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Original Articles

miR-374a-5p promotes tumor progression by targeting *ARRB1* in triple negative breast cancer

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A B S T R A C T

Triple negative breast cancer (TNBC) has higher aggressiveness and poorer outcomes compared with other subtypes of breast cancer. However, the genomic and molecular aberrations of TNBC are largely unknown. In this study, miR-374a-5p was discovered as a novel TNBC-specific miRNA and its functions and the molecular mechanisms involved were investigated. Combined gene expression profiling of miRNA-microarray and human transcriptome dataset analysis revealed that miR-374a-5p is specifically upregulated in TNBC patients. Functional studies using *in vitro* and *in vivo* models indicated that upregulated miR-374a-5p promotes tumor progression in TNBC. miR-374a-5p was also found to directly target arrestin beta 1 (*ARRB1*) that is specifically downregulated in TNBC patients in several human genomic datasets. Overexpressed ARRB1 reduced TNBC cell growth and migration, and the ARRB1 expression level is inversely correlated with the histological grade of the breast cancer and positively associated with TNBC patient survival, suggestive of a tumor-suppressive function of ARRB1 in breast cancer. Interestingly, increased ARRB1 activates AMPK in TNBC cells, associated with the expression of miR-374a-5p. Taken together, the findings suggest that miR-374a-5p is a potential prognostic marker of TNBC.

1. Introduction

Breast cancer is a histologically and clinically heterogeneous disease that is classified into at least four major types based on molecular characteristics: luminal A (Lum A), luminal B (Lum B), human epidermal growth factor receptor 2^+ (HER2), and triple negative/basal-like breast cancer [1,2]. Among them, triple negative breast cancer (TNBC) is the subtype lacking the expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 [3]. They are reported to account for 12–17% of all breast cancer patients with a higher incidence in younger patients [4,5]. Molecularly, they have hyper-activation of cell proliferation-related signaling pathways, such as PI(3)K/AKT, and MYC pathways [6,7]. Moreover, they show significantly higher aggressiveness and poorer outcomes compared with other breast cancer subtypes, evidenced by an increased risk of metastasis and recurrence, and a lower survival rate [8–10]. However, due to the lack of the expression of endocrine receptors, which serve as targets of endocrine therapy, the current treatment for TNBC remains limited [11,12].

microRNAs (miRNAs) are single-stranded, non-protein-coding RNAs of 19–25 nucleotides in length [13]. The miRNA complexed with RNA-induced silencing complex (RISC) recognizes target messenger RNAs (mRNAs) and activates RISC to hinder their translation and expression, which is the reason why miRNAs are considered to be gene regulators [14]. As this regulation is mediated by incomplete complementarity of

Abbreviations: TNBC, Triple-negative breast cancer; miRNA, microRNA; 3′UTR, 3′-untranslated region; Lum A, Luminal A breast cancer; Lum B, Luminal B breast cancer; HER2, human epidermal growth factor receptor 2 (HER2); ARRB1, arrestin beta 1

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miRNA and mRNA sequences, a single miRNA can regulate multiple target genes [15]. Considering the biological functions of miRNAs, it is apparent that they are involved in cancer, and many of them have been reported to be dysregulated in human cancers. These miRNAs work as either oncogenic miRNAs (oncomiRs) or tumor suppressor miRNAs by suppressing the expression of tumor suppressor genes or oncogenes [15,16].

Arrestin beta 1 (ARRB1), a member of the arrestin/beta-arrestin family, mediates the desensitization of G-protein-coupled receptors (GPCRs) [17]. After transducing signals, activated GPCRs are phosphorylated at the cytosolic portion by GPCR kinases (GRKs), which subsequently enables ARRB1 to bind to GPCRs [18]. This binding sterically blocks further interaction between GPCRs and G-protein, leading to inactivation of the receptor and termination of the signaling [19]. Interestingly, beyond the desensitization of GPCRs, ARRB1 can regulate signaling transduction of GPCRs because ARRB1 can serve as a scaffold that connects the activated GPCRs with a variety of intracellular kinases [20,21]. It has been found that ARRB1 regulates cellular mechanisms by directly interacting with several kinases, such as WNT, proto-oncogene tyrosine-protein kinase Src (SRC), AKT, and mitogen-activated protein kinases (MAPKs) [22–25]. Therefore, ARRB1 has been implicated in human disease, including cancer, and recent studies have demonstrated that dysregulation of ARRB1 expression promotes cancer phenotypes and poorer outcomes in many types of cancer [26–28].

In this study, miR-374a-5p was identified as a TNBC-specific miRNA; moreover, its function and the molecular mechanisms involved were investigated. miR-374a-5p was specifically upregulated in TNBC, compared with in other breast cancer subtypes; furthermore, suppression of miR-374a-5p attenuated cancer cell growth and migration, as well as tumor progression. Additionally, the aforementioned results revealed that miR-374a-5p directly targeted *ARRB1*, a downregulated gene in TNBC, and that *ARRB1* regulated cancer cell growth and migration, as well as were involved in AMPK activation. These results indicated that upregulation of miR-374a-5p contributed to tumor progression in TNBC by regulating *ARRB1* expression, suggesting that miR-374a-5p may serve as a novel therapeutic target for TNBC.

2. Materials and methods

2.1. Cell culture

MDA-MB-231, MDA-MB-468, MDA-MB-157, MCF7, SK-BR-3 cells were tested using short tandem repeat markers for DNA fingerprinting analysis (Korean Cell Line Bank, South Korea). ZR-75-1 and T47D cells were purchased from American Type Culture Collection (ATCC®, USA). HS578T, BT-20, HCC-1187, HCC-1395, and HCC-1937 cells were purchased from Korean Cell Line Bank. MDA-MB-468, MDA-MB-157, MDA-MB-231, HS578T, SK-BR-3, T47D, MCF7 cells, and HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; WelGENE, South Korea), and other cell lines were grown in Roswell Park Memorial Institute 1640 Medium (RPMI, WelGENE) with 10% fetal bovine serum (FBS, Gibco, USA) at 37 °C, 5% CO_2 atmosphere. For AMPK activation, pre-transfected cells were cultured in medium with different concentrations of glucose (0 or 25 mM) and 10% FBS for 9–18 h or in medium with different concentrations of AICA ribonucleotide (AICAR; LC laboratories, USA; 0 or 1 mM) and 10% FBS for 6 h.

2.2. Transfection

To regulate miRNA expression, cells were transiently transfected with 30 nM of miR-374a-5p inhibitor (Ambion) or negative control inhibitor (Ambion) using siPORT™ NeoFX™ transfection reagent (Ambion) as indicated by the supplier. Cells were transfected with *ARRB1* constructs (OriGene; Rockville, USA) or empty vector constructs using FuGENE® HD transfection reagent (Madison, USA) according to the manufacturer's instructions. For RNA interference of *ARRB1*, cells were transfected with 15–30 nM of *ARRB1* siRNA (Dharmacon, USA) or control siRNA (Dharmacon) using Lipofectamin® RNAiMAX Transfection Reagent (Invitrogen, USA) according to the manufacturer's recommendation. All transfection was conducted for 24–48 h.

2.3. Cell viability assay

Cell viability was measured using a WST-8 assay. TNBC cells were pre-transfected with miRNA, overexpression constructs, or siRNA for 24 h. After that, the cells were detached and plated again at a density of 6×10^4 cells/ml. Following cell attachment, WST-8 labeling mixture (Enzo Life Sciences, USA) was added to a single well of each group and incubated for 30 min at 37 °C. The absorbance at 450 nm was measured using a multi-well spectrophotometer (Synergy HTX, BioTek Instruments). Cell viability was measured for 4–5 days. Under normal conditions, cells were cultured in DMEM with 25 mM of glucose and 1% FBS, while glucose-free DMEM with 5% FBS was used for glucose starvation.

2.4. BrdU incorporation assay

Cells were pre-transfected with miRNA, overexpression constructs, or siRNA for 24 h, and then replated followed by further incubation for 24 h. Bromodeoxyuridine (BrdU) incorporation was measured using the Cell Proliferation ELISA, BrdU (colorimetric) Kit (Roche) according to the manufacturer's instruction, and the absorbance at 370 nm was measured using a multi-well spectrophotometer (BioTek Instruments).

2.5. Wound healing assay

Cells were pre-transfected with miRNA, overexpression constructs, or siRNA for 24 h and then replated at 100% confluence. After overnight incubation in 10% FBS media, confluent cells were scratched by manual scratching with a 1000 μl pipette tip, washed with PBS, and further incubated in FBS 0–5% media for 48 h.

2.6. Statistical analysis

All experiments were repeated more than three times independently. Statistical analyses were assessed using GraphPad Prism 5 software (GraphPad, USA). The data were analyzed by a two-tailed Student's t-test and values are presented as the mean ± Standard Deviation (SD). P-values < 0.05 were considered statistically significant. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

3. Results

3.1. TNBC-specific upregulation of miR-374a-5p

To identify potential miRNAs that could be used as specific molecular markers of TNBC, miRNA-microarray analysis was performed. First upregulated miRNAs were identified in the tumor tissues by comparison with the corresponding non-tumor lesions for each subtype breast cancer patients (Supplementary Fig. S1A). These tumor tissue-upregulated miRNAs were further compared across breast cancer subtypes to discover miRNAs that are more expressed in TNBCs than other subtypes, with 23 miRNAs displaying significantly upregulated expression in TNBC (Fig. 1A and Supplementary Fig. S1B).

Based on our analysis, miRNAs with fold-change values of more than 3 in TNBC tumor compared to those in adjacent normal tissues were profiled by TCGA dataset analysis using cBioPortal [29,30]. Among the selected miRNAs, hsa-miR-374a, the pre-miRNA of miR-374a-5p, had highly upregulated expression in basal-like patients, a result that was more significant when the expression was compared between TNBC and other subtypes (Fig. 1B). Moreover, miR-374a-5p

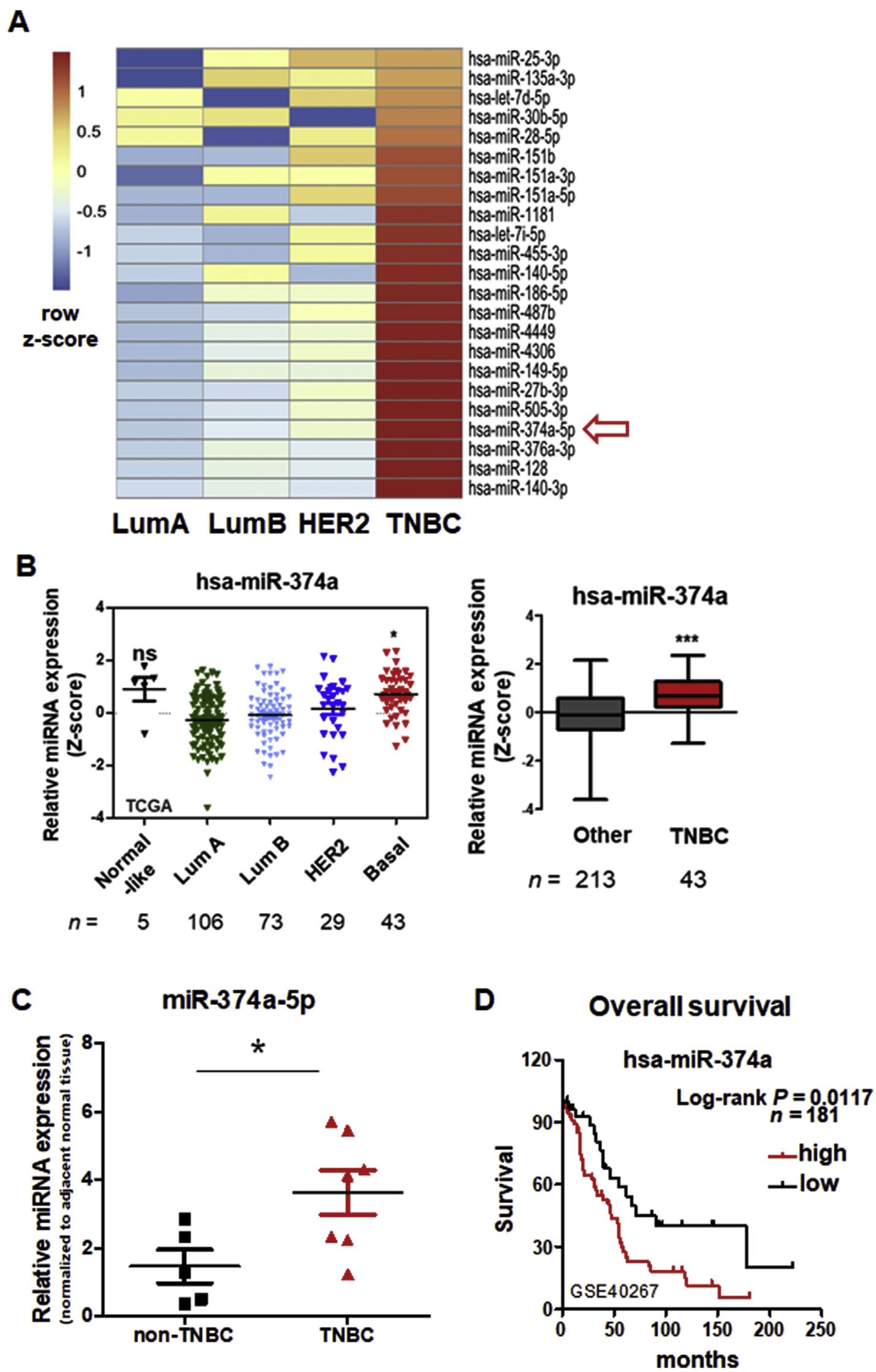


Fig. 1. miR-374a-5p is specifically upregulated in TNBC. (A) The heatmap of miRNAs with higher expression in the TNBC patients (n = 3) compared with other subtypes of patients (Lum A; n = 2, Lum B; n = 2, and HER; n = 3). Expression levels are represented by colored bars; higher (red) or lower (blue). (B) TCGA dataset analysis showing the expression profiles of hsa-miR-374a; left, the relative hsa-miR-374a expression across PAM50 subtypes and right, differential expression between TNBC and other subtypes. (C) qRT-PCR analysis showing the relative miR-374a-5p expression in the tissues of TNBC patients and other subtypes. miR-374a-5p level of tumor tissues was normalized to that of corresponding normal tissues in each breast cancer subtype and then miR-374a-5p levels of TNBC patients were normalized to that of patients with other subtypes. (D) Kaplan-Meier plots of breast cancer patients classified by the expression of *hsa-miR-374*. The graph data represent the mean ± SEM The graph data represent the mean ± SEM (*$P < 0.05$, ***$P < 0.001$; ns, non-significant difference).

expression in TNBCs patients was higher than in other types of cancer, supporting that miR-374a-5p might be a key miRNA in TNBC (Supplementary Fig. S1C). miR-374a-5p expression in tumor tissues was normalized to adjacent normal tissues and further compared across breast cancer subtypes. As expected, miR-374a-5p expression was increased about 3.5-fold in TNBC patients (Fig. 1C). Furthermore, Kaplan-Meier analysis, exclusively comprised of data from ER negative patients [31], revealed that breast cancer patients with high expression of *hsa-miR-374a* had significantly worse overall survival rates compared with the rest of the cohort (Fig. 1D). Collectively, these results suggest that miR-374a-5p is specifically upregulated in TNBC in comparison with other breast cancer subtypes. Furthermore, since miR-374a-5p has barely been studied in TNBC, miR-374a-5p was selected as a potential TNBC-specific molecular marker and conducted further experiment.

3.2. miR-374a-5p promotes cell survival, proliferation, and migration in TNBC cells

To explore the function of miR-374a-5p in TNBC, the biological effect of miR-374a-5p suppression in TNBC cell lines was investigated. miR-374a-5p expression was suppressed in human TNBC cells, using anti-miR-374a-5p inhibitor (Fig. 2A), and the cellular processes were assessed, including cell survival, proliferation, and migration. miR-374a-5p suppression impaired TNBC cell growth, demonstrated by a relative decrease in cell viability and BrdU incorporation, or cell proliferation in miR-374a-5p knockdown cells (Fig. 2B and C). In addition,

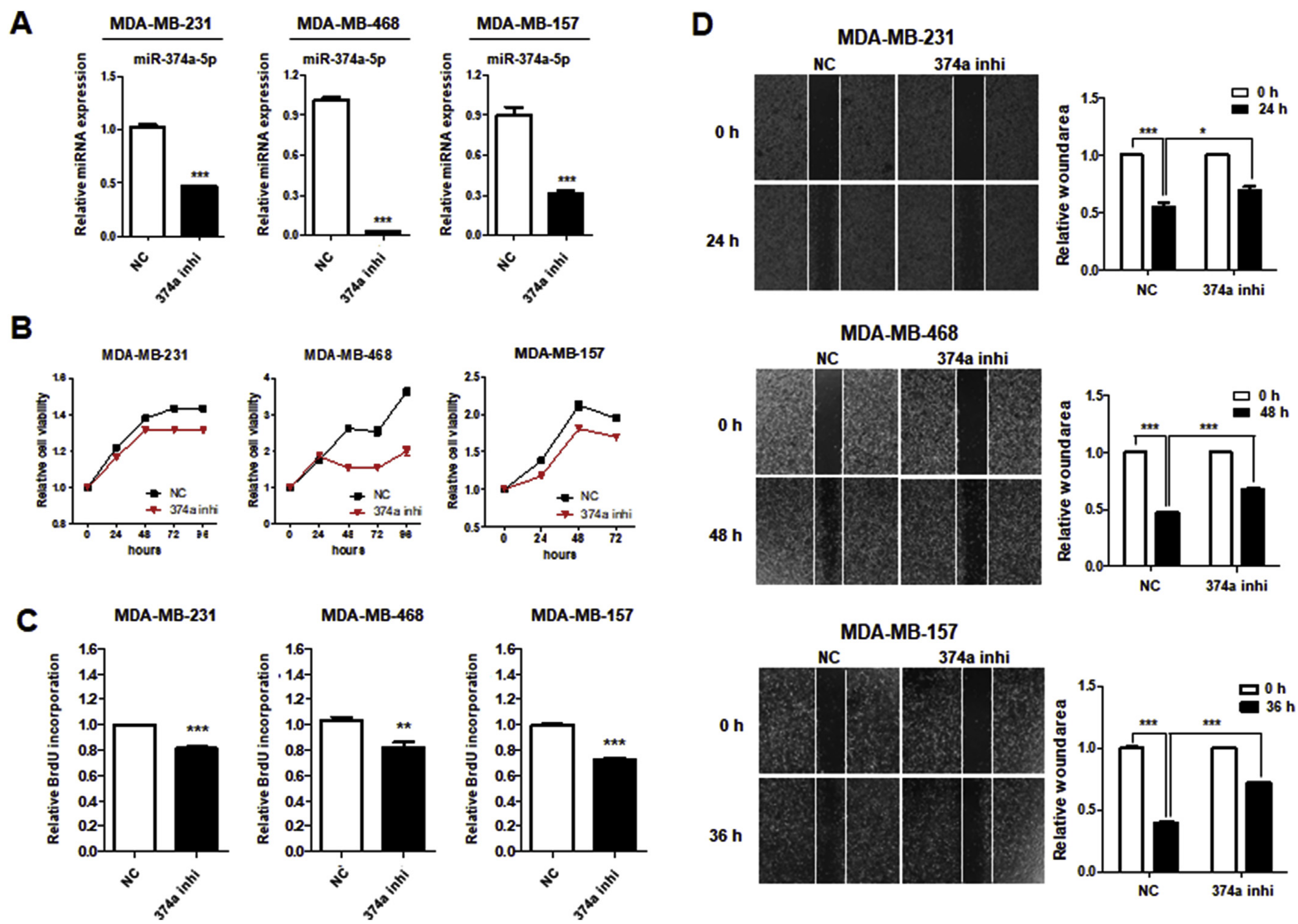


Fig. 2. Knockdown of miR-374a-5p attenuates cell survival, proliferation, and migration in TNBC cell lines. (A) qRT-PCR analysis showing miR-374a-5p knockdown efficiency in TNBC cell lines after the transfection with negative control (NC) or miR-374a-5p inhibitor (374a inhi). (B) Cell viability of the control and miR-374a-5p-knockdowned cells. Pre-transfected cells were cultured in DMEM with 1% FBS, and the cell viability was determined using WST8 reagent. (C) Relative BrdU incorporation was measured 48 h after cells were transfected with NC or miR-374a-5p inhibitor. (D) Wound-healing assay conducted in NC or miR-374a-5p inhibitor-transfected cells. Representative images at indicated time point (× 40) and bar graphs of the relative wound area are shown. The graph data represent the mean ± SEM (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, non-significant difference; n = 3 independent experiments).

the wound closure rate of miR-374a-5p-suppressed cells was decreased in comparison with the control, suggesting that miR-374a-5p inhibition attenuated the migration ability of TNBC cells (Fig. 2D). These results indicate that miR-374a-5p knockdown suppresses the cell survival, proliferation, and migration of TNBC cells, implying that high expression of miR-374a-5p might be important for cellular cancer mechanisms in TNBC cells.

3.3. miR-374a-5p induces tumor progression in vivo

To further explore the effect of miR-374a-5p suppression on tumor progression, MDA-MB-231 cells stably expressing anti-miR-374a-5p inhibitor or negative control were established using lentiviral vector (Fig. 3A). These cells were subcutaneously injected into nude mice to generate xenograft mouse models with differential expression of miR-374a-5p (Fig. 3B). As expected, the downregulation of miR-374a-5p dramatically suppressed tumor growth in the xenograft mice, evidenced by a decrease in weight, volume, and size of the tumors (Fig. 3C–E). Additionally, immunohistochemistal analysis indicated that the level of cell proliferating marker Ki-67 was decreased in miR-374a-5p-suppressed tumors relative to the control tumors (Fig. 3F). These results indicate that miR-374a-5p downregulation attenuated tumor progression *in vivo,* supporting that miR-374a-5p might function as an oncomiR in TNBC.

3.4. miR-374a-5p directly targets ARRB1, which is specifically downregulated in TNBC

The previous results prompted the identification of downstream targets of miR-374a-5p in TNBC. A total of 5118 candidate genes retaining miR-374a-5p-binding sequences were discovered using TargetScan (http://www.targetscan.org). We identified 30 expected target genes with miR-374a-5p-binding sequence, which were downregulated in adjacent normal tissues versus TNBC tissue by using GSE113865 dataset. By comparison to other carcinomas and other breast cancer subtypes, we finally selected 4 genes that were especially downregulated in TNBC (Supplementary Fig. S2A). The luciferase activity of the 3′UTR construct of *ARRB1*, one of the 4 candidate genes, was highly attenuated by miR-374a-5p overexpression, whereas that of the 3′UTR of the other candidate genes was not decreased by miR-374a-5p (Fig. 4B and Supplementary Fig. S2B). Fig. 4a illustrates the sequence of miR-374a-5p and the complementary binding site in the 3′UTR of *ARRB1*. To further verify the post-transcriptional regulation of *ARRB1* by miR-374a-5p, the effect of miR-374a-5p suppression was assessed in TNBC cell lines. As expected, the RNA and protein levels of ARRB1 were enhanced under miR-374a-5p downregulation (Fig. 4C). However, the expression of the other candidate target genes were hardly changed (Supplementary Fig. S2C). Furthermore, *ARRB1* expression was markedly higher in the miR-374a-5p-suppressed tumors of

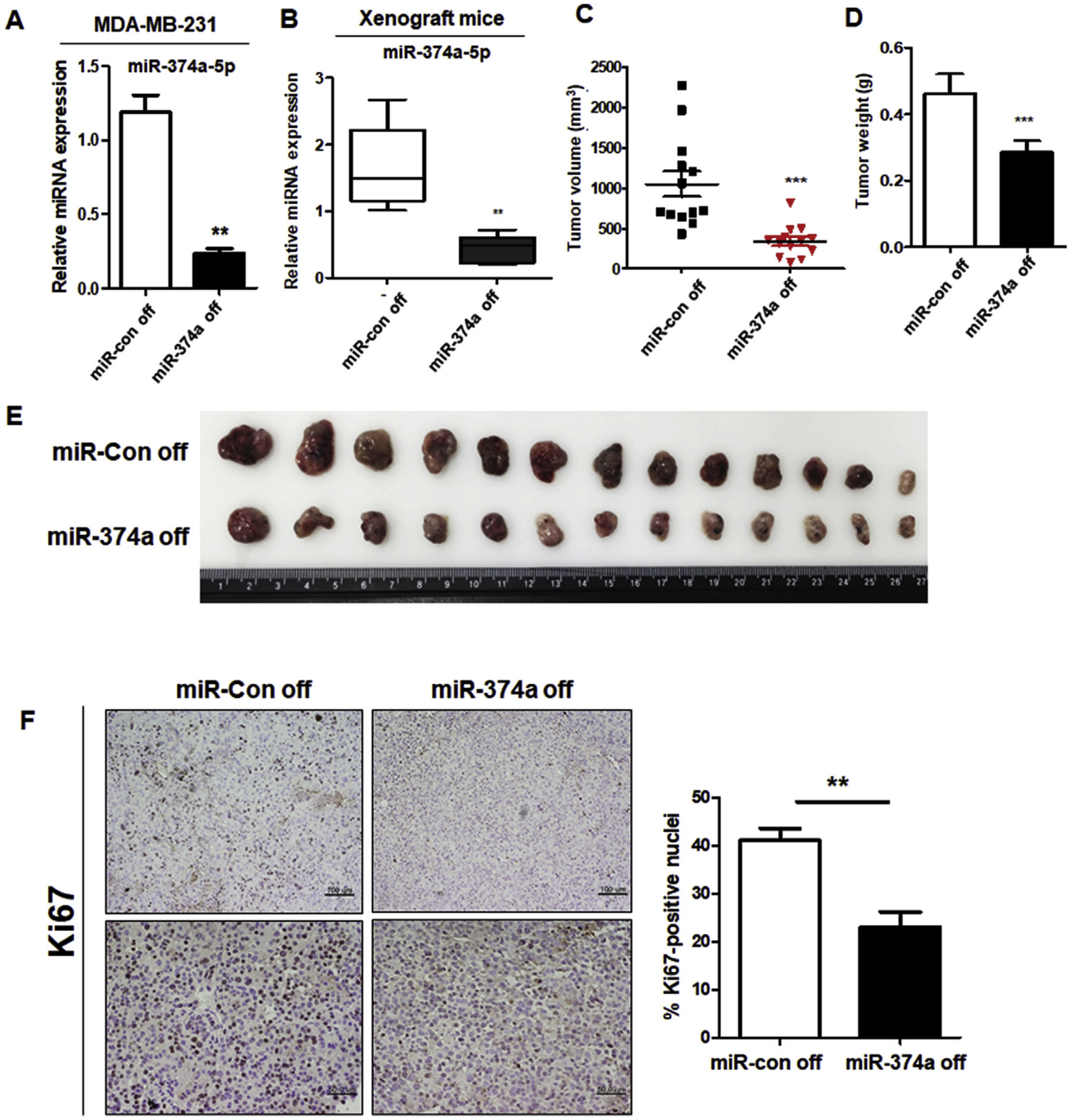


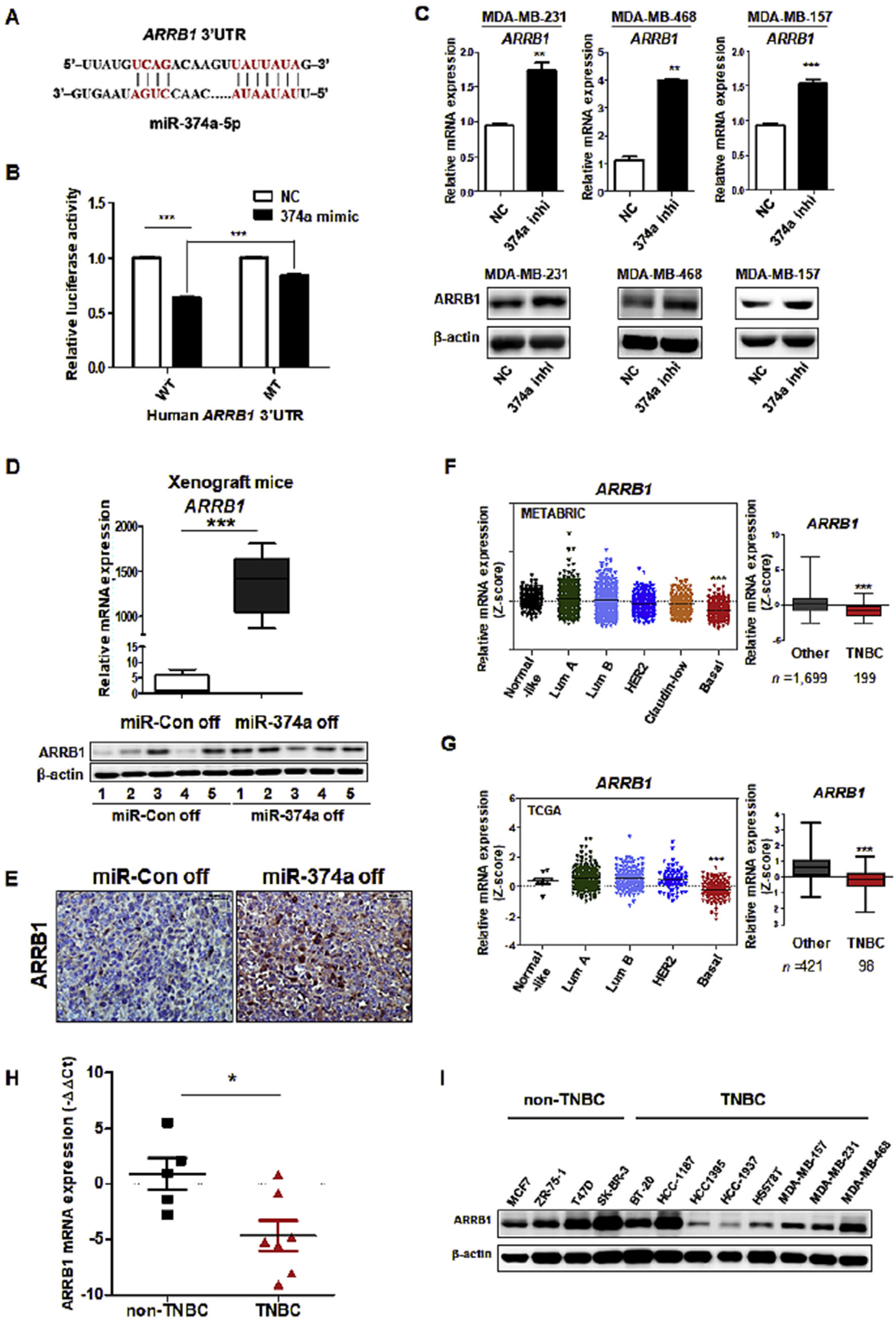
Fig. 3. miR-374a-5p knockdown inhibits tumor growth *in vivo*. (A) The expression of miR-374-5p expression in lenti-miR-control cells and lenti-miR-374-5p inhibited cells (B) Relative miR-374a-5p expression in control tumors and miR-374a-5p suppressed tumors of xenograft mice (n = 5 per group). (C and D) Graphs showing the decrease in tumor volume and weight in miR-374a-5p-suppressed tumors compared with the control. (E) The image of actual tumors isolated from xenograft mice after the sacrifice(F) Representative images showing immunohistochemically staining with anti-Ki67 in miR-374a-5p-knockdowned or control tumors (left) Bars: upper, 100 μm; bottom, 50 μm Ki67-positive cells (%) in two groups (right). (**P < 0.01, ***P < 0.001; ns, non-significant difference; n = 3 independent experiments).

xenograft mice compared with the control tumors (Fig. 4D). In addition, ARRB1 expression was highly upregulated in miR-374a-5p inhibited xenograft samples (Fig. 4E). These results supported that *ARRB1* was regulated by miR-374a-5p. Taken together, these data suggested that *ARRB1* was a direct target of miR-374a-5p.

Since we previously confirmed that miR-374a-5p was upregulated in TNBC, the *ARRB1* expression patterns were assessed in breast cancer patients using human genomic datasets. As shown from the relative *ARRB1* expression levels classified into PAM50 subtypes using the METABRIC [32] and TCGA datasets (Fig. 4F and G), *ARRB1* was markedly downregulated in the basal-like patients in both datasets. Moreover, the METABRIC dataset showed that *ARRB1* expression in claudin-low subtype patients was relatively decreased compared with other subtypes except from basal-like patients. A substantial 61–71% of claudin-low cases belong to TNBC subtype and 25–39% of TNBC tumors are claudin-low subtype [33], which is probably the reason why *ARRB1* was downregulated in claudin-low subtype as well. Likewise, The *ARRB1* expression was significantly reduced in TNBC patient in comparison with non-TNBC (Fig. 4H). Finally, the ARRB1 protein level was markedly decreased in TNBC cell lines compared with other subtypes (Fig. 4I). Taken together, these results demonstrated that *ARRB1*, the target of miR-374a-5p, is specifically downregulated in TNBC and has a negative correlation with miR-374a-5p expression.

3.5. ARRB1 is involved in AMPK activation

To investigate the underlying mechanism of ARRB1 as a tumor suppressor, we considered ARRB1 might interact with tumor suppressive kinases, especially AMP-activated protein kinase (AMPK) because ARRB1 was reported to regulate cellular process by interacting with



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variety of kinases. As shown in Fig. 5A, ARRB1 overexpression significantly enhanced glucose starvation-induced AMPK activation in MDA-MB-231 cells. More specifically, AMPK activation was barely induced by energy starvation in empty vector-transfected cells, while ARRB1 overexpression enhanced the phosphorylation of AMPK even under normal conditions. In addition, endogenous ARRB1 expression

Fig. 4. *ARRB1* is a direct target of miR-374a-5p and downregulated in TNBC. (A) The sequence alignment of miR-374a-5p and the 3′UTR of *ARRB1* containing binding site identified by TargetScan. (B) Luciferase activity of the wild-type or mutant 3′UTR construct of *ARRB1* in HEK293T cells after the transfection with miR-374a-5p mimic (374a mimic) or NC. (C) qRT-PCR and western blotting analysis showing that mRNA and protein expression of ARRB1 were increased after the transfection with miR-374a-5p inhibitor in TNBC cells. (D) qRT-PCR and western blotting analysis to compare ARRB1 expression between miR-374a-5p-suppressed tumors and the control isolated from xenograft mice. (E) The representative images showed staining of ARRB1 in xenograft models, comparing control tumor with miR-374a-5p inhibited tumor. The scare bar represented 50 μm. (F) *ARRB1* expression profiles analyzed with METABRIC dataset. *ARRB1* expression across the PAM50 subtypes (left) or between TNBC and other breast cancer subtypes (right). The sample sizes were as follows: Normal-like, n = 140; Lum A, n = 679; Lum B, n = 461; HER2, n = 220; Claudin-low, n = 199; Basal, n = 199. (G) *ARRB1* expression profiles analyzed with TCGA dataset as in (F). The sample sizes were as follows; Normal-like, n = 8; Lum A, n = 230; Lum B, n = 125; HER2, n = 58; Basal, n = 96. (H) qRT-PCR analysis showing the relative *ARRB1* expression in the tissues of TNBC patients and other subtypes. ARRB1 mRNA expression of tumor tissues was normalized to that of corresponding normal tissues in each breast cancer subtype and then miR-374a-5p levels of TNBC patients were normalized to that of patients with other subtypes (I) Western blotting analysis showing ARRB1 protein levels of human breast cancer cell lines. β-actin was used as the loading control in all western blotting analysis. The graph data represent the mean ± SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ns, non-significant difference; n = 3 independent experiments).

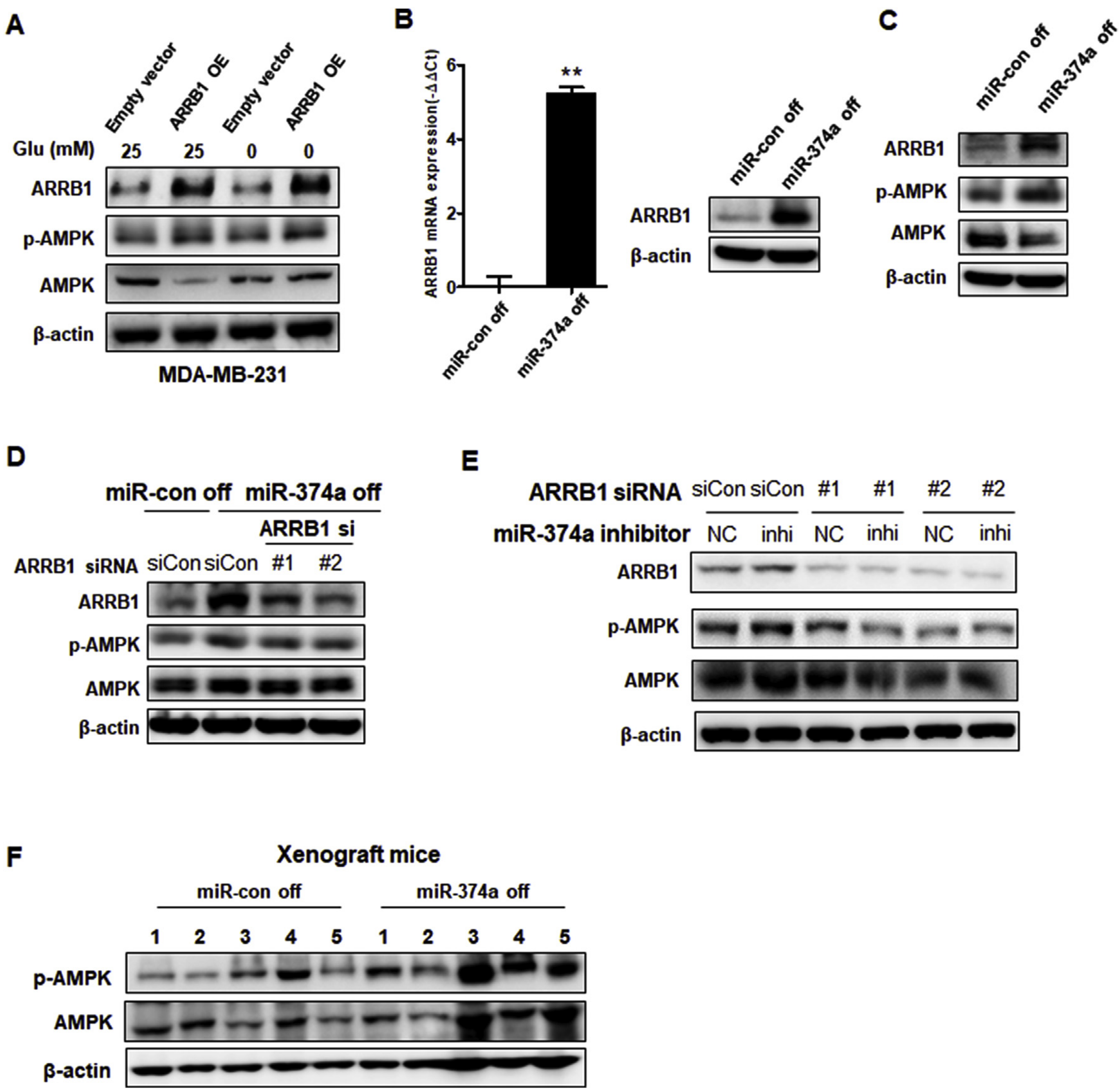

Fig. 5. ARRB1 is involved in AMPK activation. (A) TNBC cells were transfected with empty vector or ARRB1 constructs and further cultured in culture medium containing 25 or 0 mM of glucose (Glu) for 9 h. Cell lysates were subjected to western blotting analysis. (B) The ARRB1 mRNA and protein expression in stably miR-374a-5p inhibited MDA-MB-231 cells. (C) Western blotting analysis of ARRB1 phosphorylation (Thr172) in stably miR-control or miR-374a-5p inhibited MDA-MB-231 cells. (D) The AMPK phosphorylation by treatment with ARRB1 siRNA or control siRNA (siCon) in miR-374a-5p stably reduced cells or control cells. (E) The ARRB1, p-AMPK and AMPK expression was analyzed by western blotting, treating miR-374a-5p inhibitor (inhi) or negative control (NC) after that incubating ARRB1 siRNA or control siRNA. (F) Phosphorylated AMPK expression was analyzed by western blotting between miR-374a-5p-suppressed tumors and the controls isolated from xenograft mice. The graph data represent the mean ± SEM (**$P < 0.01$; n = 3 independent experiments).

was suppressed in TNBC cells using ARRB1-targeting siRNA, which resulted in the attenuation of AMPK activation under glucose starvation (Supplementary Fig. S3A). Furthermore, the phosphorylation of AMPK was suppressed when ARRB1 was suppressed even under treatment with AICAR (AICA ribonucleotide, an AMPK activator [34]) in MDA-MB-231 cells when *ARRB1* was suppressed (Supplementary Fig. S3B).

Next, to demonstrate reduced expression of ARRB1 by targeting miR-374a-5p in TNBC, we confirmed the phosphorylation of AMPK in

MDA-MB-231 cells stably suppressed miR-374a-5p as described in Fig. 3A. The ARRB1 mRNA and protein expression was highly upregulated and phosphorylated AMPK levels also increased in stably miR-374a-5p suppressed (Fig. 5B and C). Moreover, the reduction of the AMPK phosphorylation when treated siRNA that target ARRB1 but not the 3′UTR was observed in cells that stably suppressed expression of miR-374a-5p (Fig. 5D). It was also observed that the activation of AMPKα was reduced upon treatment with ARRB1 siRNAs (Supplementary Fig. 3C). In addition, activated phosphorylation of AMPK was suppressed by treatment with ARRB1 siRNA, despite transient inhibition of miR-374a in MDA-MB-231 cells (Fig. 5E). These results indicated that the phosphorylation of AMPK was regulated by the axes of miR-374a-5p/ARRB1 in TNBC cells. Additionally, the protein level of phosphorylated AMPK was assessed in the tumors of xenograft mice, as described in Fig. 4D, and phosphorylated AMPK was found to be increased in miR-374a-5p-suppressed tumors (Fig. 5F). These results suggest that ARRB1 is involved in AMPK activation which further implies that AMPK might be an important mediator of the function of ARRB1 and ARRB1 might function as a tumor suppressor in TNBC.

3.6. ARRB1 is involved is involved in TNBC prognosis in suppression of cell survival, proliferation, and migration

As mentioned above, ARRB1 was assumed to serve as a tumor suppressor in TNBC considering that ARRB1 was highly downregulated in TNBC. For validation, whether the upregulation of ARRB1 attenuated cancer cellular mechanisms was examined. Transfection with constructs containing *ARRB1* significantly enhanced ARRB1 levels in TNBC cell lines (Supplementary Fig. S4A). The previous data indicated that AMPK activation was affected by the regulation of *ARRB1* expression. Thus, the effect of *ARRB1* overexpression on cellular mechanisms was assessed, both under normal conditions and glucose-free conditions where AMPK was activated. The viability and proliferation of TNBC cell lines were relatively suppressed by ARRB1 overexpression both under normal and glucose-free conditions (Fig. 6A and B), implying that upregulation of ARRB1 inhibited cancer cell growth. In addition, a wound healing assay was performed (migration ability was assessed only under normal conditions since the cells did not migrate under glucose-starvation regardless of the expression of ARRB1), and cell migration was significantly suppressed under ARRB1 overexpression (Fig. 6C). These results suggest that upregulation of ARRB1 might inhibit tumor suppression in TNBC. For further validation, the effects of ARRB1 downregulation were assessed in TNBC cell lines. As expected, cell proliferation and migration were more extensively facilitated when *ARRB1* was knocked down (Supplementary Figs. S4C–D). Next, *ARRB1* expression was profiled among the histological grades of breast cancer using the METABRIC dataset, and the analysis demonstrated that *ARRB1* expression was inversely correlated with a high histological grade (Fig. 6D). Finally, Kaplan-Meier analysis showed that TNBC patients with low expression of *ARRB1* had markedly worse overall survival and distant metastasis free survival (DMFS) than those with high *ARRB1* expression (Fig. 6E). Taken together, these results indicate that ARRB1 might function as a tumor suppressor in TNBC.

4. Discussion

Breast cancer is one of the most common type of cancer leading to 14% of cancer deaths each year [35]. The major cause of the mortality of breast cancer is the heterogeneity of the disease. Molecular, histological, and clinical features are different depending on the breast cancer subtypes so understanding and targeting the specific characteristics of each subtype is required for effective therapies. However, therapeutic options for advanced TNBC remain limited as there is a lack of endocrine receptors to determine the prognosis and therapeutic responses and the genomic aberrations are poorly understood [12,36]. Therefore, the identification of TNBC-specific molecular markers is required for effective diagnosis and targeted therapies.

The present study discovered miR-374a-5p upregulated in TNBC. hsa-miR-374a has been reported to be upregulated in several types of cancer, such as gastric cancer, ovarian cancer and breast cancer [37–41], but the specific functions of miR-374a in breast cancer remains to be determined. In this work, miRNA expression profiling was performed in breast cancer patient tissues, finding that miR-374a-5p was significantly upregulated in TNBC tissues compared with other subtype tissues. A transcriptome data analysis using the TCGA dataset also supported that TNBC patients have markedly higher expression of miR-374a-5p than other subtypes of patients. These findings demonstrate the availability of miR-374a-5p as a molecular marker of TNBC. Moreover, the functional analysis of miR-374a-5p using TNBC cell lines and mouse models revealed that miR-374a-5p suppression inhibited tumor progression and growth, indicative of functions as a TNBC-specific oncomiR. These results are consistent with recent reports that miR-374a promotes cancer cell proliferation and invasion in gastric and esophageal cancers [42,43]. One persisting question is whether miR-374a-5p plays an oncogenic role in other subtypes of breast cancer, seeing as they had relatively low expression of miR-374a-5p compared with TNBC. Therefore, further investigation using the appropriate models for each subtype will be required to more specifically define the functions of miR-374a-5p in breast cancer. Instead, the current study using *in vitro* and *in vivo* TNBC models confirmed that miR-374a-5p promotes cancer progression, at least in TNBC.

This study also identified the molecular mechanism underlying miR-374a-5p function in TNBC by discovering a direct target gene, *ARRB1*. Recently, *ARRB1* has been reported to be upregulated and to promote cancer cell growth and metastasis in some cancer types [23,25,44,45]. Meanwhile, reduced expression of ARRB1 has been reported to be associated with the initiation of the production of cancer stem cells and poor overall survival in some cancers [46,47]. In the present study, several transcriptome dataset analyses demonstrated that *ARRB1* is significantly downregulated in TNBC. The overexpression of *ARRB1* attenuated the growth and migration of TNBC cell lines, suggesting that *ARRB1* is a potential tumor suppressor in TNBC. Moreover, the strong correlation between the low expression of *ARRB1* and the poor prognosis of TNBC patients also supports the tumor-suppressive role of ARRB1. Notably, the presented study showed that *ARRB1* expression was associated with glucose starvation-induced AMPK activation in TNBC cell lines, suggesting that normal *ARRB1* expression might be crucial for AMPK activation. Recent studies reported that phosphorylated AMPK is downregulated in breast cancer specimens and is inversely correlated with the histological grade [48,49]. This study showed that phosphorylated AMPK levels were positively correlated with ARRB1 protein levels in the xenograft mice (Fig. 5F) and that *ARRB1* expression was inversely correlated with the histological grade of breast cancer (Fig. 6E). These results prompted the assumption that dysregulation of AMPK activation in breast cancer might by caused by downregulation of *ARRB1*. Also, considering that AMPK attenuates tumor progression in many types of cancer [50,51], AMPK might be an important mediator of the tumor-suppressive functions of ARRB1, which is exemplified by that ARRB1 downregulation enhanced TNBC cell viability only under glucose starvation where AMPK is activated. Moreover, by treatment with ARRB1 siRNA in miR-374a-5p stably suppressed cells, activated AMPK phosphorylation was decreased (Fig. 5D), implying a more specific molecular mechanism for miR-374a-5p is involved in TNBC —the miR-374a-5p/ARRB1/AMPK axis. Since the specific mechanisms underlying the relationship between ARRB1 and AMPK are still unknown, further studies on ARRB1 regulatory mechanisms in AMPK activation are required.

In conclusion, this study showed that miR-374a-5p promoted tumor progression in TNBC by regulating *ARRB1*. Integrative miRNA expression profiling of microarrays and analysis of human genomic dataset revealed the upregulation of miR-374a-5p and downregulation of *ARRB1* in TNBC. Moreover, functional studies and survival analyses

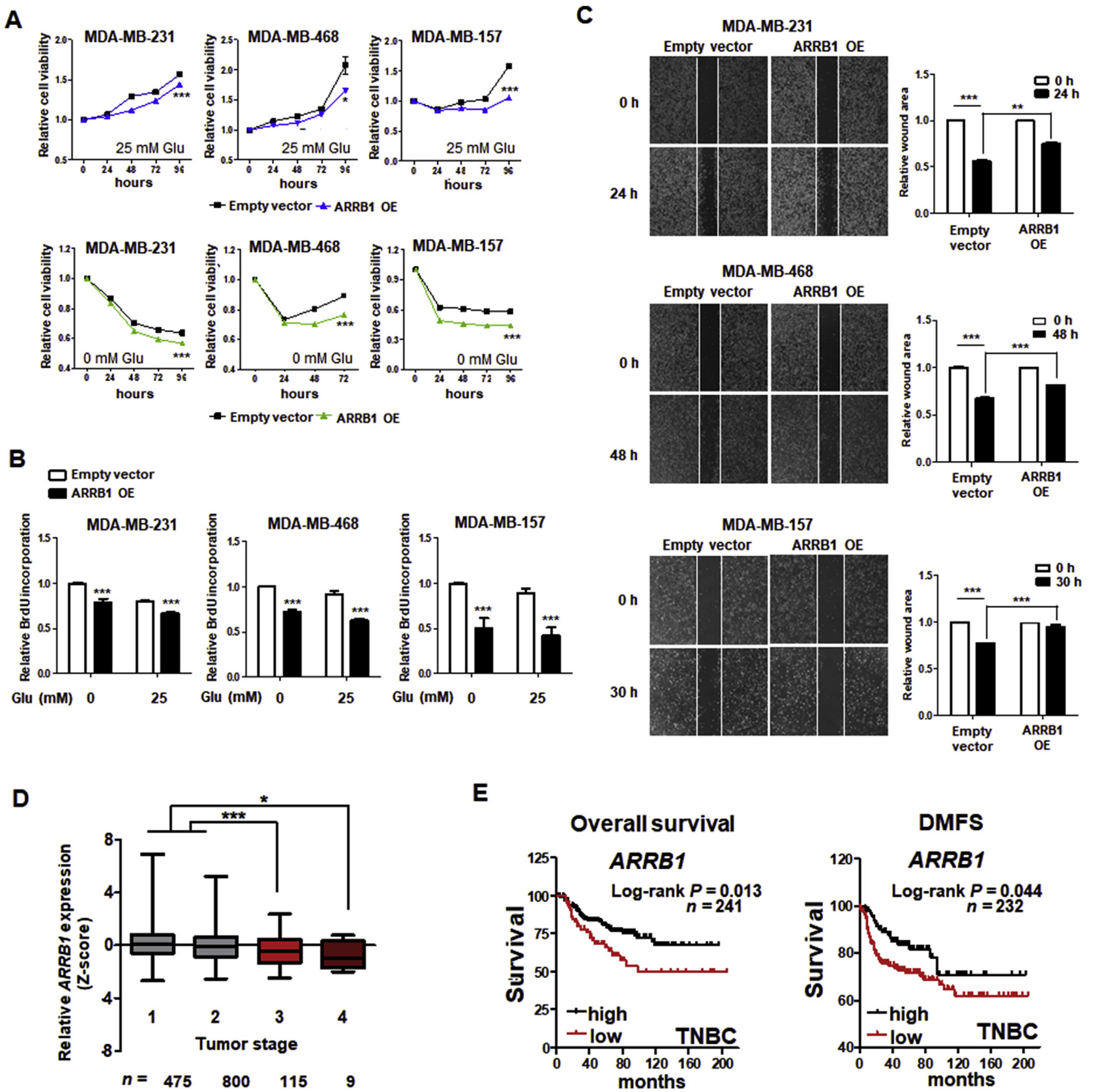

Fig. 6. ARRB1 regulates cell survival, proliferation, migration and is involved in prognosis of TNBC patients. (A) ARRB1-overexpressing cells and the control cells were cultured in DMEM with 25 (up) or 0 mM (down) of glucose and the cell viability was measured at indicated time point. (B) TNBC cells expressing ARRB1 or empty vector constructs were cultured in DMEM with 25 or 0 mM of glucose for 18 h and then subjected to BrdU incorporation assay. (C) Wound-healing assay was performed with ARRB1-overexpressing cells or the control cells. Representative images (× 10) and bar graphs showing the relative wound area at indicated time point are shown. (D) Relative *ARRB1* mRNA expression of the clinical tumor stage of breast cancer patients from METABRIC dataset. (E) Kaplan-Meier plots showing the overall survival and distant-metastasis free survival (DMFS) rates compared between TNBC patients with high and low expression of *ARRB1*. The graph data represent the mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001; ns, non-significant difference; n = 3 independent experiments).

provided evidence of the oncogenic function of miR-374a-5p and the tumor-suppressive role of *ARRB1* in TNBC. Taken together, these results suggested that miR-374a-5p may serve as a novel prognostic marker and therapeutic target for TNBC.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.04.006.

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