Targeting the Vav1/miR-29b axis as a potential approach for treating selected molecular subtypes of triple-negative breast cancer

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Abstract. MicroRNA (miR)-29b has been reported to play a controversial role in breast cancer, particularly triple-negative breast cancer (TNBC). Based on our previous data revealing that the PU.1-mediated expression of miR-29b in cells from acute myeloid leukemia is sustained by Vav1, the potential role of this multidomain protein in modulating miR-29b levels in breast tumor cells, in which Vav1 is ecstopically expressed and shows a nuclear accumulation, was investigated. Breast cancer cell lines with various phenotypes and patient-derived xenograft-derived TNBC cells were subjected to Vav1 modulation and reverse transcription quantitative PCR of miR-29b levels. The recruitment of CCAAT enhancer binding protein α (CEBPα) to miR-29b promoters was investigated by quantitative chromatin immunoprecipitation assays. It was found that Vav1 was essential for the recovery of mature miR-29b in breast cancer cell lines, and that it promoted the expression of the miRNA in TNBC cells of the mesenchymal molecular subtype by sustaining the transcription of the miR-29b1/a cluster mediated by CEBPα. The present results suggest that Vav1 is a crucial modulator of miR-29b expression in breast tumor cells, and this finding may help identify strategies that may be useful in the management of TNBC by targeting the Vav1/miR-29b axis, as there is a lack of molecular-based treatments for TNBC.

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Abbreviations: DNMT, DNA methyltransferase; TNBC, triple-negative breast cancer; AML, acute myeloid leukemia; RT-qPCR, reverse transcription quantitative PCR; Q-ChIP, quantitative chromatin immunoprecipitation

Key words: breast cancer, Vav1, miR-29b, triple-negative breast cancer, CEBPα

Introduction

The microRNA (miR)-29 family consists of the miR-29a, miR-29b and miR-29c members, whose aberrant expression is involved in tumorigenesis, as their specific target genes include oncogenes and members of the DNA methyltransferase family (1,2). miR-29b dysregulation has been reported in breast cancer (3-5), in which low miR-29b expression has been found to be positively correlated with large tumor size and advanced cancer stage (6,7). Accordingly, miR-29b has been reported as a sensitive marker for predicting patient outcome in all breast tumor subtypes (7), including triple-negative breast cancer (TNBC) (8). In TNBC cell lines, miR-29b has been shown to suppress viability and migration and increase sensitivity to chemotherapeutic agents (2,9), suggesting that the restoration of miR-29b levels may be crucial for this unfavorable breast cancer subset. More recently, the predominantly expressed mature miR-29b-3p and the less expressed miR-29b1-5p have been reported to have opposite effects on tumor cells with a triple-negative phenotype (9-13); therefore, the role of miR-29b in TNBC remains elusive.

A number of positive or negative transcriptional modulators of miR-29b expression have been identified (14), but little is known concerning the regulation of miR-29b in breast cancer (15). The multidomain protein Vav1 has been found to play a peculiar role in miR-29b expression in acute myeloid leukemia (AML) cells, in which the PU.1-mediated expression of miR-29b is almost completely dependent on adequate levels of Vav1 inside the nuclear compartment (16). Vav1 is ectopically expressed in the majority of breast carcinomas, in which it displays a prevalent localization inside the nucleus, which is positively correlated with a low incidence of relapse (17). In TNBC cells, Vav1 negatively regulates invasiveness *in vitro* and metastatic efficiency *in vivo* by affecting the expression of genes involved in the invasion and/or metastasis of breast tumors (17,18).

The aim of the present study was to assess the role of Vav1 in the regulation of miR-29b levels in breast cancer cells. This study focused on cells with a triple-negative phenotype, for which target-based therapies are not currently available, and sought to determine whether Vav1 can promote the miR-29b transcriptional process.

Materials and methods

All reagents were purchased from Merck KGaA unless otherwise specified.

Cells and treatments. The non-transformed MCF10A cells (RRID: CVCL_0598) and the malignant MCF7 (RRID: CVCL_0031), MDA-MB-453 (RRID: CVCL_0418), MDA-MB-468 (RRID: CVCL_0419) and MDA-MB-231 (RRID: CVCL_0062) cell lines were purchased from the American Type Culture Collection. BT-474 cells (RRID: CVCL_0179) were obtained from Interlab Cell Line Collection.

MCF10A cells were cultured in DMEM-F12 (Thermo Fisher Scientific, Inc.) containing 10 μg/ml bovine insulin, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone, 20 ng/ml recombinant human epidermal growth factor, and 10% horse serum. The BT-474 cell line was cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS), 1 mM Na pyruvate, and 0.01 mg/ml bovine insulin. MCF7, MDA-MB-453, MDA-MB-468 and MDA-MB-231 cell lines were maintained in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (17-19).

Established cell lines from breast tumor patient-derived xenografts (PDXs) with a triple-negative phenotype (HBCx-2, HBCx-9, HBCx-17, HBCx-39 and T174) were provided by Xentech and cultured in Gibco™ Advanced DMEM-F12 (Thermo Fisher Scientific, Inc.) supplemented with 8% FBS (Thermo Fisher Scientific, Inc.), 1% penicillin- streptomycin solution and 20 μM Rho-associated kinase inhibitor Y-27632 (DBA ITALIA SRL).

All cell lines were cultured at 37˚C in a humidified atmosphere of 5% CO_2 in air, and were tested for mycoplasma and other contaminations monthly.

In all cell lines, the upregulation and downregulation of Vav1 were performed as previously described (18).

Immunochemical analysis. Total lysates (50 μg protein) were separated on polyacrylamide denaturing gels, blotted to nitrocellulose membranes (GE Healthcare), and treated with primary antibodies against Vav1 (diluted 1:500, cat. no. sc-8039), CEBPα (diluted 1:250, cat. no. sc-61) (both from Santa Cruz Biotechnology, Inc.), GATA3 (diluted 1:500, cat. no. ab199428; Abcam), and β-tubulin (diluted 1:1,000, cat. no. T4026; Merck KGaA), as previously described (18). The membranes were then incubated with appropriate peroxidase-conjugated secondary antibodies (goat anti-mouse, diluted 1:2,000, cat. no A4416; goat anti-rabbit, diluted 1:2,000, cat. no. A6154; Merck KGaA) and visualized using the ECL system (PerkinElmer, Inc.). Chemiluminescence images of the bands were captured by ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare), and densitometric analysis was performed using ImageQuant TL version 7.0 software (RRID:SCR_018374; GE Healthcare).

Reverse transcription quantitative PCR (RT-qPCR). High-quality RNA, including small RNAs, was extracted from all cell lines using miRNeasy Micro Kit (Qiagen SpA Italia), according to the manufacturer's instructions.

miR-29b and CEBPα expression were evaluated by RT-qPCR using TaqMan Assays (ID 000413; ID Hs05650633_s1: Thermo Fisher Scientific, Inc), as previously described (16,19,20). miR-29b and CEBPα expression levels were normalized to U6 snRNA (ID 001973, Thermo Fisher Scientific, Inc.) and to RPL13A (ID Hs03043885_g1, Thermo Fisher Scientific, Inc.), respectively.

To measure Vav1 mRNA in PDX-derived cell lines, and pri-miR-29b1 and pri-miR-29b2 in all cell lines, RT-qPCR was performed using the iTaq Universal SYBR-Green SuperMix on a CFX96™ Real-time detection system (RRID:SCR_018064; Bio-Rad Laboratories Inc.). The following primers were used: Vav1, 5'-ACGTCGAGGTCAAGCACATT-3' forward and 5'-GGCCTGCTGATGGTTCTCTT-3' reverse; pri-miR-29b1, 5'-AAATGGCAGTCAGGTCTCTG-3' forward and 5'-GCA ATGCAAATGTATGCAAAT-3' reverse; pri-miR-29b2, 5'-TTGAGTGTGGCGATTGTCAT-3' forward and 5'-ATC AACGCCGAATACTCCAG-3' reverse.

Levels of Vav1, pri-miR-29b1 and pri-miR-29b2 were normalized to RPL32 content (5'- CATCTCCTTCTCGGC ATCA-3' forward and 5'-AACCCTGTTGTCAATGCCTC-3' reverse).

All reactions were performed in triplicate, and the experiments were repeated 3 times.

Quantitative chromatin immunoprecipitation (Q-ChIP) assay. Q-ChIP assays were performed as previously described (16). Samples were immunoprecipitated with antibodies against CEBPα or with a non-specific IgG antibody (cat. no. sc-53344; Santa Cruz Biotechnology, Inc). qPCR of a 131-bp DNA fragment (primers: 5'-GCAGGTTTTCAGTTGGTGGTTT-3' forward and 5'-GCCGTGACAGTTCAGTAGGA-3' reverse), encompassing the putative CEBPα binding site at -89/+42 bp from the transcriptional start in the pri-miR29a/b1 promoter on Chr 7q32.3 was performed using an iTaq Universal SYBR-Green SuperMix. PCR products were separated on Tris-acetate 1% agarose gels, stained with ethidium bromide and visualized by a UV light apparatus.

Statistical analysis. The association between Vav1 mRNA, pre-miR-29b1 and pre-miR-29b2 levels was evaluated within each of the 918 invasive ductal carcinomas of 'The Cancer Genome Atlas' (TCGA; cancergenome.nih.gov). Pearson's correlation coefficient (r) and Spearman's rank correlation coefficient (ϱ) were calculated for all values (https://jasp-stats.org/). All miR-29b values in cell lines are expressed as mean ± standard deviation and analyzed by one-way ANOVA followed by Dunnett's multiple comparison test for more than two groups, using GraphPad Prism 6.0 statistical package (RRID: SCR_002798; GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results and Discussion

The involvement of the multidomain protein Vav1 in gene transcription has been demonstrated in both myeloid leukemia and

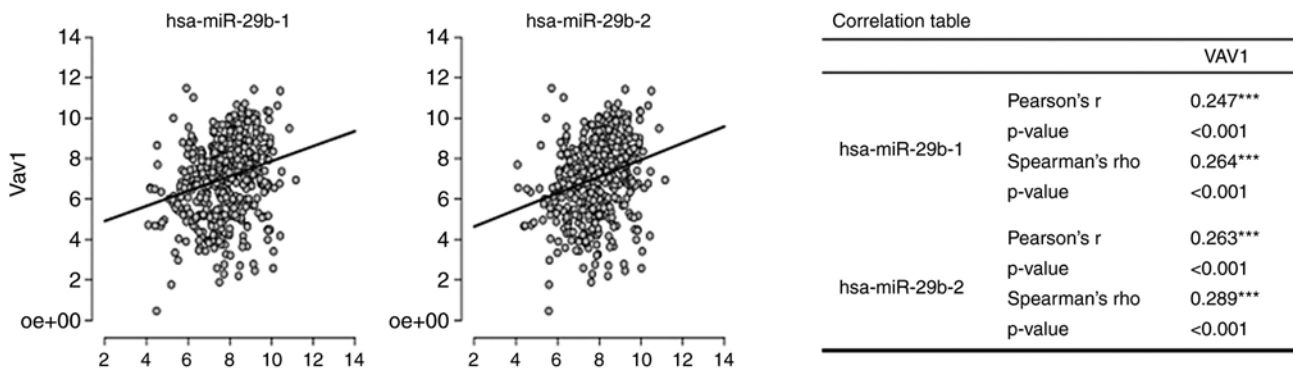

Figure 1. Correlation between Vav1 and miR-29b in invasive breast cancer tissues. The expression levels (reads per million) of pre-miR-29b1 (hsa-mir-29b-1) and pre-miR-29b2 (hsa-mir-29b-2) in tissues from the TGCA cohort (n=918) of invasive breast cancers were log2-transformed and correlated with Vav1 mRNA expression of the same tissue samples using scatterplot graphs. The Pearson's and the Spearman's correlation coefficients and their respective P-values are reported in the Correlation Table. ***P<0.001. TGCA, The cancer Genome Atlas.

breast cancer cells. In acute promyelocytic leukemia cells, it has been shown that Vav1 promotes the access of the transcription factor PU.1 to its consensus regions on DNA (16,21), and that adequate levels of Vav1 are essential for the PU.1-mediated expression of miRNAs, including that of miR-29b (16).

Vav1 has been found to be ectopically expressed in breast cancer, where it has been reported to accumulate inside the nuclear compartment of tumor cells and to be involved in gene expression (17); these findings were similar to those observed in leukemia cells (16,21). The positive correlation (P<0.001) between Vav1 mRNA and the levels of both pre-miR-29b1 and pre-miR-29b2 contributors to mature miR-29b that was observed in the well-characterized TCGA cohort of invasive breast tumors (Fig. 1) suggest a potential regulatory role of Vav1 in the expression of miR-29b in breast cancer cells, on which this miRNA has been shown to have controversial effects (9-13, 22).

The association between Vav1 and miR-29b was first investigated in the BT-474, MCF7, MDA-MB-453, MDA-MB-468, and MDA-MB-231 breast cancer cell lines, with different phenotypes, representative of the most common breast tumors (23). As expected, based on our previous results (13,17), the examined cell lines exhibited various levels of Vav1, which were not correlated with the tumor phenotype, and the protein expression was absent in the non-malignant MCF10A cell line (Fig. 2A). At variance with Vav1 expression, the level of miR-29b was significantly higher in cell lines with a triple-negative phenotype (MDA-MB-468 and MDA-MB-231), as compared with the non-malignant MCF10A cell line; while the expression of the miRNA remained low in the luminal BT-474 (luminal B) and MCF7 (luminal A) cell lines (Fig. 2B).

Regardless of the apparent lack of a correlation between the basal levels of the two molecules, Vav1 was found to be essential for the expression of miR-29b in all examined cell lines (Fig. 2C), which suggest a crucial role of this multidomain protein in the mechanism(s) leading to the production of mature miRNA in breast cancer cells, independent of their phenotype. However, the forced expression of Vav1 induced miR-29b only in MDA-MB-231 cells (Fig. 2C), suggesting a phenotype-related mechanism involving Vav1 in the expression of this miRNA in breast cancer. Despite the fact that no experimental evidence or predictive analysis has suggested Vav1 as a direct target of miR-29b, mimic and inhibitors were used in the present study to exclude any effects of the miRNA on Vav1 levels (data not shown).

Since Vav1 is involved in miRNA expression as a facilitator of transcription factors in AML cells (16,21), the role of Vav1 in regulating miR-29b in breast cancer cells was explored at the transcriptional level. The investigation excluded the transcription factor PU.1, with which Vav1 acts synergistically in AML cells (16,21,24), as it was not expressed in the examined cell lines (25). GATA binding protein 3 (GATA3), the only transcription factor known to regulate miR-29b in breast tumor cells (15), was also excluded from the investigation, since it was found to be expressed in the cell lines examined except in MDA-MB-231 (Fig. 3A), confirming previous data in MCF7 and MDA-MB-231 cell lines (26). Therefore, CCAAT enhancer binding protein α (CEBPα), the main regulator of miR-29b in hematopoietic cells, which interacts specifically with its promoter on chromosome 7 and is responsible for the transcription of the miR-29a/b1 locus (27), was taken into consideration. It was revealed that, of the examined cell lines, only MDA-MB-231 exhibited a low expression of CEBPα (Fig. 3A), which, substantiating the high expression of the pri-miR-29b1 observed in this cell line (Fig. 3B), was actually recruited by the miR-29b promoter on Chr 7 (Fig. 3C). The overexpression of Vav1 in MDA-MB-231 cells induced an association between CEBPα and DNA, which was significantly reduced by the silencing of the protein (Fig. 3D), justifying the effects of the upregulation and downregulation of Vav1 on miR-29b levels in this cell line. The evaluation of CEBPα expression in MDA-MB-231 cells, in which Vav1 was forcedly regulated, excluded any effects of Vav1 on this transcription factor (Fig. 3E).

These data suggest that, in breast cancer cells, similar to AML cells, Vav1 can regulate the interaction of transcription factors with their DNA consensus sequences. In particular, they highlight the specific role of Vav1 in regulating the CEBPα-dependent expression of miR-29b in MDA-MB-231 cells, while clearly suggesting the existence of other mechanisms through which Vav1 supports the production of miR-29b in breast tumor cells. Furthermore, the lack of effects of Vav1 overexpression on the miR-29b levels in MDA-MB-453

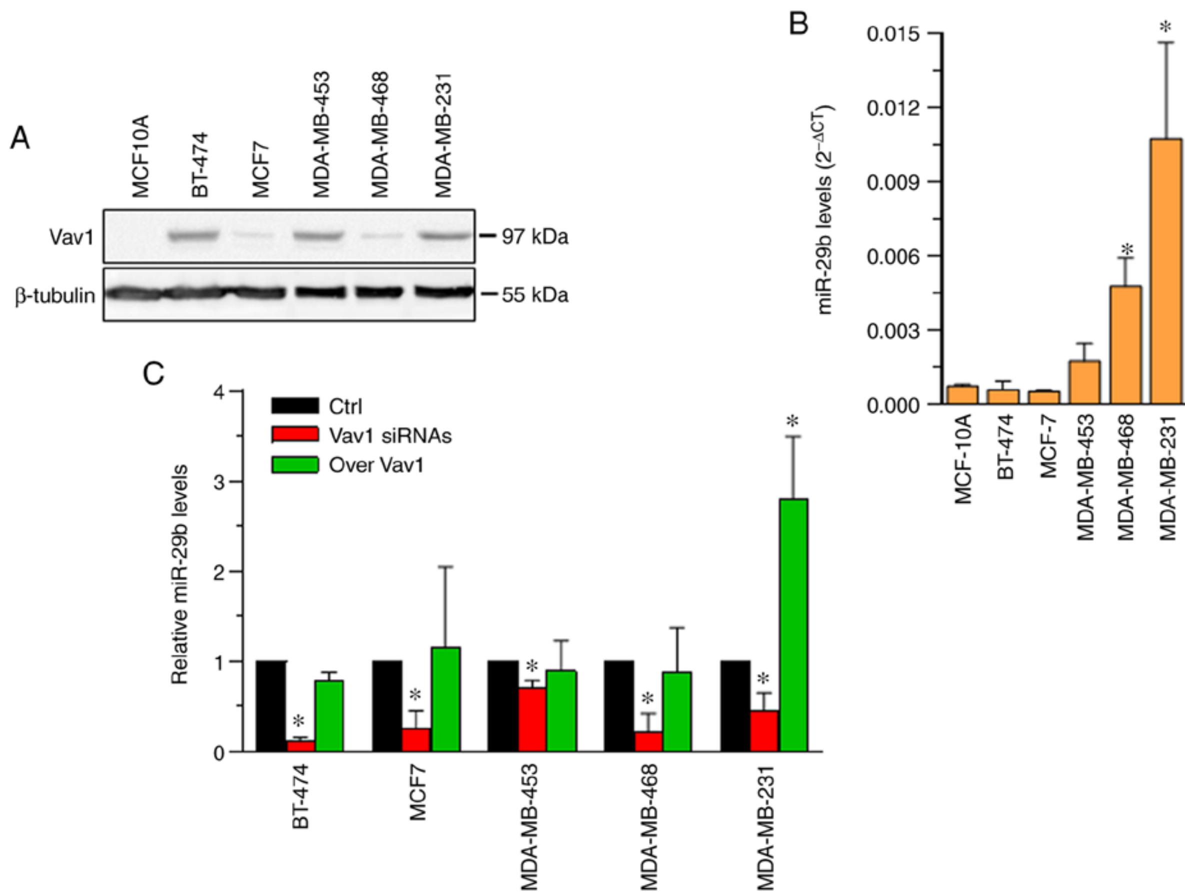

Figure 2. Correlation between Vav1 and miR-29b in breast cancer cell lines. (A) Western blot analysis of Vav1 and (B) RT-qPCR analysis of miR-29b levels ($2^{-\Delta CT}$ method) in MCF10A, BT-474, MCF7, MDA-MB-453, MDA-MB-468 and MDA-MB-231 cell lines. (C) RT-qPCR analysis of miR-29b in cell lines transfected with siRNAs specific for Vav1 (Vav1 siRNAs) or with a construct expressing the full-length human Vav1 (Over Vav1). Values obtained from cells transfected with control siRNAs or empty vectors (Ctrl) were taken as 1. Relative transcript levels were determined using the $2^{-\Delta\Delta Ct}$ method. Data are presented as the means of 3 separate experiments ± SD. *P<0.05 compared to the respective controls. RT-qPCR, reverse transcription quantitative PCR.

and MDA-MB-468 cells, which exhibited a triple-negative phenotype (28) but a lack of CEBPα (Fig. 3A), support the Vav1/CEBPα cooperation but highlight the need to examine this phenomenon in depth in TNBC, which is highly heterogeneous (29) and characterized by controversial roles of miR-29b (9-13).

The study was then extended to triple-negative cell lines selected on the basis of their molecular subtype, according to the Lehmann classification (30) which, considering age at diagnosis and local and distant disease progression, may prove useful for identifying appropriate targeted therapies for patients with TNBC (30). The HBCx-2, HBCx-9, HBCx-17, HBCx-39 and T174 PDX-derived TNBC cell lines of the mesenchymal (M), basal-like 1 (BL1), basal-like 2 (BL2) and luminal androgen receptor (LAR) subtypes, which expressed various and clearly not correlated basal levels of Vav1 and miR-29b (Fig. 4A), were investigated to determine the association between Vav1 and miR-29b. The overexpression of Vav1 (Fig. S1A) was found to be sufficient to increase miR-29b levels only in the HBCx-9 and HBCx-17 cell lines (Fig. 4B), which were characterized by a mesenchymal phenotype and expressed CEBPα (Fig. 4C), similar to the MDA-MB-231 cell line, which also had a mesenchymal molecular phenotype (2,28). Possibly due to the low amount of Vav1 in these cell lines, its silencing (Fig. S1B) allowed to reveal a significant decrease in miR-29b only in the HBCx-9 cells (Fig. 4B).

The overexpression of Vav1 was ineffective in the other examined PDX-derived cell lines that did not express the transcription factor (Fig. 4B and C), supporting the existence of a Vav1/CEBPα cooperation in TNBC cells. On the other hand, the silencing of Vav1 reduced the levels of miR-29b (Fig. 4B) in PDX-derived cells with a BL1 phenotype (HBCx-39) that did not express CEBPα (Fig. 4C); this finding was similar to that in the MDA-MB-468 cell line, which is also classified as BL1 (28). No effects of Vav1 on miR-29b were observed in PDX-derived cell lines of the less frequent BL2 and LAR molecular subtypes (Fig. 4B), which expressed relatively high basal levels of the two molecules (Fig. 4A) and were negative for CEBPα (Fig. 4C). This bulk of data clearly indicate that, in TNBC cells, Vav1 plays a phenotype-specific role, which is mainly correlated with the expression of CEBPα but also suggests that Vav1 may sustain the activity of other transcription factors regulating miR-29b. This latter hypothesis was supported by the presence of both miR-29b primary transcripts in all examined TNBC cell lines (Figs. 3B and 4D), indicating that transcription factors other than CEBPα, which is only responsible for miR-29b1 expression, are involved in positive or negative transcriptional regulation of this miRNA in breast cancers. Given the multiple roles that Vav1 can play

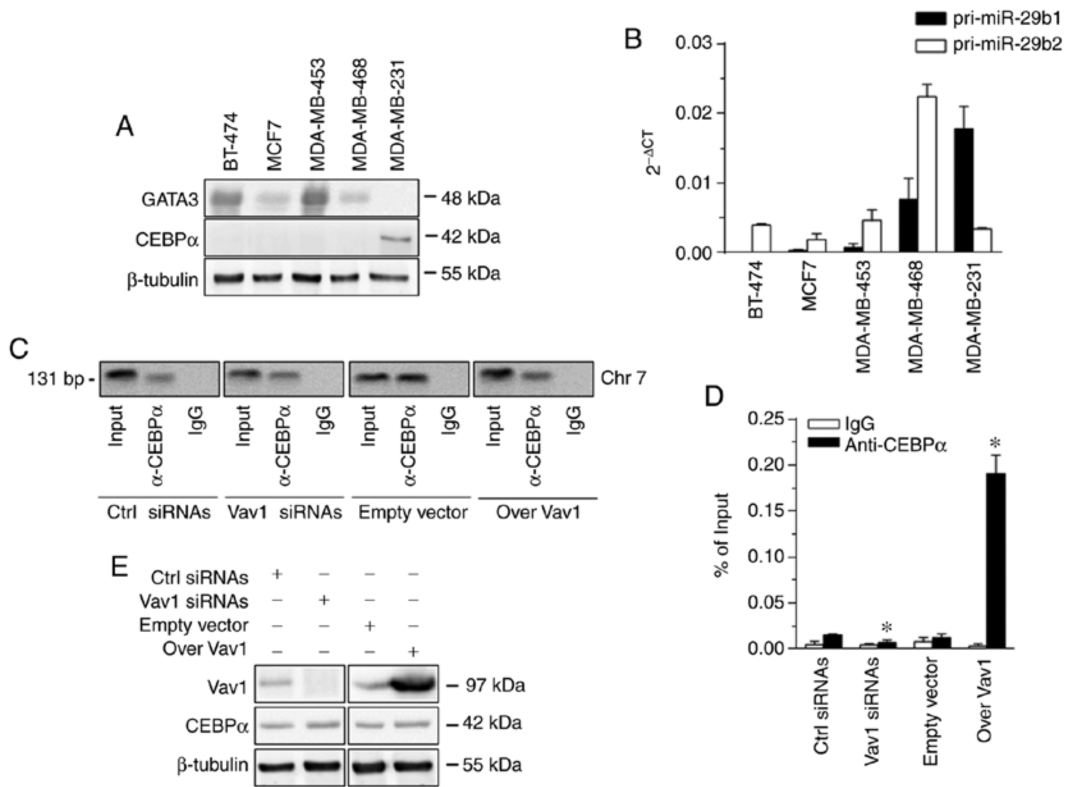

Figure 3. *In vivo* interaction of CEBPα with miR-29b promoters. (A) Western blot analysis of GATA3 and CEBPα and (B) RT-qPCR analysis of pri-miR-29b1 and pri-miR-29b2 in cells grown in control conditions. (C) Representative analysis of *in vivo* recruitment of CEBPα to miR-29b promoter following chromatin immunoprecipitation with an antibody directed against CEBPα in MDA-MB-231 cells, in which Vav1 was downregulated (Vav1 siRNAs) or overexpressed (Over Vav1). Bands correspond to PCR products obtained by amplifying a fragment encompassing the CEBPα binding site within the miR29a/b1 promoter on Chr 7. Input, genomic DNA not subjected to immunoprecipitation (positive control); IgG, samples immunoprecipitated with a non-specific antibody (negative control). (D) ChIP-qPCR values are presented as a percentage of the Input. Data are presented as the mean of 3 separate experiments ± SD. *P<0.05 compared to the respective controls. (E) Western blot analysis of Vav1 and CEBPα in MDA-MB-231 cells in which Vav1 was downregulated or overexpressed. RT-qPCR, reverse transcription quantitative PCR; CEBPα, CCAAT enhancer binding protein α.

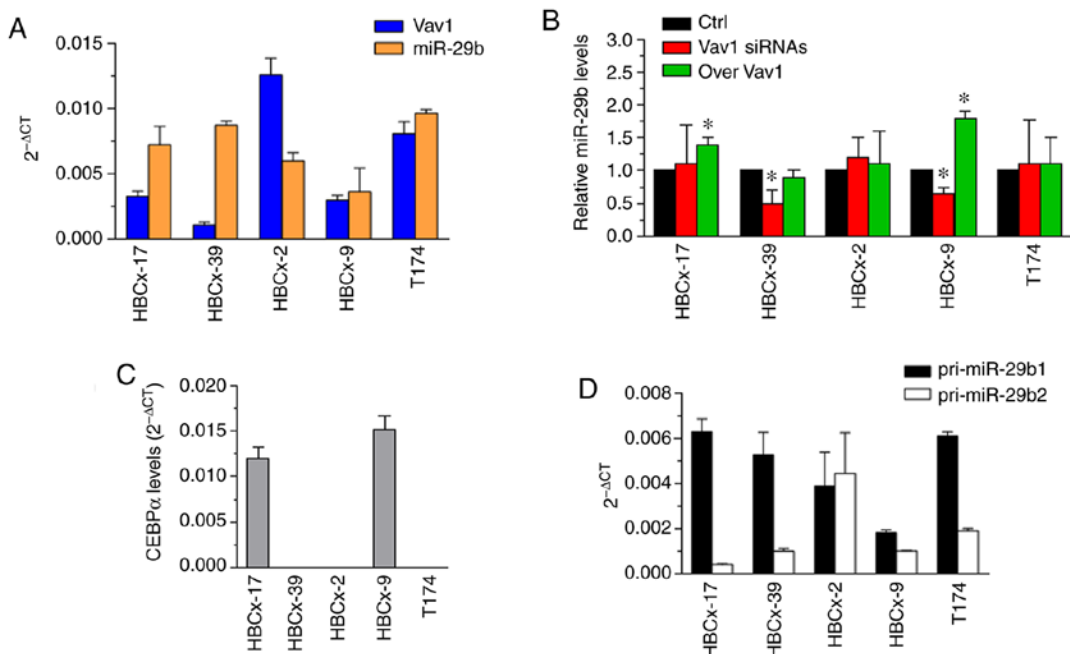

Figure 4. Correlation between Vav1 and miR-29b in cell lines from triple-negative PDXs. (A) RT-qPCR analysis of Vav1 mRNA and miR-29b in PDX-derived cell lines. (B) RT-qPCR analysis of miR-29b in PDX-derived cell lines in which Vav1 was silenced (Vav1 siRNAs) or overexpressed (Over Vav1). The values are relative to those of cells transfected with control siRNAs or empty vectors (Ctrl), respectively, taken as 1. Data are presented as the means of 3 separate experiments ± SD. *P<0.05 compared to the respective controls. RT-qPCR analysis of (C) CEBPα mRNA and (D) pri-miR-29b1 and pri-miR-29b2 in PDX-derived cell lines grown in control conditions. RT-qPCR, reverse transcription quantitative PCR; PDX, patient-derived xenograft; CEBPα, CCAAT enhancer binding protein α.

in cells, both cytoplasmic and nuclear, and the controversial role of miR-29b in breast cancer that takes into account the 3p and 5p variants, our results were unable to establish the functional meaning of the Vav1/miR-29b axis in the different subtypes of breast cancer.

Considering that miR-29b affects multiple oncogenic characteristics of breast tumors, a better knowledge of the machinery that regulates its expression could constitute an important contribution to the management of this type of cancer. Although the complex positive and negative regulation of miR-29b precursors requires further study to understand the mechanisms involved, the present results indicated that, in breast cancer cells, Vav1 is involved in the regulation of miR-29b levels at the transcriptional level. Of note, the forced modulation of Vav1 expression affects the levels of mature miR-29b in specific molecular subtypes of TNBC, suggesting that approaches targeting Vav1 may be useful in tumor subsets for which there is lack of targeted therapies, and the patient prognosis is generally unfavorable despite the high chemosensitivity.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

VB was responsible for the study concept, and supervised all the experiments. VB, SC, MDM and JGJ integrated the results. SG, FV and FB performed experiments and prepared the figures. SV performed the statistical analysis. VB and MDM drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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