated that miR-200a level is negatively correlated with the level of a predicted target, the EPH receptor A2 (*Epha2/EPHA2*), during HC11 differentiation (12). However, whether miR-200a can regulate EPHA2 expression and corresponding mechanism are still unknown.

EPHA2 is located in the cell membrane, where it binds ephrin-A ligands (encoded by the genes EFNA1-A5) and regulates cell–cell interaction. In the normal mouse mammary gland, EphA2 is located in the terminal end buds of the gland and is required for their development and branching (22). Elucidation of its mechanism of action has shown that ligands provide the receptor with bi-directional functions (23). Binding of its ligand, ephrin-A1, to EPHA2 causes receptor degradation, suppresses migration and inhibits proliferation, whereas in the absence of ligand, EPHA2 accumulates and promotes invasiveness (24). Studies in mice have shown that Epha2 expression promotes mammary tumorigenesis and metastatic progression (25,26). Immunohistochemical staining of clinical samples has demonstrated that expression of this receptor is increased in malignant breast tissue specimens compared to normal breast tissue (27) and that the level of expression is negatively correlated with OS in human breast cancer (28). Thus, multiple data suggest a tumorigenic role for EPHA2 in breast cancer progression. Also, the ligand Ephrin-A1 is undetectable in TNBC cells while EPHA2 is overexpressed and thus, EPHA2 is suggested to promote tumor invasiveness in TNBC (29,30). Therefore, EPHA2 has been proposed as a potential therapeutic target in TNBC (3,31). Pre-clinical studies show that expression of ephrin-A1 can reduce xenograft tumor growth of a mouse mammary cancer cell line (MT1A2) (32) and that a small molecule agonist is efficient in suppressing migration of breast cancer cells (33). Thus, approaches to inhibit unliganded EPHA2 function hold promise for improved TNBC therapeutics.

Materials and methods

In silico survival analysis

mRNA levels of *EPHA2* and corresponding patient survival were analyzed in large-scale breast cancer datasets (34) using the online analysis tool http://kmplot.com. OS in basal-like, Luminal A, Luminal B and Her2-positive breast cancer subtypes was analyzed. Hazard ratio and log-rank test were calculated for the significance testing.

Cell culture

HC11 cells were obtained from Dr Groner's group where the cell line is originally established and authenticated (20) and further characterized by us (12,21). Cells were maintained in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, L-glutamine, 5 μg/ml insulin, 10 ng/ml epidermal growth factor and 50 μg/ml gentamicin (all from Sigma, Saint Louis, MO, USA). MDA-MB-231 (purchased from and validated by ATCC, Manassas, VA, USA) and SUM159 (purchased from and validated by Asterand, Detroit, MI, USA) cells were kept in 1:1 Dulbecco's modified Eagle's medium (DMEM):F12 (Gibco) or DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Sigma), respectively, and also characterized in our previous article (35).

Transfections

Cells were placed on a six-well (or 24-well) plate at a density of 16×10^4 (or 3×10^4) cells/well for HC11; and 33×10^4 (or 6×10^4) cells/well for MDA-MB-231 and SUM159. Cells were transfected with miR-200a mimic or non-targeting miRIDIAN miRNA mimic control (Dharmacon, Pittsburgh, PA, USA) at a final concentration of 25 nM for expression assay and 50 nM for functional assays using DharmaFECT 1 (Dharmacon). The sequence for miR-200a is the same in human and mouse species. On-TARGETplus SMARTpool human siRNA targeting *EPHA2* and On-TARGETplus SMARTpool non-targeting siRNA control (Dharmacon) were transfected at a final concentration of 100 nM using DharmaFECT 1. To overexpress EPHA2, 800 ng of *EPHA2* open reading frame (ORF) expression clone and negative control vector (both from Genecopoeia, Rockville, MD, USA) were transfected using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer instruction. To silence AMP-activated protein kinase (AMPK), two different siRNA sequences, siR-AMPK-1 (Sigma) and siR-AMPK-2 (Invitrogen), respectively, were transfected into MDA-MB-231 cells at a final concentration of 100 nM using DharmaFECT 1 according to the manufacturer's instructions. Total RNA was collected 24 h post-transfection. For functional assays, transfection medium was replaced with antibiotic-free medium after 24 h and incubated according to assay requirements.

Cell-based 3′UTR reporter assay

EPHA2 3′untranslated region (UTR) gene construct (Genecopoeia, HmiT004657) containing the predicted miR-200a targeting site, mutant miR-200a targeting site or a target control (Genecopoeia, CmiT000001) cloned into luciferase reporter vector p-EZX-MT01 were used. HEK-293 cells were placed on a 24-well plate at a number of 10^5. Cells were co-transfected with 800 ng reporter gene construct and 100 nM miR-200a mimic or controls using Lipofectamin 2000 (Invitrogen). After 24 h transfection, cells were collected and subjected to measurement of reporter activity using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Expression analysis

Total RNA, including the miRNA population, was extracted using Trizol (Invitrogen) and miRNeasy kits (Qiagen, Valencia, CA, USA) as described previously (12). Quantitative and qualitative analysis of RNA was performed using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA) and the Agilent 2100 Bioanalyzer (Agilent technologies, Palo Alto, CA, USA), respectively. One microgram of total RNA was used as

template for cDNA synthesis, using SuperScript III First-Strand Synthesis kit and random hexamers. qPCR was performed in triplicates using Fast SYBR Green SuperMix (Life Technologies, Grand Island, NY, USA), on an ABI PRISM 7500 series real-time PCR machine (Life Technologies). ΔΔCT method was applied for calculation of relative levels of gene expression. Student's unpaired two-tailed *t*-test was used for significance testing, and differences considered significant when $P \leq 0.05$.

To determine relative levels of proteins, 50 μg of protein extracts were separated on a 7.5% SDS-PAGE following established protocol (36). Antibodies against EPHA2 (C-20) and E-cadherin (H-108, Santa Cruz Biotechnology, Santa Cruz, CA, USA), VIM (SC-32322, Santa Cruz Biotechnology), AMPK (2523S, Cell Signaling Technology, Danvers, MA, USA) and pAMPK (Thr172-2531S, Cell Signaling Technology) were used at concentration 1:1000, and against β-Actin (AC-15) and GAPDH (Sigma) at 1:5000, followed by horseradish peroxidase conjugated secondary antibodies (Cell Signaling Technology) at 1:5000 and 1:10 000, respectively. Immunoreactive bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Proliferation assays

For colony-formation assay, cells were placed on a 50-mm Petri dish at a number of 1×10^3. After 10 days, cells were fixed with methanol: acetic acid (2:1) and stained with crystal violet (0.5% crystal violet in 25% methanol). Colonies were counted using ImageJ and images were taken under Olympus 1x51 inverted microscope. For cell counting, cells were transfected, trypsinized after 48 h and combined with Trypan Blue in a 1:1 ratio. Viable cell numbers were counted using Countess Automated Cell Counter (Invitrogen). For Hoechst dye staining, cells were transfected and stained using blue fluorometric double-stranded DNA quantification kit (Life Technologies). The fluorescence intensity was measured at excision 360 nm and emission 460 nm. For BrdU staining, cells were transfected and serum starved for 48 h in 0.5% bovine serum albumin DMEM-F12 medium. Full-serum medium was added back for 36 h before the cells were incubated with BrdU (30 uM) for 60 min. Cells were then fixed in 70% ethanol and washed with phosphate-buffered saline, 2N HCl/ Triton X-100, tetraborate and incubated with fluorescein isothiocyanate-conjugated BrdU antibody (BD Biosciences, San Jose, CA, USA) for 30 min, followed by fluorescence-activated cell sorting analysis. Propidium iodide staining was used to test cell cycle distribution. Cells were transfected and synchronized in 0.5% bovine serum albumin. After 48 h, full-serum medium was added and cells were allowed to grow for 36 h before fixation (70% ethanol) and staining by propidium iodide (50 ug/ml, Sigma-Aldrich). The cell cycle distribution of G0/G1, S and G2/M phase was examined using BD FACSAria III (BD Biosciences).

Migration assays

For wound-healing assays, a scratch was made to the confluent cell monolayer 24 h post-transfection, or as otherwise indicated, and the medium replaced with serum-free medium. Images were taken with Olympus 1x51 inverted microscope at 8, 12 and 24 h (HC11 cells) and at 0–12 h (TNBC cells). Distance of migration and area covered by migrating cells were quantified by using Olympus cellSens digital imaging software and ImageJ. For the trans-well migration assay, migration was measured using Boyden Chambers (Cell Biolabs Inc). At 48 h after transfection, cells were starved in DMEM, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 0.1% bovine serum albumin starvation medium for additional 24 h. Upon trypsinization, cells were seeded in fibronectin-coated chambers and chemoattractant (fetal bovine serum) was placed in the bottom. After 6 h, cells at the bottom layer were fixated with 2% formaldehyde, 0.2% glutaraldehyde in phosphate-buffered saline and washed with phosphate-buffered saline. Final staining with crystal violet was performed and ImageJ software was used for image analysis.

Results

EPHA2 expression correlates with poor survival outcome in TNBC

EPHA2 expression has previously been linked to poor survival among breast cancer patients (28). To investigate whether *EPHA2* expression correlates with clinical outcome in the TNBC subtype specifically, we correlated mRNA levels of EPHA2 with survival of patients with different subtypes of breast cancer, using publicly available large-scale breast cancer datasets (34). Higher *EPHA2* expression was significantly associated with lower survival within the basal-like subtype, which is primarily TNBC, but not within the luminal A, luminal B or HER2-positive subtypes (Figure 1A). On the contrary, in luminal A tumors, high expression of *EPHA2* was correlated with a better survival. This indicates that EPHA2 may be linked to metastatic potential and adverse outcome specifically in TNBC patients.

The 3′ UTR of *EPHA2* contains a predicted and conserved target site for miR-200a

To explore if EPHA2 may be targeted by miR-200a, we used target prediction software which indicated a potential binding site of miR-200a in the 3′ UTR of *EPHA2* (Figure 1B). Eight nucleotides at the *EPHA2* 3′UTR target site showed complete complementarity to the seed sequence of miR-200a. Moreover, we found the target site to be conserved among distant species of vertebrates (Figure 1B), indicating *EPHA2* is a potential target of miR-200a regulation. For the related miR-200b, the complementary sequence was seven nucleotides, with a mismatch in the center of the seed sequence.

EPHA2 expression is downregulated by miR-200a in mouse and human cell lines

We have previously reported that the transcriptome profile of human TNBC clusters with the gene expression profile of undifferentiated murine HC11 cells (21). Interestingly, the differentiation-induced miR-200a was correlated with reduced mRNA levels of EPHA2 in these cells (12). As EPHA2 has a predicted miR-200a-binding site that is conserved between mouse and human (Figure 1B), we hypothesized that miR-200a could regulate *EPHA2* in both species. To demonstrate whether EPHA2 protein levels were significantly repressed by miR-200a in mouse, we performed single and double transfection with miR-200a mimic or control in undifferentiated HC11 cells followed by western blot analysis. We observed a clear decrease of EPHA2 protein levels within 48 h of transfection with miR-200a compared to control, and after two consecutive transfections (72 h), the EPHA2 protein was decreased to barely detectable level (Figure 1C). Previous studies have shown that in TNBC cell lines, similar to that in the undifferentiated stage of HC11, miR-200a expression is low (12,14) while the *EPHA2* expression is high (29). To determine whether miR-200a could also silence *EPHA2* in TNBC cells, we transfected two human TNBC cell lines, MDA-MB-231 and SUM159, with miR-200a mimic or control and confirmed increased miR-200a levels using qPCR (Supplementary Figure 1A, available at *Carcinogenesis* Online). Within 24 h of the miR-200a mimic transfection, the mRNA levels of *EPHA2* were reduced by 46% in MDA-MB-231 cells ($P = 0.001$) and by 35% in SUM159 cells ($P = 0.004$) compared to control (Figure 1D). A clear decrease in corresponding protein levels was also noted 48 h after transfection with the miR-200a mimic (Figure 1E). Collectively, these data show that miR-200a decreases EPHA2 protein levels in a conserved manner in both undifferentiated murine mammary epithelial cells and in human TNBC cell lines.

EPHA2 is a direct target of miR-200a

To test whether *EPHA2* is directly targeted by miR-200a through binding to its 3′UTR, we cloned wild-type human *EPHA2* 3'UTR downstream of the firefly luciferase reporter gene, using the same construct but with mutated miR-200a binding site (CAGTGTTA→CACACATA) as control (Figure 2A). Additionally, a control vector without the 3′UTR sequence was also included.

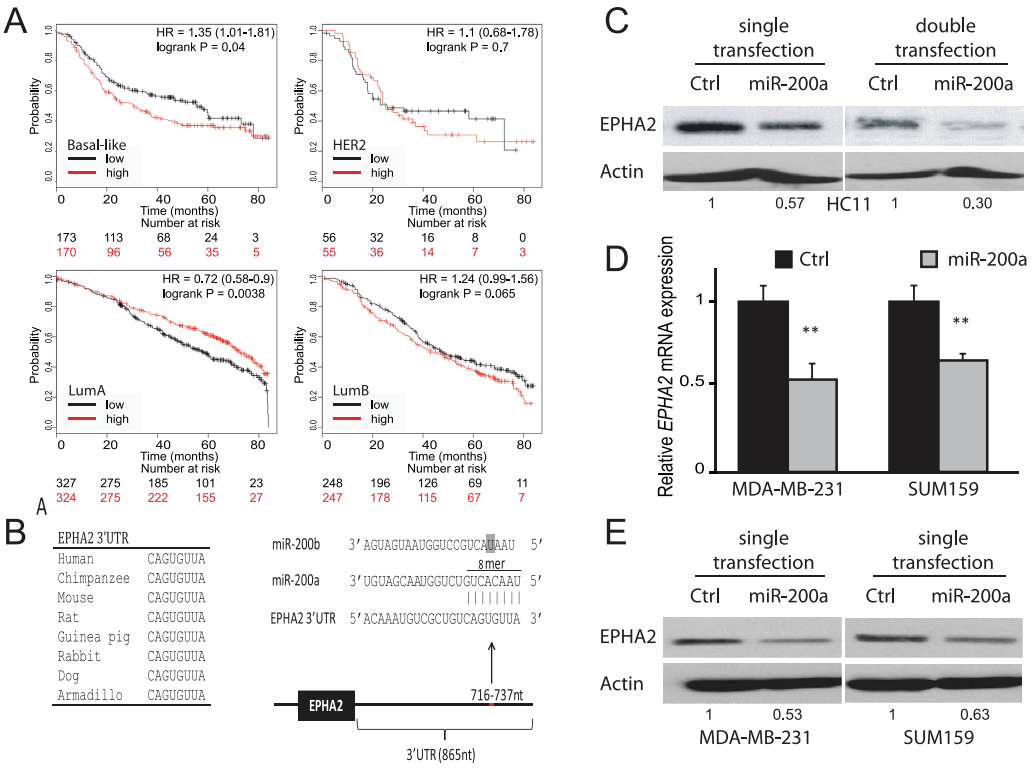

Figure 1. *EPHA2* is correlated with poor clinical outcome in TNBC and is a predicted target of miR-200a. (**A**) Kaplan–Meier survival plots show that high level of *EPHA2* mRNA is significantly correlated to a lower overall survival in a cohort of basal-like patients ($N = 343$). This correlation is not significant for Luminal B ($N = 495$), or HER2-positive ($N = 111$) subtype patients. For Luminal A ($N = 651$) patients, high levels of EPHA2 correlate with better survival. (**B**) A potential target site for miR-200a was identified in the 3′UTR sequence of *EPHA2* at nucleotides 716–737, using target prediction software TargetScan. Eight nucleotides within the seed sequence of miR-200a showed complete complementarity with the 3′UTR sequence of *EPHA2*. These nucleotides were 100% conserved between various vertebrate species. (**C**) Western blotting of undifferentiated HC11 cells transfected with miR-200a mimic (25 nM) or control in a single (left), or double transfection (right), shows decreased levels of EPHA2 protein by miR-200a. β-Actin was used as a loading control. (**D**) Significant reductions of *EPHA2* mRNA levels were noted within 24 h after miR-200a mimic (25 nM) treatment in MDA-MB-231 and SUM159 cells, compared to control mimic. (**E**) Western blotting of MDA-MB-231 and SUM159 cells, 48 h after transfection with miR-200a mimic (25 nM) or control mimic, shows that miR-200a reduced expression of EPHA2 protein in both cell lines. β-Actin was used as a loading control. Student's *t*-test, two-tailed distribution was performed and $P \leq 0.05$ was considered statistically significant.

HEK293 cells were cotransfected with one of these constructs and with miR-200a, miR-9 (non-related miRNA control) or control mimic. As shown in Figure 2B, only cells with the wild-type *EPHA2* 3′UTR-containing construct exhibited significant decrease of luciferase activity when transfected with miR-200a mimic ($P = 0.007$). Mutation of the miR-200a binding site abolished the ability of miR-200a to silence luciferase expression, suggesting that this putative binding site is essential for silencing *EPHA2*. In contrast, non-related miR-9 and control mimic had no significant effect on the luciferase expression. Furthermore, we noted that endogenous *EPHA2* in HEK293 cells was simultaneously downregulated (by 40%, $P = 0.001$) by miR-200a mimic (Figure 2C). Collectively, our results demonstrate that the 3′UTR of EPHA2 mRNA is directly targeted by miR-200a.

miR-200a inhibits migration and proliferation in both HC11 and TNBC cells

miR-200a has been shown to repress migration, proliferation and mammosphere formation in various mammary epithelial cells and in breast cancer cells (14,37,38). To explore the functional impact of the miR-200a-*EPHA2* pathway, we first investigated miR-200a functions in the HC11 model. We used the migratory undifferentiated stage of HC11 where the level of miR-200a is low and *Epha2* is high (12,21). Transfection with miR-200a mimic significantly decreased colony-formation capacity ($P = 0.012$, Figure 3A). Trypan blue staining and cell counting showed that proliferation was decreased by 30% upon miR-200a treatment ($P = 0.016$, Figure 3B). Using wound-healing assays, miR-200a was found to decrease HC11 cell migration ($P = 0.013$ at 12 h, $P = 0.033$ at 24 h, Figure 3C). Next, we corroborated and quantified the previously reported effects that miR-200a has in the TNBC breast cancer cells, using cell line MDA-MB-231. As shown in Figure 3D and E, we observed that proliferation and migration of the TNBC cell line MDA-MB-231 were significantly decreased after transfection with miR-200a. Cell counting indicated that proliferation was reduced by 50% ($P = 0.002$) 48 h after transfection with miR-200a (Figure 3D). BrdU staining confirmed the reduction of proliferation ($P = 0.004$, Supplementary Figure 1B, available at *Carcinogenesis* Online), while FACS analysis showed no change in cell cycle distribution by miR-200a in MDA-MB-231 (Supplementary Figure 1C, available at *Carcinogenesis* Online). Migration was assessed using trans-well migration assay and miR-200a transfected cells exhibited significantly less migration 48 h post-transfection ($P = 0.03$, Figure 3E). Together, our results clearly show that miR-200a reduces proliferation and migration in both mammary stem-like HC11 cells and TNBC cell line MDA-MB-231 and hence are suitable models for studies of miR-200a functions.

A novel miR-200a-*EPHA2* pathway represses migration but not proliferation

In order to determine the functional impact of miR-200a-mediated silencing of *EPHA2* in TNBC cells, we silenced *EPHA2* using siRNAs or overexpressed it using ORF clone in both MDA-MB-231

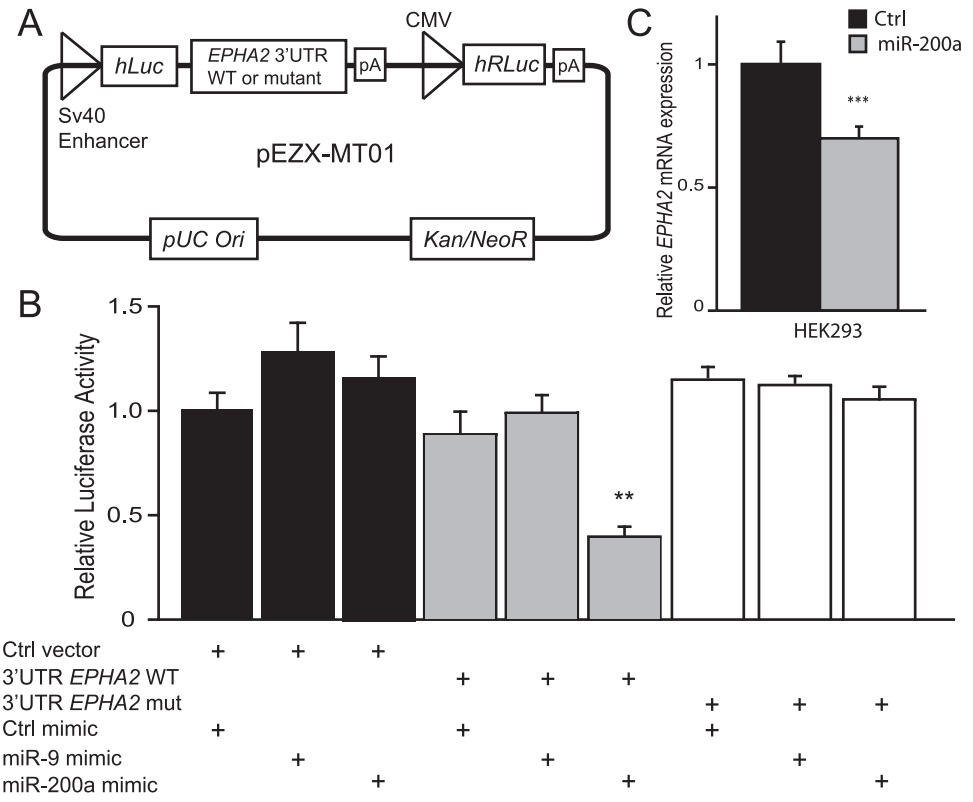

Figure 2. miR-200a directly targets the 3′UTR of *EPHA2*. (**A**) The constructs contain *EPHA2* 3′UTR sequence encompassing the predicted miR-200a site or control with (a) this site mutated, and (b) no 3′UTR sequence, cloned into luciferase reporter vectors. The constructs contains hLuc and hRLuc under the control of Sv40 and CMV promoter. hRLuc is endogenous control of construct expression. (**B**) HEK239 cells were transfected with plasmid containing WT or mutated *EPHA2* 3′UTR, or control plasmid, and either miR-200a mimic, control mimic or miR-9 mimic. Luciferase activity was measured 24 h post transfection. Only the construct containing the predicted target site significantly decreased its luciferase activity, and only when miR-200a was added. (**C**) *EPHA2* mRNA levels were reduced also in HEK239 cells. The cells were transfected with miR-200a mimic (25 nM) or control mimic and collected 24 h post transfection. *EPHA2* and housekeeping gene *36B4* mRNA levels were measured using qPCR and data analyzed using the ΔΔCT method. For significance, Student's *t*-test, two-tailed distribution was used.

and SUM159 cells. Successful silencing of *EPHA2* was examined by measuring the mRNA levels 24 h after the transfection (Supplementary Figure 1D, available at *Carcinogenesis* Online), and analysis of corresponding protein levels 48 h after transfection of siRNA or ORF clone confirmed corresponding reduction or upregulation of EPHA2 protein in both MDA-MB-231 and SUM159 cell lines (Supplementary Figure 1E, available at *Carcinogenesis* Online). Next, we performed migration assays following changes in *EPHA2* expression. Using wound-healing assays, we observed that MDA-MB-231 and SUM159 cell migration was significantly decreased ($P = 0.004$ and $P = 0.04$, respectively) upon silencing of *EPHA2* (Figure 4A, middle), similar to that after the miR-200a mimic treatment (Figure 4A, top). In contrast, overexpression of *EPHA2* promoted gap closure in both TNBC cell lines (Figure 4A, bottom). More importantly, overexpression of *EPHA2* could rescue the anti-migratory effects of miR-200a in both MDA-MB-231 and SUM159 cells (Figure 4C), suggesting that *EPHA2*, as a direct target of miR-200a, is critical for the anti-migratory role of miR-200a. We did not observe any significant changes of cell proliferation upon neither *EPHA2* silencing nor EPHA2 overexpression (Supplementary Figure 1F–G, available at *Carcinogenesis* Online). Collectively, these results demonstrate that miR-200a-mediated silencing of *EPHA2* represses TNBC cell migration independently of the effect miR-200a has on cell proliferation.

E-cadherin is not involved in the miR-200a-EPHA2-mediated anti-migratory effects

Previous studies have shown that miR-200a indirectly upregulates E-cadherin by silencing several E-cadherin inhibitors, thereby repressing migration (18). It is possible that the above described miR-200a-mediated silencing of EPHA2 converges with E-cadherin upregulation and subsequent effects on migration in breast cancer cells. To explore if EPHA2 signaling modulated E-cadherin levels, we evaluated the expression levels of E-cadherin upon miR-200a, si-*EPHA2* and *EPHA2*-ORF overexpression. As previously established, E-cadherin protein levels increased significantly upon miR-200a expression (Figure 5A). However, when we silenced *EPHA2*, and noted reduced migratory potential (Figure 4, middle), E-cadherin levels were not changed (Figure 5A). Likewise, overexpression of EPHA2 increased migratory potential (Figure 4, bottom) but did not affect E-cadherin levels (data not shown). We also found the decreased expression level of mesenchymal marker Vimentin mediated by miR-200a (18) in MDA-MB-231 cells but not in SUM159 (Supplementary Figure 1H, available at *Carcinogenesis* Online, SUM159 data not shown). Such decrease of Vimentin was not affected by EPHA2 in either triple-negative cell line. Collectively, our data demonstrate the regulation of miR-200a-EPHA2 on TNBC cell migration is a novel pathway of action distinct from the E-cadherin mechanism.

The miR-200a-*EPHA2* pathway contributes to AMPK activation

Women with higher body mass index or type II diabetes are thought to be at a higher risk of developing TNBC (39). Previous studies have shown that treatment with metformin, an antidiabetic drug, inhibits TNBC cell proliferation (40). Metformin is

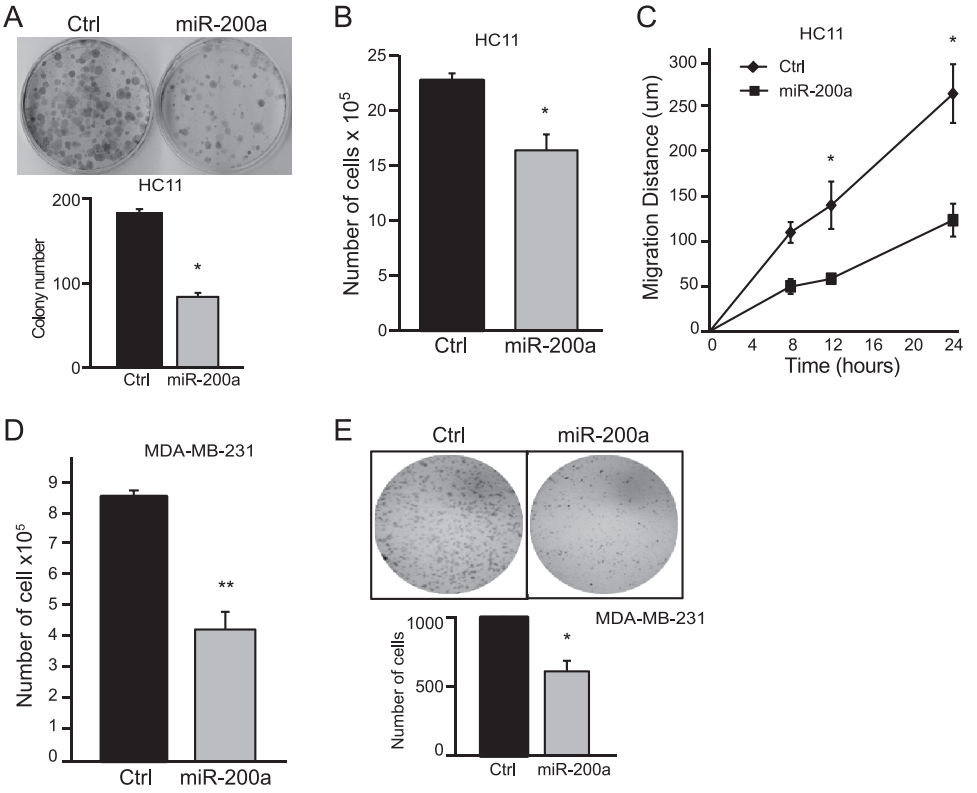

Figure 3. miR-200a reduces migration and proliferation of undifferentiated murine non-tumorous HC11 cells and human TNBC cells. (**A**) Colony formation is reduced in undifferentiated HC11 cells transfected with mimic-200a (50 nM). Cells were seeded in triplicates and incubated for 14 days, and colonies were stained with crystal violet and counted and measured using ImageJ. (**B**) Proliferation of HC11 cells were reduced upon treatment with miR-200a mimic (50 nM) compared to mimic control. The figure illustrates the combined result of three repeated experiments, performed 72 h after transfection. (**C**) HC11 cell migration was reduced after miR-200a mimic (50 nM) transfection compared to control mimic. Migration distance was measured from 0 to 24 h. The figure illustrates the combined result of three repeated experiments. (**D**) Proliferation assays of MDA-MB-231 cells after treatment with miR-200a mimic (50 nM) or mimic control showed that miR-200a significantly decreased proliferation ($P = 0.002$) within 48 h, using cell counting. (**E**) Transwell migration assay demonstrate that miR-200a significantly reduces migratory potential ($P = 0.03$) in MDA-MB-231 cells. Upon 24 h starvation of cells, fetal bovine serum was used as chemoattractant at the bottom of the chamber 48 h post transfection with miR-200a mimic (50 nM) or control mimic. Cells were allowed to migrate for 6 h prior to staining. Statistical significance was calculated using Student's *t*-test, two-tailed distribution, and significance is represented as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

widely used for type II diabetes treatment and some of its biological effects are attributed in part to AMPK activation. Other AMPK agonists, such as phenformin or demethyxocurcumin, have also been tested in TNBC cells and show similar results (41,42). One of the targets of AMPK is Acetyl-CoA carboxylase (ACC). AMPK can phosphorylate ACC at Ser79 and thereby inhibit enzymatic activity which blocks fatty acid synthesis. ACC activation correlates with TNBC invasion (43,44). Previous work has shown that AMPK can regulate the assembly of epithelial tight junctions (45) and AMPK activation/ACC deactivation reduces the invasion potential of the cells via regulation of invadopodia formation (43). In addition, EPHA2 receptor ligand-independent action supports migration via AKT activation (24), which could result in reduced AMPK phosphorylation levels (46). Thus, we sought to investigate whether mir-200a-*EPHA2* pathway has an effect on AMPK phosphorylation, whereby it modulates the TNBC migration. AMPK activation can be assessed by the phosphorylation of Thr172 located on the activation loop of the α catalytic subunit of AMPK. Thr172 phosphorylation was increased upon silencing of EPHA2 or upon miR-200a mimic transfection (Figure 5B), suggesting the miR-200a-*EPHA2* pathway regulates AMPK activity. Also, inhibition of AMPK activity via treatment with the AMPK inhibitor Compound C (Supplementary Figure 1I, available at *Carcinogenesis* Online) and specific siRNAs (Supplementary Figure 1J, available at *Carcinogenesis* Online) can both increase the tumor migration (Figure 5C and Supplementary Figure 1J, available at *Carcinogenesis* Online).

Discussion

Previous studies have reported a role for the oncogenic *EPHA2* in the invasiveness of breast cancer (29,30) and found an inverse correlation between its expression and total breast cancer survival (28). Here, we investigated the relation between *EPHA2* and survival in different breast cancer subtypes, and found that high *EPHA2* expression predicts poor prognosis in TNBC patients specifically, while this is not significant for other molecular subtypes of breast cancer (Figure 1A). We demonstrate that miR-200a can suppress migration in TNBC cells through the direct silencing of *EPHA2* (Figure 2B, and 4A-C). While both the miR-200 family and EPHA2 are known to affect migration and invasiveness in TNBC, a direct crosstalk between them in breast cancer has not been previously demonstrated. Our finding that EPHA2 is directly regulated by miR-200a in both mouse and human is novel. Furthermore, we find this miR-200a-EPHA2 regulation could activate downstream AMPK pathway and thereby contribute to reduced tumor cell migration (Figure 5B and C). Our established miR-200a-EPHA2 axis demonstrates a novel mechanism, besides the miR-200a-E-cadhein pathway, of anti-tumorigenic function of miR-200a in TNBC (Figure 6).

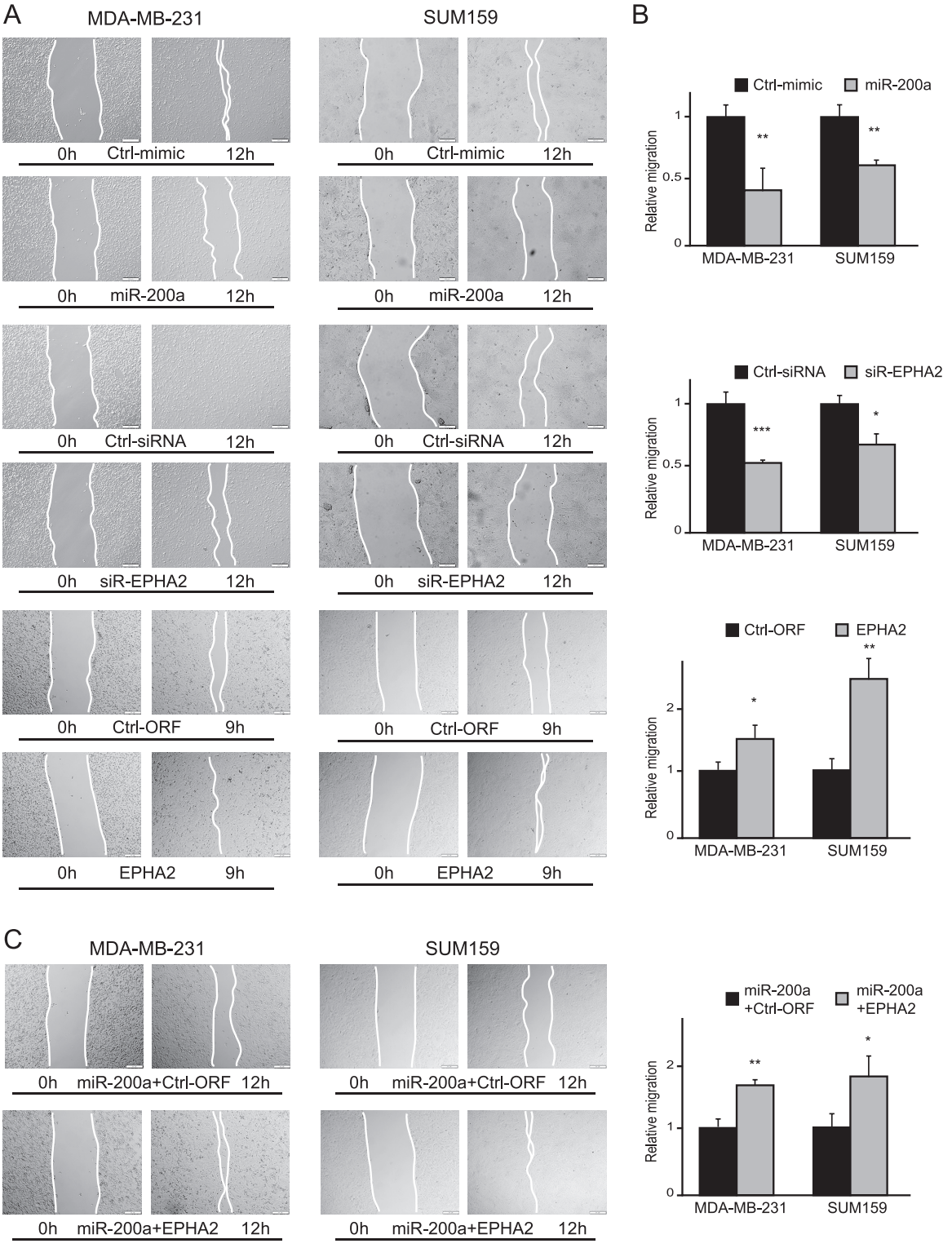

Figure 4. miR-200a reduces migration of TNBC cells through EPHA2 regulation. (**A**) The effect of miR-200a and EPHA2 on migration was measured in MDA-MB-231 and SUM159 cells. Cells transfected with miR-200a mimic (50 nM), *si-EPHA2* (100 nM), *EPHA2*-ORF (800 ng) or corresponding control for 48 h were subjected to wound-healing assay. Images were taken at 0 and 12 h (or 9 h) after wound introduction. The white line highlights the wound edge at 0 and 12 h (or 9 h). (**B**) Quantification of relative cell migration in MDA-MB-231 and SUM159 transfected as shown in A. Distance of gap closure was measured using ImageJ. Data are presented as mean ± SD from two independent experiments. Unpaired two-tailed student's *t*-test was used for significant testing, and presented as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. (**C**) Overexpression of EPHA2 rescued the anti-migratory effect of miR-200a in MDA-MB-231 and SUM159 cells. Cells were cotransfected with miR-200a mimic and *EPHA2*-ORF or control ORF and subjected to wound-healing assay.

For many years, the role of EPHA2 in migration was controversial (47–49), but recent studies have clarified that EPHA2 enhances migration in absence of its ligand; while in presence of its ligand (EFNA1) EPHA2 is degraded, preventing migration (24). Contrary to immortalized MCF-10A and luminal A breast cancer cells, TNBC cell lines express low to undetectable

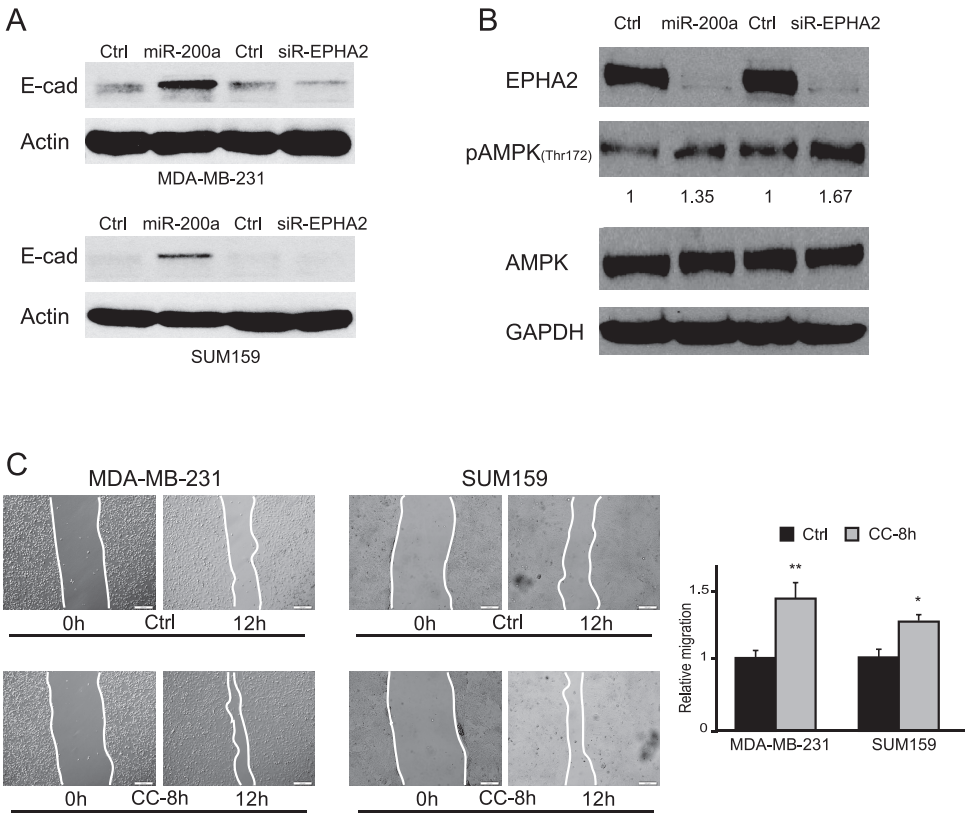

Figure 5. EPHA2 does not affect E-cadherin levels but increases AMPK phosphorylation. (**A**) E-cadherin levels are independent on EPHA2 in TNBC cells. MDA-MB-231 and SUM159 cells were transfected with miR-200a mimic (50 nM), s-i*EPHA2* or corresponding control were subjected to western blot for E-cadherin. β-Actin was used as a loading control. (**B**) AMPK phosphorylation is enhanced by miR-200a-*EPHA2* pathway. MDA-MB-231 cells were transfected and followed by western blot for pAMPK and total AMPK. (**C**) Inhibition of AMPK promotes cell migration in TNBC cells. Cells were treated with Compound C for 8 h followed by wound-healing assay. Images were taken at 0 and 12 h after wound introduction and relative migration distance was quantified using ImageJ.

levels of EFNA1, whereas EPHA2 is highly expressed and largely unphosphorylated, thus acting migratory (29). Accordingly, high EPHA2 levels correlated with poor prognosis for the basal-like (TNBC) subtype but with better prognosis for the luminal A subtype (Figure 1A). Using functional assays, we demonstrate that repression of EPHA2 reduces migration significantly in TNBC cells, and contributes to the anti-migratory effect of miR-200a in TNBCs. Expression of EPHA2 lacking the 3'UTR can rescue the cells from anti-migratory effects of miR-200a (Figure 4C). Interestingly, this mechanism acts separately from the previously determined effect of miR-200a on E-cadherin regulation, as EPHA2 levels did not influence E-cadherin levels. We propose that the lack of miR-200a, and subsequent increase in EPHA2 levels, are important factors for the invasiveness of TNBC cells. This new signaling pathway adds a new layer to our understanding of the roles miR-200a has in cancer involving regulators of migration and EMT.

E-cadherin is a marker of epithelial cells, and its downregulation indicates EMT and increased ability to metastasize. We have previously demonstrated that miR-200a and miR-200b enhance E-cadherin expression in the HC11 cells, but only miR-200a affected EPHA2 mRNA levels (12). miR-200a differs from miR-200b only by one nucleotide (C to U) in the middle of its 5′ seed sequence. This one-nucleotide difference can be sufficient to separate certain targets genes. Other genes, such as ETS1 and PLCG1, are also differentially targeted by these two miRNAs (50,51). The ability of miR-200a to regulate two different key mechanisms of cell adhesion enhances its ability to reverse EMT. Our finding thus suggests a biological rational to why miR-200a was upregulated to a higher degree than miR-200b during HC11 mammary cell differentiation. Since they are both expressed from one polycistronic pri-miRNA transcript at chromosomal location 1p36 (52), this differential regulation of their mature levels is likely to be specifically regulated at the miRNA processing level. miRNA processing and post-transcriptional regulation is complex, mainly including pri-miRNA cleavage by Drosha-DGCR8, pre-miRNA nuclear export, cleavage by Dicer-TRBP, RISC loading and guide strand selection (53). Different miRNAs are processed differently by specific factors, which provide multiple opportunities for post-transcriptional regulation of miRNA expression (54,55). Hence, it will be important to define how miR-200a and miR-200b biogenesis is altered in cancer.

As demonstrated here, miR-200a targets two separate pathways: enhancing E-cadherin and reducing EPHA2, both of which lead to reduced migration (Figure 6). In addition, it has been shown that in non-cancerous MCF-10A cells upregulation of E-cadherin can modulate the phosphorylation and localization of EPHA2 (56). Thus, miR-200a can potentially affect EPHA2 activity through multiple mechanisms: indirectly through the regulation of E-cadherin and subsequent effects on EPHA2 phosphorylation, and directly through post-transcriptional regulation by targeting its 3′UTR (Figure 6C and D). We also determined that EPHA2-activated AMPK can contribute to migration.

Therapeutic delivery of miR-200a could provide an advantageous way for stabilization of adherent junctions, reduction of EMT and prevention of metastasis. As miR-200a has multiple anti-metastatic actions including targeting *ZEB1*, *ZEB2*, *SUZ12* and *EPHA2* directly, tumor cells will need multiple major changes to avoid these effects

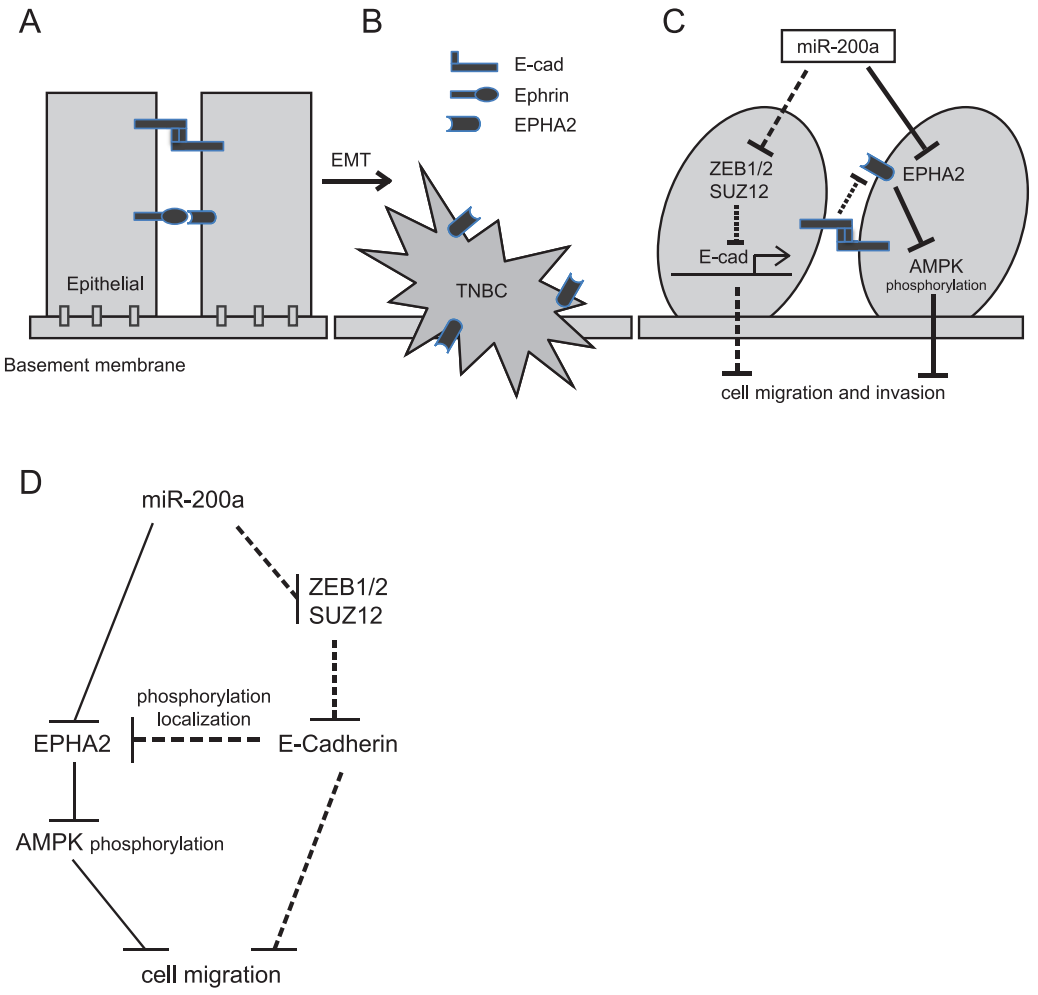

Figure 6. Model illustrating how miR-200a regulates TNBC migration. (**A**) Differentiated epithelial cells expressing E-cadherin, EPHA2 and Ephrin. (**B**) TNBC cells exhibit downregulation of E-cadherin and lack of Ephrin along with a migratory and invasive phenotype. (**C**) miR-200a regulates both the E-cadherin and EPHA2 pathways, resulting in increased levels of E-cadherin and reduced levels of EPHA2 along with inhibited migratory and invasive capacities. (**D**) Proposed pathways that miR-200a can repress migration through dual pathways: by direct targeting the 3′UTR of *EPHA2* (solid lines, as demonstrated in this study) and through the indirect upregulation of the E-cadherin (dashed lines, as reported in literature).

and will have difficulties developing resistance. Thus, miR-200a could be a promising candidate for miRNA-replacement therapy. In our previous study, we investigated miR-200a's role in mammary differentiation of murine stem cell-like cells (12). We reported that in differentiated cells miR-200a is highly expressed and inhibits ZEB1, ZEB2 and SUZ12 resulting in high levels of E-cadherin, whereas in mammary stem cell-like cells miR-200a and E-cadherin levels are low. In this study, we characterized the role of miR-200a in TNBC and show that it directly targets the oncogene EPHA2. This regulation results in decreased cell migration. As such, it is important to further explore the therapeutic potential of this miRNA.

Supplementary material

Supplementary Figure 1 and Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

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