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ARTICLE

miR-1204 targets VDR to promotes epithelial-mesenchymal transition and metastasis in breast cancer

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Abstract

Plasmacytoma variant translocation 1 (PVT1) is an lncRNA that plays vital roles in breast cancer (BC) pathogenesis. Increasing evidence suggests that miRNAs that reside in the PVT1 locus are the main driver of the oncogenic roles of PVT1 in cancer. However, the oncogenic role and underlying mechanism of miR-1204, located in the PVT1 locus, in human cancer is still unclear. In this study, we discovered that increased expression of miR-1204 is associated with poor prognosis in BC. Moreover, miR-1204 promotes proliferation, epithelial-mesenchymal transition and invasion of BC cells both in vitro and in vivo. Mechanistic investigations demonstrated that VDR is a novel target gene of miR-1204. Interference of VDR restored miR-1204-mediated BC cell proliferation, tumorigenesis, and metastasis. Collectively, our results demonstrated that the miR-1204-VDR pathway exerts oncogenic effects in BC with potential therapeutic applications in blocking BC development and progression.

Introduction

Breast cancer (BC) is the second leading cause of cancer death in women [1]. Although many dysregulated molecular pathways have been identified in BC, the extensive transcriptional and genomic heterogeneity has limited the development of broad therapeutic strategies for treating BC patients [2]. Therefore, identifying new therapeutic molecules that can suppress the multiple pathways supporting BC growth would be important clinical breakthroughs.

MicroRNAs (miRNA) are small non-coding RNA molecules that function as 19 to 22 nucleotide species by binding to the 3 untranslated region (3′-UTR) of target mRNA [3]. miRNA induces target mRNA degradation in a sequence complementarity-dependent fashion and/or inhibition of protein synthesis [4, 5]. There are two types of miRNA targets: mRNAs with near perfect complementarity to the miRNA and mRNAs with limited complementarity to the miRNA [6, 7]. miRNAs function in diverse physiological processes and an increasing number of studies have proven that miRNAs play vital roles in carcinogenesis by regulating gene expression through mechanisms including deletion, amplification, mutation of miRNA locus, and epigenetic silencing of miR-RNA target genes [8–11].

Genomic amplification at chromosome 8q24 is one of the most frequent genomic abnormalities in human cancers

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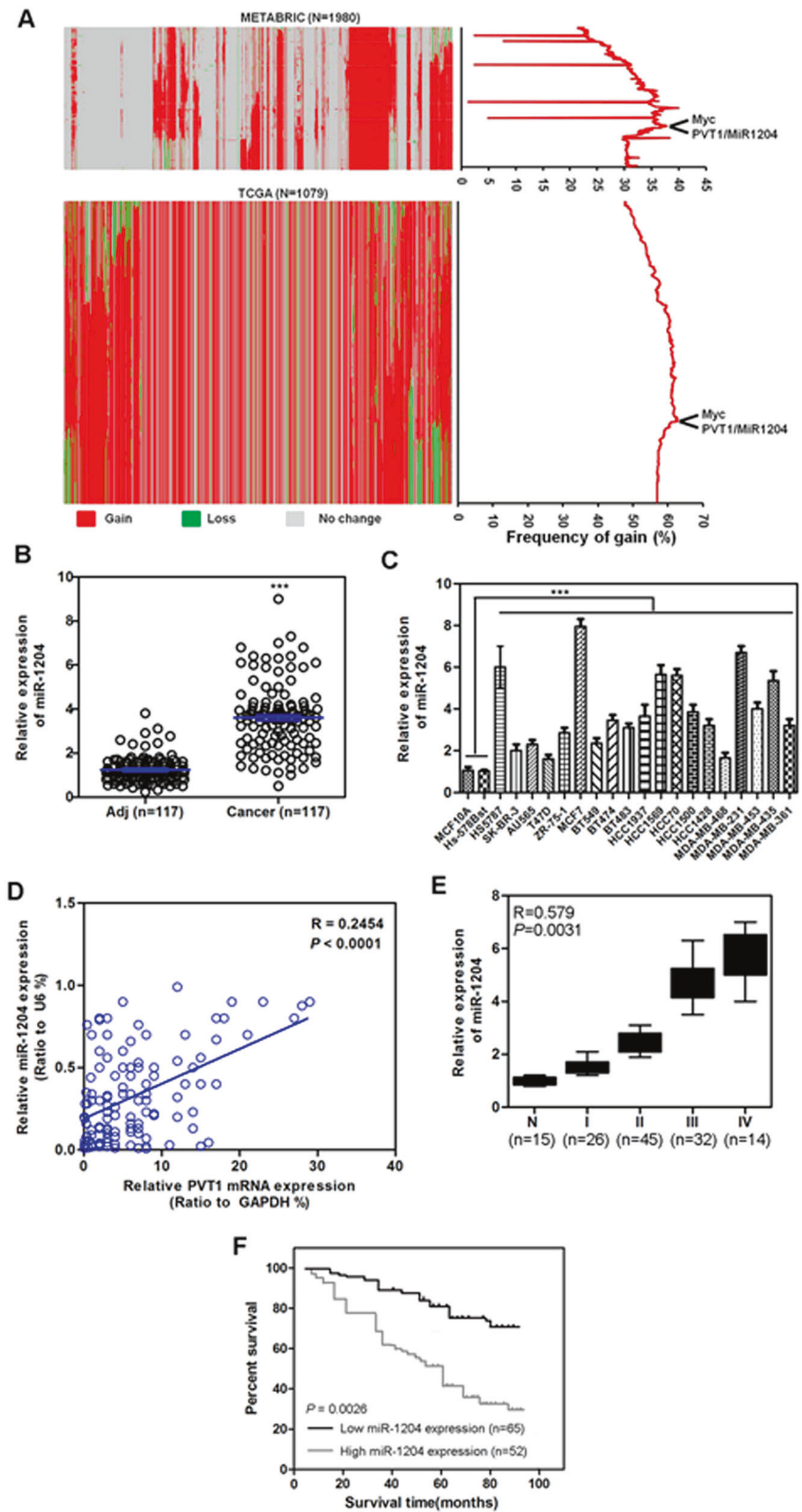


Fig. 1 Higher level of miR-1204 is associated with poor prognosis in BC. **a** The region of 8q24 near miR-1204 is commonly amplified in human breast cancer. **b** The miR-1204 expression levels in 117 paired BC tissues and corresponding non-tumor tissues were measured by qRT-PCR (Each bar represents the mean ± SD of three independent experiments. ***$P < 0.001$). **c** The miR-1204 expression levels in 19 BC cell lines and normal breast epithelial cells (MCF-10A and Hs-578Bst) were measured by qRT-PCR. **d** A significant positive correlation between the levels of miR-1204 and PVT1 expression was observed in BC specimens ($P < 0.0001$). **e** Increased miR-1204 expression correlated with increased BC differentiation. **f** Kaplan–Meier curves of breast cancer with low- versus high expression of miR-1204 ($n = 117$; $P = 0.0028$, log-rank test). **b** and **c** Transcript levels were normalized to U6 expression. Each bar represents the mean ± SD of three independent experiments. *** $P < 0.001$

[12, 13]. Consistently, the region syntenic to 8q24 in the mouse genome is a common site of tumorigenic retroviral insertion in lymphomas and a site of recurrent translocation in plasmacytomas [14]. The PVT1 locus is located in this region and has been implicated in cancer pathophysiology [15–17]. However, it is now becoming apparent that this

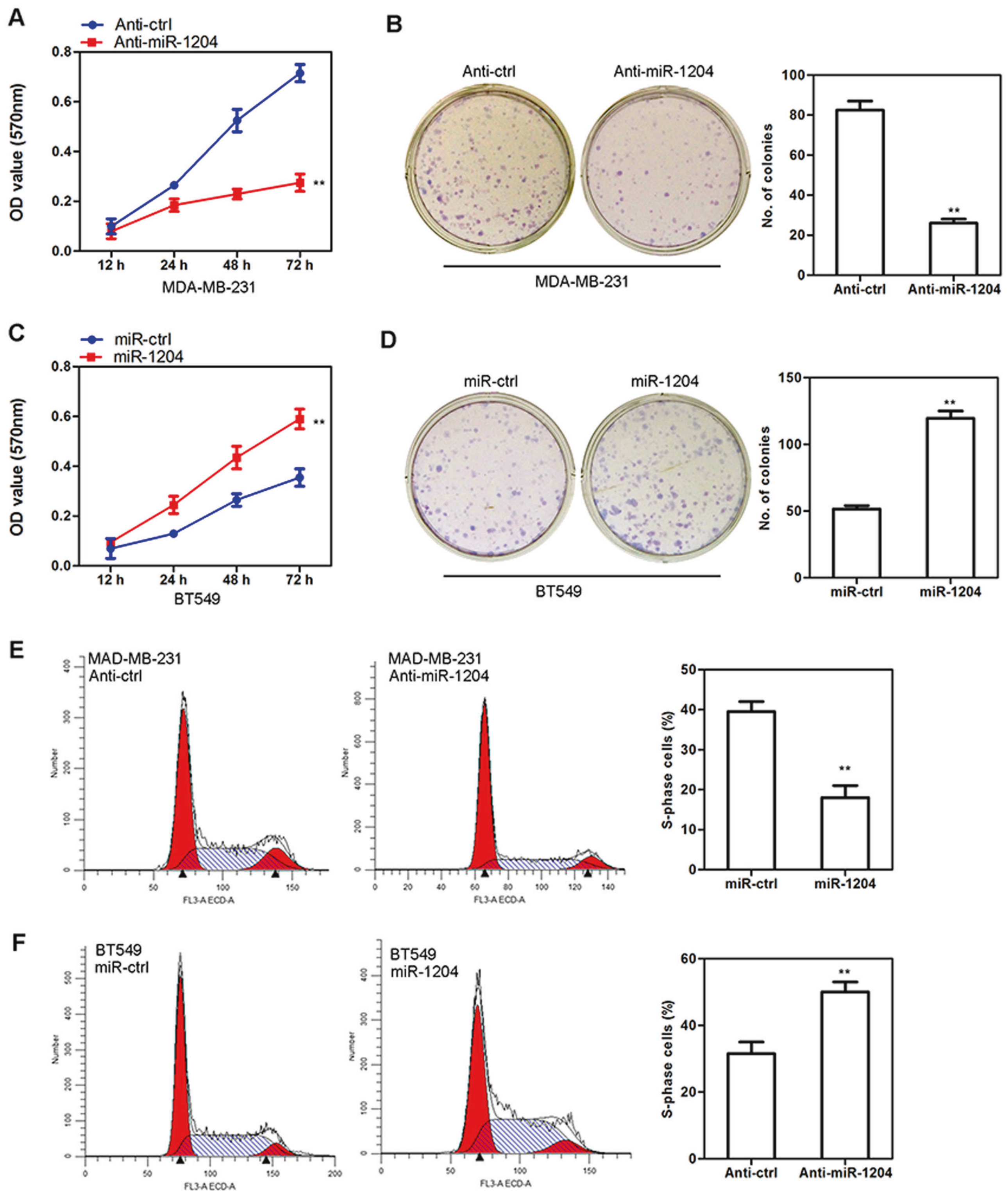


Fig. 2 miR-1204 promotes breast cancer cell proliferation in vitro. **a**–**d**, cell proliferation was examined by MTT (**a** and **c**) and colony formation (**b** and **d**) assays. F and F, Effect of miR-1204 on BC cell cycle. MDA-MB-231(**e**) and BT549 (**f**) cells transfected with anti-miR-1204 or miR-1204 and theirs control for 72 h were used in cell-cycle analysis upon propidium iodide staining. Shown are representative histograms (left) and quantification of the S-phase cells (right; mean ± SD). Error bars represent the mean ± SD of three independent experiments, with each conducted in duplicate. In all experiments, ** $P < 0.01$

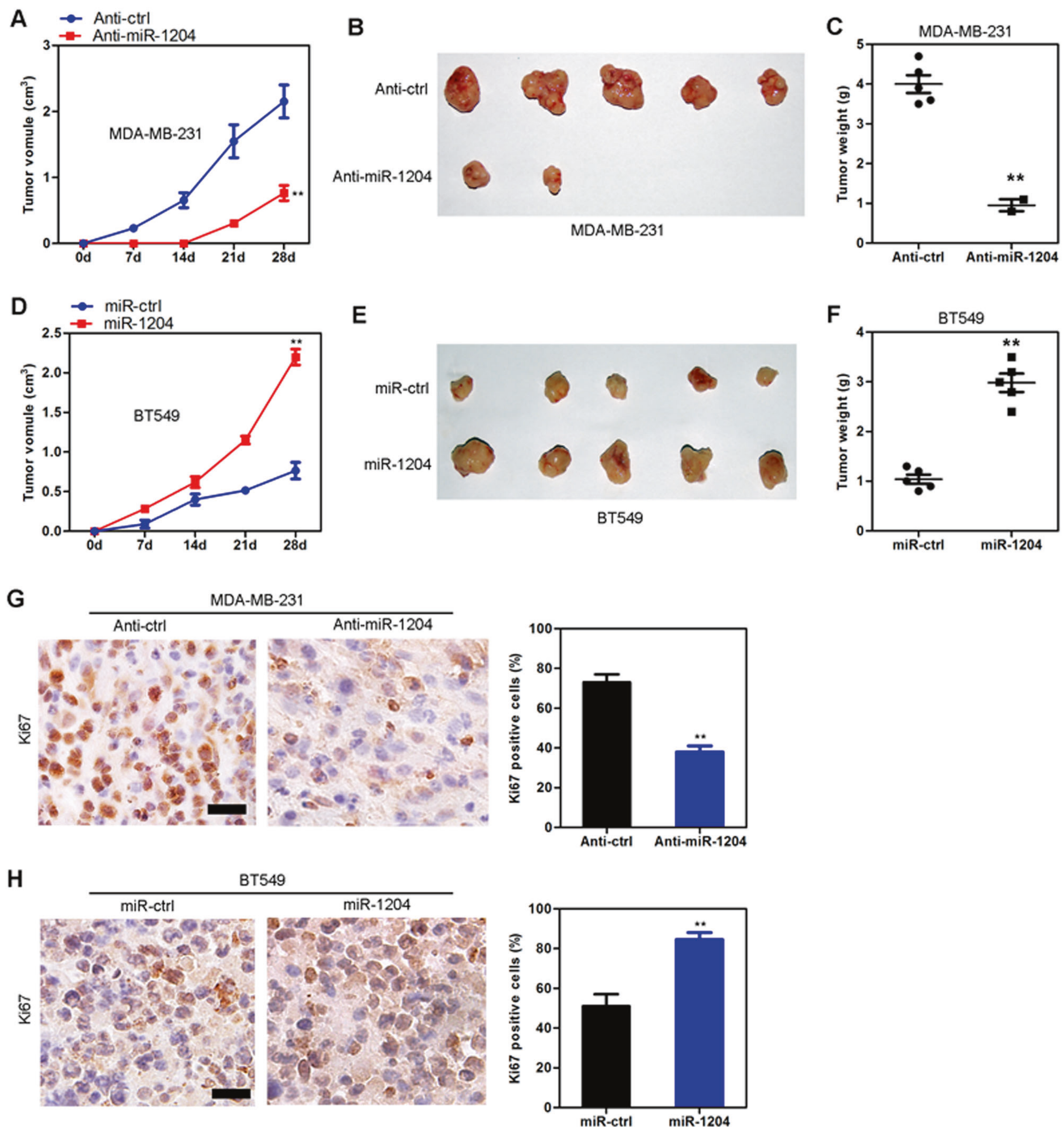


Fig. 3 miR-1204 promotes tumorigenesis of human breast cancer. **a** and **d**, Growth curve of tumors formed by MDA-MB-231-anti-miR-1204 (**a**), BT549-miR-1204 (**d**), or their control cells by subcutaneous injection. **c** and **f**, The weight of tumors formed by MDA-MB-231-anti-miR-1204 (**c**), BT549-miR-1204 (**f**), or their control cells at harvest time. **b** and **e** The tumors picture of MDA-MB-231-anti-miR-1204 (**b**), BT549-miR-1204 (**e**), or their control cells at harvest time. **g** and **h**, Representative immunohistochemical micrograhps of Ki67 in endpoint tumor tissues of MDA-MB-231-anti-miR-1204 (**g**), BT549-miR-1204 (**h**), or their control cells. Original magnification, ×500. Error bars represent the mean ± SD of three independent experiments, with each conducted in duplicate. In all experiments, ** $P < 0.01$

region encodes multiple regulatory non-coding RNAs, including PVT1 at 8q24.21 and miR-151 at 8q24.3 that may contribute to cancer pathophysiology when aberrantly expressed [17, 18]. A previous study focused on the PVT1 locus located 57–250 kb distal to MYC, since it is frequently co-amplified with MYC [19]. The mechanism by which PVT1 acts was suggested by recent reports showed that the PVT1 locus harbors multiple miRNAs including

miR-1204, suggesting that the oncogenic properties of PVT1 could be mediated through deregulation of miRNAs [18, 20]. Our hypothesis is that miR-1204 is a key cancer-causing oncogene within the often amplified genomic region on 8q24 that plays an important role in tumor development and metastasis. However, the potential oncogenic role and underlying mechanism of miR-1204 in human BC is still unclear.

Here, we identified that higher levels of miR-1204 is associated with poor prognosis in BC. Moreover, miR-1204 promotes proliferation and invasion of BC cells both in vitro and in vivo. Mechanistic investigations discovered that VDR is a novel target gene of miR-1204. Interference of VDR expression restored anti-miR-1204 induced inhibition of BC cell proliferation and tumorigenesis. Together, our results demonstrated that miR-1204-VDR pathway exerts tumorigenic and metastatic effects in BC with potential therapeutic applications in modifying BC development and progression.

Results

Higher level of miR-1204 is associated with poor prognosis in BC

We first analyzed the genomic amplification on chromosome 8q24 in BC using METABRIC and TCGA data, we found that a region of 8q24 near MYC, PVT1, and miR-1204 was commonly amplified in human BC (Fig. 1a). High-resolution analysis of DNA copy number levels of PVT1/miR-1204 and MYC in 1247 BCs and 54 BC cell lines revealed that the PVT1 and MYC genomic loci are frequently co-amplified (Supplementary Fig. S1A-B).

To investigate the role of miR-1204 in BC progression, we measured transcriptional levels of miR-1204 in 117 paired BC tissues and corresponding non-tumor tissues using qRT-PCR. The transcript levels of miR-1204 were significantly upregulated in cancerous tissues compared to adjacent non-tumor tissues (Fig. 1b). Consistent with this observation, we found that miR-1204 expression was dramatically increased in all 19 tested BC cell lines compared to two normal breast epithelial cells (MCF-10A and Hs-578Bst) (Fig. 1c). Not surprisingly, the expression level of miR-1204 is significantly positively correlated with the expression level of PVT1 (Fig. 1d). Moreover, we revealed that miR-1204 expression in human BC specimens was positively correlated with clinical stage (Fig. 1e). Furthermore, we found that patients with higher miR-1204 expression had a shorter survival time ($P = 0.0026$; Fig. 1f). Together, these results suggest that overexpression of miR-1204 could contribute to human BC progression.

miR-1204 promotes BC cell proliferation

We genetically manipulated miR-1204 in different BC cell lines to stably overexpress miR-1204 in MCF10A, BT549, and MDA-MB-468 cells, and silence miR-1204 in MDA-MB-231 and MCF7 cells (Supplemental Fig. S2A-E). Expression levels of miR-1204 were confirmed by qPCR (Supplemental Fig. S2A-E). We found that silencing miR-1204 significantly inhibited the proliferation of MDA-MB-231 and MCF7 cells (Fig. 2a; Supplemental Fig. S3A) and colony formation (Fig. 2B; Supplemental Fig. S3B), whereas miR-1204 overexpression dramatically increased proliferation of BT549 and MDA-MB-468 cells (Fig. 2c, d; Supplemental Fig. S3C and D). These results were confirmed using the BrdU incorporation assay and further by flow cytometric analysis. Silencing miR-1204 significantly inhibited BrdU incorporation in MDA-MB-231 cells (Supplemental Fig. S4A), whereas overexpression of miR-1204 significantly increased the BrdU incorporation in BC cells (Supplemental Fig. S4B). Similarly, silencing miR-1204 significantly decreased the percentage of S-phase cells compared to the control group (Fig. 2e; Supplemental Fig. S3E), whereas miR-1204 overexpression significantly increases the proportion of S-phase cells (Fig. 2f; Supplemental Fig. S3F). In addition, we found that silencing miR-1240 significantly increased the fraction of MDA-MB-231 apoptotic cells (Supplemental Fig. S5A), and overexpression miR-1204 inhibited the apoptosis in BT549 cells (Supplemental Fig. S5B). Taken together, these results suggest that miR-1204 regulates BC cell proliferation capacity.

miR-1204 promotes tumor development in a xenograft mouse model

To assess whether miR-1204 affects tumor development, we injected MDA-MB-231 cells with or without silencing miR-1204 into nude mice. MDA-MB-231 control cells rapidly formed tumors within 28 days after injection, whereas silencing of miR-1204 in MDA-MB-231 cells significantly suppressed tumor formation (Fig. 3a, b). In contrast, overexpression of miR-1204 in BT549 cells significantly promoted tumor development in vivo compared to control (Fig. 3d, e). Moreover, tumors sizes and weights were also significantly affected by miR-1204 (Fig. 3c, f). The expression level of miR-1204 in these tumors was consistent with the genetic manipulation of miR-1204 (Supplemental Fig. S6A and B). To investigate cell proliferation in these tumors, we used IHC staining to evaluate the expression levels of Ki67, and found that the fraction of Ki67 positive cells was significantly lower in tumors derived from miR-1204 knockdown cells than in control tumors (Fig. 3g). In contrast, overexpression of miR-1204

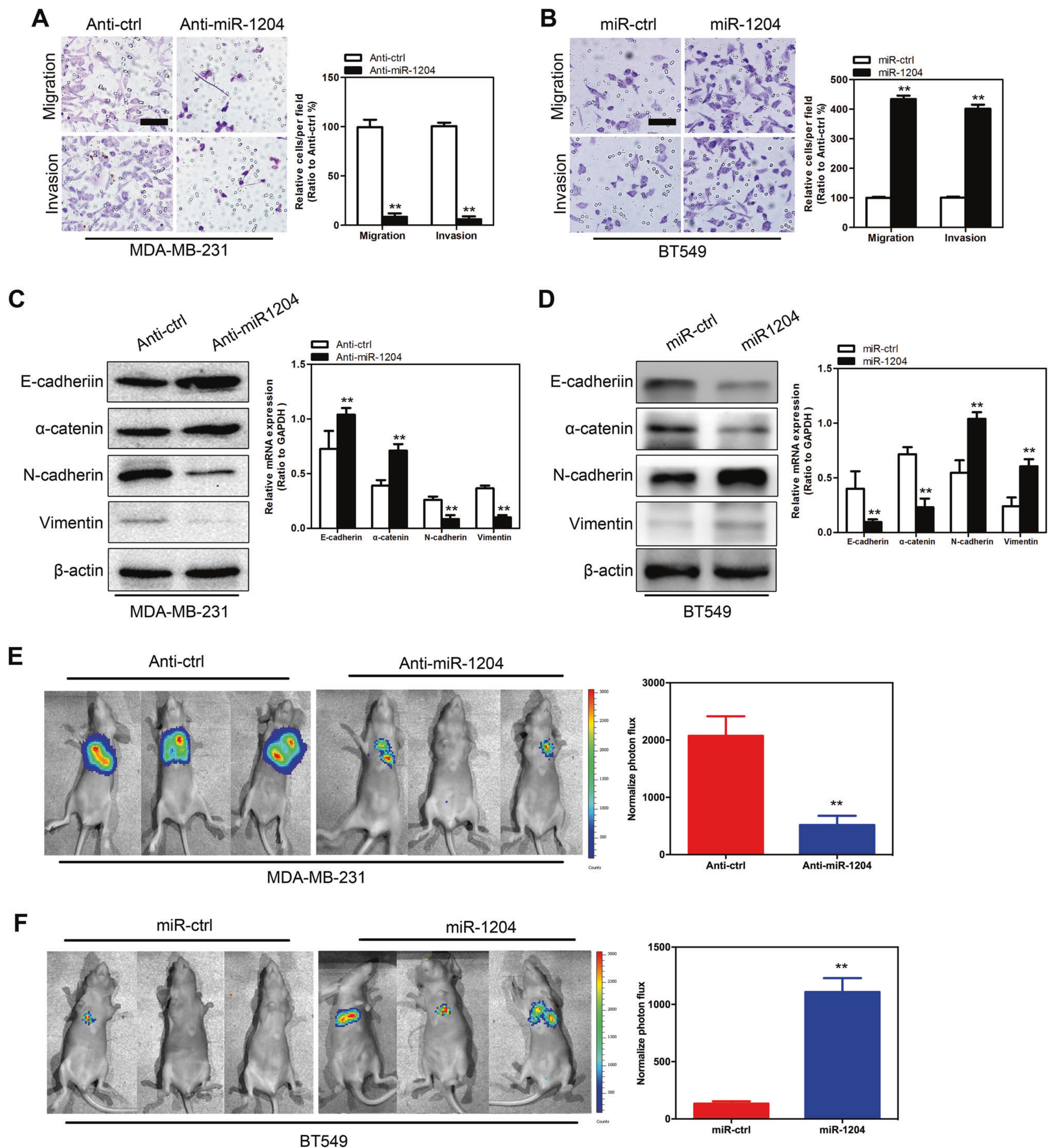
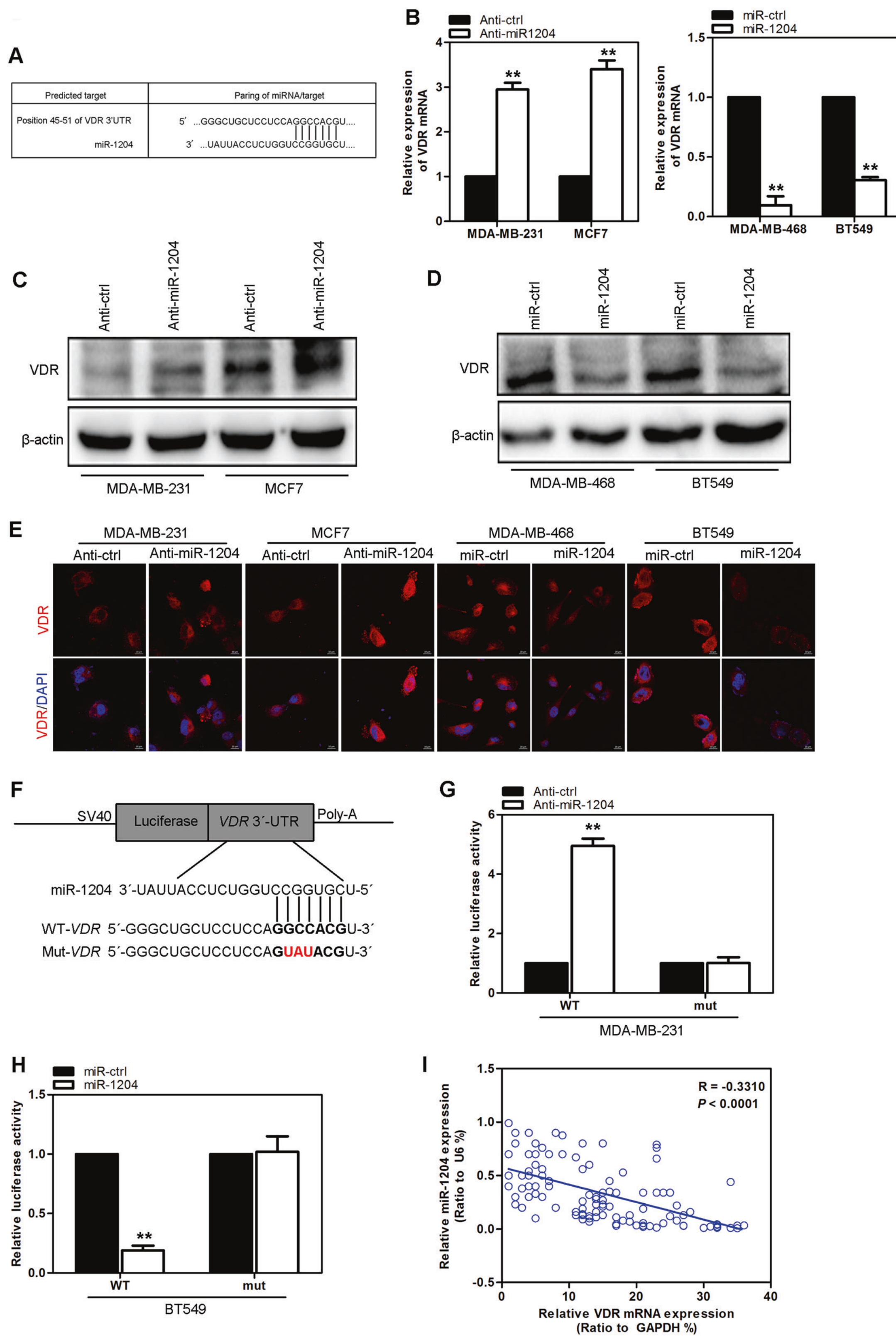


Fig. 4 miR-1204 promotes BC cells migration, invasion, EMT in vitro, and metastasis in vivo. MDA-MB-231-anti-miR-1204 and BT549-miR-1204 or control cells were subjected to transwell migration (**a** and **b**, upper panels) and Matrigel invasion assays (**a** and **b**, lower panels). **c** and **d**, Expression of epithelial and mesenchymal marker was analyzed by Western blotting (left) and qRT-PCR (right) in MDA-MB-231-anti-miR-1204(**c**) and BT549-miR-1204 (**d**) or control cells. **e**, Metastasis bioluminesence images of the MDA-MB-231-anti-miR-1204 and its control cells group (left). Chart representing the average photon counts from the MDA-MB-231-anti-miR-1204 and its control cells groups (right). **f**, Metastasis bioluminesence images of the BT549-miR-1204 and its control cells group (left). Chart representing the average photon counts from the BT549-miR-1204 and its control cells groups (right). $n = 3$ for tail-veil injection. $^{**}P < 0.01$ is based on Student's t-test. Error bars indicate standard deviation


A
Predicted target
Paring of miRNA/target
Position 45-51 of VDR 3'UTR
miR-1204
5' ...GGGCUGCUCCUCCAGGCCACGU....
3' ...UAUUACCUCUGGUCCGGUGCU....
B
Anti-ctrl
Anti-miR1204
Relative expression of VDR mRNA
MDA-MB-231
MCF7
miR-ctrl
miR-1204
MDA-MB-468
BT549
C
Anti-ctrl
Anti-miR-1204
VDR
β-actin
D
miR-ctrl
miR-1204
E
VDR
VDR/DAPI
F
SV40
Luciferase
VDR 3´-UTR
Poly-A
miR-1204 3´-UAUUACCUCUGGUCCGGUGCU-5´
WT-VDR 5´-GGGCUGCUCCUCCAGGCCACGU-3´
Mut-VDR 5´-GGGCUGCUCCUCCAGUAUACGU-3´
G
Relative luciferase activity
WT
mut
H
I
R = -0.3310
P < 0.0001
Relative miR-1204 expression (Ratio to U6 %)
Relative VDR mRNA expression (Ratio to GAPDH %)

◀ **Fig. 5** VDR is a novel target gene of miR-1204. **a** Predicted binding of miR-1204 to the 3′-UTR of VDR. **b** qRT-PCR analysis of the effect of silencing or overexpression miR-1204 on VDR mRNA levels in indicated BC cells. **c** and **d** Western blot analysis of the effect of silencing (**c**) or overexpression (**d**) miR-1204 on VDR mRNA levels in indicated BC cells. **e** Immunofluorescence staining of VDR in miR-1204 silencing or overexpression BC cells. **f** Schematic diagram of the VDR 3′-UTR pMIR-REPORT constructs. Sequences were compared between mature miR-1204 and the wild-type (WT) or mutant (Mut) putative target sites in the 3′-UTR or VDR. **g** The luciferase activity was assayed in MDA-MB-231-anti-miR-1204 and its control cells. **h** The luciferase activity was assayed in BT549-miR-1204 and its control cells. **i** a significant reverse correlation between the levels of miR-1204 and VDR expression was observed in BC specimens. $^{**}P < 0.01$ is based on Student's *t*-test. Error bars indicate standard deviation

significantly increased the fraction of Ki67 positive cells (Fig. 3h). Together, these findings suggest that miR-1204 promotes tumor development in a xenograft model.

miR-1204 promotes BC cell migration, invasion and epithelial-mesenchymal transition (EMT)

We assessed the effect of miR-1204 on cell motility. Silencing miR-1204 dramatically reduced the migratory capacity and matrigel invasion of MDA-MB-231 and MCF7 cells (Fig. 4a and Supplemental Fig. S7A). In contrast, overexpression of miR-1204 dramatically increased the migratory and invasive capacity of MDA-MB-468 and BT549 cells (Fig. 4b and Supplemental Fig. S7B). It is well known that induction of the epithelial to mesenchymal transition (EMT) is a major event that provides motility to cancer cells in order to generate metastases. Next, we assessed the effect of miR-1204 on EMT by examining the expression of EMT markers, and found that miR-1204 silencing increased the levels of epithelial markers (E-cadherin and α-catenin) and decreased the levels of mesenchymal markers (N-cadherin and vimentin) in MDA-MB-231 and BT549 cells (Fig. 4c and Supplemental Fig. S8A). Moreover, transcript levels of EMT markers correlated with the corresponding protein levels (Fig. 4c and Supplemental Fig. S8C), suggesting that miR-1204 affected the expression of epithelial and mesenchymal markers at the transcriptional level. Conversely, miR-1204 overexpression decreased levels of epithelial markers, and increased levels of mesenchymal markers (Fig. 4d and Supplemental Figs. S8B and D). Consistent with this, cells morphology was also changed accordingly (Supplemental Figs. S8E-F). Next, the key transcription factors (Slug, Snail, Twist, ZEB1, and ZEB2) were measured by WB and qRT-PCR in miR-1204 changed condition. The results shown that the expression of Snail and ZEB1 were regulated by miR-1204 (Supplemental Figs. S8G-J). These results indicate that miR-1204 confers migratory and invasive properties and induces EMT in BC cells.

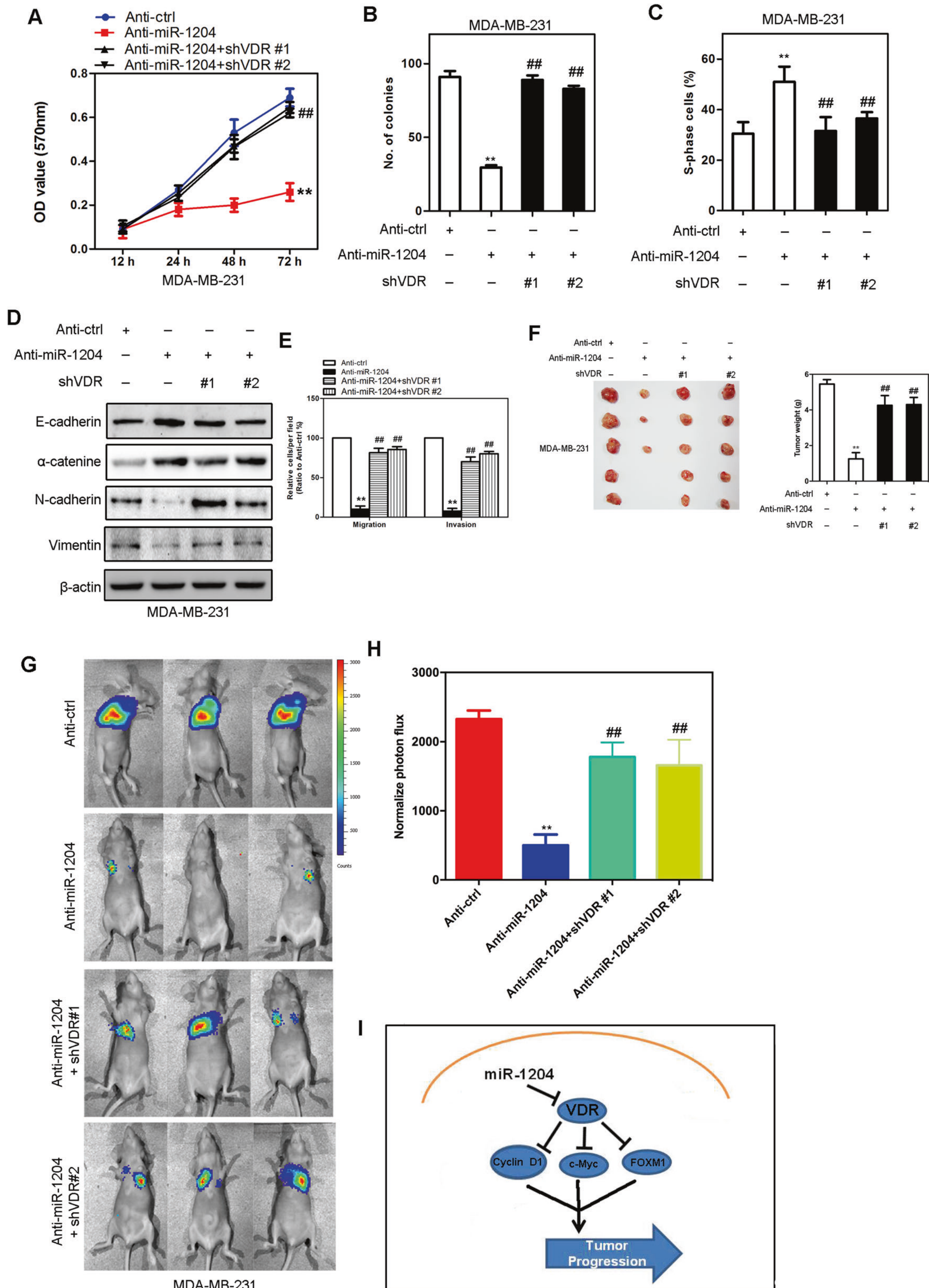
miR-1204 promotes BC cell metastasis in vivo

We then investigated whether miR-1204 was relevant for metastasis in vivo. MDA-MB-231-Anti-miR-1204, BT549-miR-1204, and their corresponding control cells were injected into nude mice through the tail vein. Silencing miR-1204 in MDA-MB-231 cells significantly decreased the distant metastasis of each mouse (Fig. 4e). miR-1204 overexpression in BT549 cells promoted metastatic behavior (Fig. 4f). Therefore, the in vivo results further demonstrate a critical role of miR-1204 in BC cell metastasis.

VDR is a novel target gene of miR-1204

To delineate the molecular mechanism of miR-1204-mediated tumorigenesis and progression, both computational and experimental approaches were employed to identify candidate miR-1204 target genes. More than 1000 mRNAs were predicted to be regulated by miR-1204 based on the representation of miR-1204 sites in their 3′untranslated regions (UTR) (Supplemental Table. S1). RNA-seq was used to identify genes whose expression was modulated after miR-1204 silencing in MDA-MB-231 cells (Supplemental Fig. S9A and B; Supplemental Table S2). We then selected the overlapping genes identified in the RNA-seq analysis and the computational prediction method. The 11 reported functional genes from the miR-1204 seed match analysis as the top 30 hsa-mir-1204 targets. We then measured the mRNA levels of these genes in MDA-MB-231 and MCF7 cells 24 h after treatment with anti-miR-1204 using real-time PCR and found that 15 out of the 30 genes were upregulated at least 2-fold compared to control in at least one cell line (Supplemental Fig. S9C). Interestingly, the most strongly downregulated gene was the putative tumor suppressor VDR.

Based on the sequence complementarity to miR-1204 seed sequence, we detected that VDR is a potential target of miR-1204 (Fig. 5a). qRT-PCR confirmed that silencing of miR-1204 in MDA-MB-231 and MCF7 cells significantly increased mRNA expression of VDR, while overexpression of miR-1204 in BT549 and MDA-MB-468 cells significantly decreased its mRNA expression (Fig. 5b). Western blotting analysis of VDR (Fig. 5c, d) and IF staining of nuclear VDR (Fig. 5e) corroborated the qPCR results. To determine whether miR-1204 directly targets the VDR gene, we cloned the 3′UTR of the putative VDR miR-1204 site into a luciferase construct (Fig. 5f). Reporter assays revealed that anti-miR-1204 increased luciferase activity (Fig. 5g), and overexpression of miR-1204 reduced luciferase activity (Fig. 5h). Mutation of miR-1204 binding sites in the VDR 3′UTR abrogated the miR-1204 effects (Fig. 5g, h). Moreover, expression of VDR mRNA was significantly negatively


A
Anti-ctrl
Anti-miR-1204
Anti-miR-1204+shVDR #1
Anti-miR-1204+shVDR #2
OD value (570nm)
12 h
24 h
48 h
72 h
MDA-MB-231
B
MDA-MB-231
No. of colonies
C
MDA-MB-231
S-phase cells (%)
Anti-ctrl
Anti-miR-1204
shVDR
D
E-cadherin
α-catenine
N-cadherin
Vimentin
β-actin
MDA-MB-231
E
Relative cells/per field (Ratio to Anti-ctrl %)
Migration
Invasion
F
MDA-MB-231
Tumor weight (g)
G
Anti-ctrl
Anti-miR-1204
Anti-miR-1204 + shVDR#1
Anti-miR-1204 + shVDR#2
MDA-MB-231
H
Normalize photon flux
Anti-ctrl
Anti-miR-1204
Anti-miR-1204+shVDR #1
Anti-miR-1204+shVDR #2
I
miR-1204
VDR
Cyclin D1
c-Myc
FOXM1
Tumor Progression

◀ **Fig. 6** Interference of VDR restored miR-1204-mediated BC cell proliferation, migration, invasion, ETM, tumorigenesis, and metastasis. **a**, **b** cell proliferation was examined by MTT (**a**) and colony formation (**b**) assays after knockdown of VDR expression in MDA-MB-231-anti-miR-1204 cells. **c** cell cycle was analyzed after knockdown of VDR expression in MDA-MB-231-anti-miR-1204 cells. **d** Expression of epithelial and mesenchymal markers was analyzed by Western blotting after knockdown of VDR expression in MDA-MB-231-anti-miR-1204 cells. **e** Expression of epithelial and mesenchymal markers was analyzed by qRT-PCR after knockdown of VDR expression in MDA-MB-231-anti-miR-1204 cells. **f** Tumor formation was assayed after knockdown of VDR expression in MDA-MB-231-anti-miR-1204 cells. **g**, **h** Tumor metastasis was assayed after knockdown of VDR expression in MDA-MB-231-anti-miR-1204 cells. **i** Model for miR-1204 mediated inhibition of VDR and its downstream effectors in the progression of BC. $^{**}P < 0.01$ is based on Student's *t*-test. Error bars indicate standard deviation

correlated with miR-1204 in BC tissues (Fig. 5i) and BC cell lines (Supplemental Fig. S9D). Taken together, these results suggest that miR-1204 can downregulate VDR expression by directly targeting its 3′UTR.

Overexpression of miR-1204 enhances the expression of VDR target proteins

Given that VDR is a negative regulator of Cyclin D1, c-Myc, and FOXM1 [21], we next determined whether miR-1204 expression affects the protein levels of these genes. As shown in Supplemental Fig. S10A, the protein levels of Cyclin D1, c-Myc, and FOXM1 were significantly decreased after silencing of miR-1204, whereas, miR-1204 overexpression markedly increased the expression of Cyclin D1, c-Myc, and FOXM1 in BC cells (Supplemental Fig. S10B). We then used IHC staining to evaluate the expression levels of Cyclin D1, c-Myc, and FOXM1 in tumors produced by MAD-MB-231 and BT549 cells in nude mice. The expression of Cyclin D1, c-Myc, and FOXM1 were significantly lower in miR-1204 knockdown tumor tissues compared to control tumor tissues (Supplemental Fig. S10C). On the other hand, overexpression of miR-1204 significantly increased the protein levels of these genes (Supplemental Fig. S10D). Moreover, expression levels of Cyclin D1, c-Myc, and FOXM1 mRNA were positively correlated with miR-1204 in BC tissues (Supplemental Fig. S10E-G).

Interference of VDR restored miR-1204-mediated BC cell proliferation, tumorigenesis, and metastasis

To test whether the miR-1204-induced proliferative and metastatic characteristics were mediated by VDR, shRNAs were used to silence VDR gene expression by virally transfecting MDA-MB-231-anti-miR-1204 cells with two distinct VDR shRNAs (Supplemental Fig. S11A and B). Knockdown of VDR expression in MDA-MB-231-anti-miR-1204 cells resulted in an increase in proliferation (Fig. 6a–c; Supplemental Fig. S12A and B). Moreover, knockdown of VDR expression in MDA-MB-231-anti-miR-1204 cells also resulted in decreased expression of the epithelial markers and increased mesenchymal markers (Fig. 6d), and was accompanied with the reduction of migratory and invasive capacities (Fig. 6e). In addition, we found that expression levels of the VDR target genes (Cyclin D1, c-Myc, and FOXM1) downregulated in MDA-MB-231-anti-miR-1204 cells were reversed after VDR silencing (Supplemental Fig. S12C). Taken together, these results showed that VDR mediates miR-1204-induced proliferation, EMT, migration, and invasion in BC cells.

To verify if VDR eventually mediates miR-1204-induced tumor formation and metastasis in vivo, MDA-MB-231-anti-miR-1204 cells with or without silenced VDR were injected into nude mice. Silencing VDR not only significantly promoted tumor formation (Fig. 6f), but also dramatically increased the metastatic lesions in the in vivo metastasis model (Fig. 6g, h). Therefore, these in vivo results further demonstrate the critical role of VDR in mediating miR-1204-promoted proliferative and metastatic behaviors in BC cells.

Discussion

In this study, we discovered a novel miR-1204/VDR signaling pathway, which plays an important functional role in BC pathogenesis. miR-1204 expression level was correlated with that of clinical stage and prognosis. Overexpression of miR-1204 promotes tumorigenic properties. The mechanistic link between miR-1204 and VDR through direct interaction, which subsequently leads to transcriptional downregulation of VDR expression and upregulation of VDR targets. These clinical and mechanistic findings strongly indicated that upregulation of the miR-1204 followed by downregulation of VDR expression and subsequent increase of VDR target genes expression promotes BC development and progression (Fig. 6i).

Genomic amplification at chromosome 8q24 is one of the most frequent genomic abnormalities in human cancers and is associated with poor survival in several human cancer types including BC [22, 23]. The well-established oncogene MYC maps to this locus and contributes to the pathophysiology of cancers in which it is amplified [24]. However, the PVT1 transcript is also located in this region and has been implicated in cancer pathophysiology [17]. Previous studies suggested that MYC and PVT1 contribute independently to BC pathogenesis [25]. miR-1204 is a PVT1-encoded miRNA located only 57 kb downstream of MYC [18]. Consistent with these reports, we showed that miR-1204 is overexpressed in many BC and overexpression

promoted BC cell proliferation and enhanced tumor formation in vivo. Interestingly, our study points to another function of miR-1204 in BC metastasis through regulating EMT.

BC cells with ectopic expression of miR-1204 displayed an EMT phenotype, and enhanced migration and invasion in vitro. Interestingly, our results indicated that miR-1204 promoted EMT, whereas silencing of miR-1204 resulted in the reversal of EMT (mesenchymal–epithelial transition). These characteristics induced by miR-1204 in vitro contributed to increased numbers of distant metastases in vivo. These findings provide a mechanistic framework to explain the clinical observations that BC patients with high levels of miR-1204 in tissue samples have a significantly shorter overall survival, and amplification of the 8q24 region encompassing miR-1204 is associated with the aggressive basal BC subtype [26, 27].

The roles of several transcription factors as EMT regulators have been extensively reported [28–31]. In our effort to elucidate the mechanism how miR-1204 modulates EMT in BC cells, we identified VDR as an effective mediator of miR-1204-induced EMT. The trans-acting transcriptional regulatory factor VDR binds to the active form of Vitamin D, 1,25-dihydroxyvitamin D3 (1,25D) [32] and then forms a heterodimer with the retinoid X receptor, which binds the Vitamin D response element (VDRE) and executes transcription of targeted genes [32]. Moreover, there is a study showing that VDR can regulate the level of nuclear β-catenin to impact oncogenic mutations on activation of the Wnt/β-catenin pathway [33]. The mechanistic connection between miR-1204 and VDR was previously unknown. In this study, we showed that miR-1204 transcriptionally controlled expression levels of VDR. Thus, we conclude that miR-1204 acts as an oncogene by directly targeting VDR, and consequently promoting BC cell proliferation and EMT in vitro and metastasis in vivo.

In summary, our results demonstrated that miR-1204 regulates proliferation and EMT and that the miR-1204-induced processes are reversible when miR-1204 expression is suppressed. These results provide a novel therapeutic avenue through manipulating miR-1204 levels in clinical practice. Further investigations are warranted to prove value of miR-1204 as a biomarker for patient outcome and as a therapeutic target for BC patients with amplification of chromosome 8q24.

Materials and methods

Bioinformatics analysis

The METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) data (http://molonc.bccrc.ca/aparicio-lab/research/metabric/) and TCGA breast cancer and cell line data were used to analyze the genomic copy changes of MYC and PVT/miR-1204. The detail of using METABRIC is according to the previous report [34]. Analysis of TCGA data were carried out using USDSC Xena method (http://xena.ucsc.edu).

Cell lines and cell culture

All cell lines listed in the supplementary Table S3 were maintained according to ATCC's protocol within 6 months after purchasing.

Patients and specimens

The RNAs was isolated from 117 BC and para-cancerous tissues for qRT-PCR analysis of miR-1024 and VDR expression levels. Samples were randomly collected from BC patients who underwent curative resection with informed consent between 2010 and 2012 at the Qilu Hospital, Shandong University. All tissues were collected immediately after tumor resection, snap-frozen in liquid nitrogen, and then stored at −80 °C. Follow-up data were summarized at the end of December 2016, with a median observation time of 75.4 months. Study protocols were approved by the Hospital Ethics Committee of Shandong University, and written informed consent was obtained from patients based on the Declaration of Helsinki.

Histological analysis

Mouse lungs and livers were collected for histological analysis of metastasis. Briefly Fixed tissues were embedded in paraffin wax following standard procedures. Sections with 5 µm thickness were stained with hematoxylin and eosin for histological analysis.

Vectors, retroviral infection, and transfection

All plasmids used in this study were listed in supplementary Table S3. The retroviral infection and transfection of cells were same as described in our previous studies [35].

Western blot

Cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail. The Bio-Rad Protein Assay kit was used to measure protein concentration. The same amounts of proteins were resolved by an 8% or 10% polyacrylamide gel and immunoblotted with indicated primary antibody (supplementary Table S3).

qRT-PCR

RNA was extracted using TRIZOL (Supplementary Table S3), while isolation of small was used miRcute miRNA Isolation Kit (Supplementary Table S3). Specifically, we designed a ploy (A) RT primer hybridizing with mature miR-1204 or U6. The reverse transcription reaction for miRcute miRNA was used First-Strand cDNA Synthesis Kit (Supplementary Table S3) and for mRNA was RevertAid First Strand cDNA Synthesis Kit (Supplementary Table S3). Real-time quantitative PCR reaction was performed with SYBR green. Primers used in this study are listed in Supplementary Table S4. The relative expression levels of miRNAs in each sample were calculated and quantified using the $2^{-\triangle\triangle Ct}$ method after normalization for expression of the positive control.

VDR shRNA knockdown

VDR knockdown cells with miR-1204 silence were obtained by infecting MDA-MB-231-anti-miR-1204 cells with lentiviral vector carrying a shRNA construct to knockdown VDR

(#1:5′-CCGGCCTGGCTGATCTTGTCAGTTACTC-GAGTAACTGACAAGATCAGCCAGGTTTTT-3′; #2:5′-CCGGCTCCTCAAACTCTGATCTGTACTCGAGTACA-GATCAGAGTTTGAGGAGTTTTT-3′) and a geneticin selection marker. Cells were selected in puromycin (5 μg/mL, Sigma) for 10 days. The expression levels of VDR were assessed by qRT-PCR and Western blotting analysis.

Dual luciferase reporter assay

A total of 1.0×10^5 cells was seeded in a 24-well plate, after cells attachment, cotransfected with 0.1 ug the reporter vector with 3 UTR-VDR or with mutant-3 UTR-VDR, 0.4 ug plasmid with pre-miR-1204 or anti-miR-1204, and 0.02 μg pMIR-*Renilla* expression vector. After cultured for 48 h, the luciferase activities of cells were measured using the dual luciferase reporter assay system (Promega) following the manufacturer's recommendations.

Cell cycle, proliferation, invasion, motility, and clonogenic assay

The reagents for cell cycle, proliferation, invasion, motility, and clonogenic assay are listed in supplementary Table S3. The detail of each assay was described in our previous studies [31].

Flow cytometry for apoptosis

Apoptosis was measured by the Annexin V apoptosis detection kit (Supplementary Table S3) according to manufacturer's protocol. The data were analyzed by Flowjo software.

Immunohistochemistry

The fixed tissues were sectioned at 4-μm thickness. Tissue sections were stained with Biotin-Streptavidin HRP Detection Systems according to manufacturer's protocol. Briefly, sections were deparaffinized and antigen repaired with sodium citrate, and then, incubated with 3% hydrogen peroxide for 10 min at room temperature. Tissue sections were blocked with goat serum for 15 min at 37 °C and incubated with indicated antibody for overnight at 4 °C. The areas of positive and negative staining were determined using Image-Pro Plus 5.1 software (Media Cybernetics, Silver Spring, MD, USA)

Confocal immunofluorescence microscopy

Cells were plated on culture slides for 24 h. The fixed cells were permeabilized using 0.5% Triton X-100. After blocking for 30 min in 10% BSA cells were incubated with primary antibodies overnight at 4 °C. After washing with PBS three times, the slides were incubated in the dark with FITC-conjugated secondary antibodies for 1 h. After three further washes, the slides were stained with DAPI for 5 min to visualize the nuclei, and examined using a confocal imaging system (Supplementary Table S3).

In vivo tumorigenesis and metastasis assay

For tumorigenesis assay, BC cells were infected with miR-Ctrl- or miR-1204 expressing lentiviruses for 72 h. A group of 6 Balb/c nude mice (Supplementary Table S3) were injected subcutaneously with infected cells into the left and right flanks. The tumor size was assessed by measuring tumor dimensions with calipers for up to 42 days [31]. For metastasis assays, 10^7 cells suspended with 0.1 ml PBS were injected into tail veins of nude mice. All the mice were euthanized 60 days after injection. All animals were maintained under the guidelines of Shandong University and under evaluation and approval of the Institutional Animal Care and Use Committee (Shandong University, Jinan, China). Food and water were provided ad libitum.

RNA-seq analysis

For RNA-seq analysis, three batches RNAs were isolated from MDA-MB-231-anti-miR-1204 and control cells using the PerfectPure RNA tissue kit. And 1 μg RNA was used to prepare libraries. Libraries were prepared using the Illumina TruSeq1 RNA Sample Preparation v2 according to manufacturer's instructions. Indexed samples were sequenced on

an Illumina HiSeq 2500 in a single read mode. The sequencing depth was 50×. The obtained reads, 50 bp long, were mapped to the genome assembly using TopHat2. Gene-level expression was quantified by applying HTSeq (version 0.6.1), and using the known genes from UCSC in gtf format as annotation. Differential expression was calculated utilizing the DESeq2 software (version 1.2.10).

Statistical analysis

Statistical analysis was performed using the SPSS statistical software program (IBM. Armonk, New York, USA). The results were presented as mean ± S.D. Association between miR-1204 and VDR in BC specimens was assessed by the X^2 test. Student two-tailed *t*-test was used to determine the comparisons between different groups. The differences with $P < 0.05$ were considered statistically significant.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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