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Parvin α and Parvin β Are Upregulated in Metastatic Murine Breast Tumor Cells and Correlate with Enhanced Cell Migration

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Background: Alterations in cell–extracellular matrix (ECM) interactions are pivotal events that drive key hallmarks of breast cancer metastasis, including anchorage-independent growth, resistance to apoptosis, and enhanced cellular motility. Parvin alpha (Parva) and Parvin beta (Parvb), both localized at focal adhesions, are critical regulators of cell adhesion, migration, and cytoskeletal dynamics. This study aimed to investigate the potential role of Parvins in promoting the metastatic capacity of breast tumor cells.Methods: Primary (4T1T) and metastatic (4T1B and 4T1L) breast tumor cells were isolated from a murine model of metastatic breast cancer. Cell viability and migration were evaluated using MTT and wound-healing assays, respectively. Quantitative real-time PCR (RT-qPCR) was employed to analyze the expression levels of Parva and Parvb.Results: No significant differences in cell viability were observed between primary and metastatic tumor cells under both 2D and 3D culture conditions. In contrast, metastatic cells demonstrated substantially greater migratory capacity. Notably, RT-qPCR analysis revealed a significant upregulation of Parva and Parvb expression in metastatic tumor cells compared with their primary counterparts.Conclusion: Parvin α (Parva) and Parvin β (Parvb) are significantly upregulated in highly metastatic 4T1 breast tumor cells compared with their primary counterparts. This upregulation correlates with increased migratory capacity in vitro. These preliminary findings suggest a potential association of Parvins with metastatic properties; however, functional studies are required to establish causality and therapeutic relevance.

Introduction

Breast cancer remains the most frequently diagnosed malignancy and a leading cause of cancer-related deaths among women worldwide, with over 2.3 million new cases and 685,000 deaths reported in 2020 (1). The high mortality is largely attributed to metastatic dissemination to distant sites, particularly the lungs and brain, which accounts for over 90% of fatalities (2). Metastatic dissemination accounts for the vast

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majority of deaths in breast cancer patients, largely due to its complex and multifaceted nature (3). This process involves a cascade of biological and physical events, including detachment from the extracellular matrix, intravasation into the bloodstream, survival in circulation, extravasation into distant tissues, and subsequent colonization (4). Understanding the cellular and molecular mechanisms driving tumor progression depends on identifying key proteins that enhance the dissemination and tumorigenic potential of breast cancer cells (5).

Integrins, a prominent family of heterodimeric cell surface receptors, play a central role in orchestrating the signaling pathways that drive breast cancer metastasis (6). These receptors are critically involved in mediating both cell–cell and cell–extracellular matrix (ECM) adhesion, essential processes in tumor progression and dissemination (7). Upon binding to their ligands, integrins initiate a cascade of intracellular signals by recruiting various kinase proteins to the plasma membrane (8, 9).

Despite lacking intrinsic kinase activity, integrins initiate downstream signaling by forming complexes with signaling-competent kinases and adaptor proteins such as integrin-linked kinase (ILK), focal adhesion kinase (FAK), and the Parvin family proteins (Parva and Parvb). These molecules are integral to the transmission of integrin signals that regulate key cellular processes, including migration, adhesion, and survival (11, 12).

Parva (α-parvin), a member of the adaptor protein family with broad tissue distribution, was initially identified through its interaction with ILK (13). Recent studies have demonstrated that α-parvin plays a pivotal role in modulating tumor cell morphology, migration, invasion, and survival signaling pathways in cultured cells (14, 15). Similarly, Parvb (β-parvin), a focal adhesion-associated protein, has been implicated in integrin-mediated cell adhesion by its binding to ILK (16).

Given the critical role of integrin signaling in metastasis and the emerging importance of parvins in regulating cell motility and adhesion, further investigation into their function in breast cancer is warranted. Here, we test the hypothesis that PARVA and PARVB are upregulated at the mRNA level in metastatic 4T1-derived breast tumor cells (4T1B and 4T1L) compared with their primary counterparts (4T1T). We further hypothesize that this upregulation correlates with enhanced migratory potential in vitro, providing correlative evidence for a possible role of Parvins in breast cancer metastasis.

Materials and Methods

Cell Culture

Primary (4T1T), lung metastatic (4T1L), and brain metastatic (4T1B) breast tumor cells were isolated and characterized based on our previously published study (17). NIH-3T3 mouse embryo cell line was attained from the Pasteur Institute of Iran approximately six months prior to the initiation of this study. Cells were seeded in DMEM (10% FBS, 100 U/ml Penicillin, and 100 ug/ml Streptomycin) (Gibco, USA).

2D and 3D Tumor Cell Culture

To establish 2D cultures, tumor cells were seeded at a density of 1×10^4 cells per well in standard 96-well tissue culture plates and incubated in DMEM at 37°C with 5% CO_2.
For 3D spheroid culture, cells were seeded at the same density in ultra-low attachment 96-well plates (UAM, Corning, USA) and cultured in a spheroid-forming medium containing 0.5% FBS and 2% Pen-Strep for six days under identical incubation conditions.

MTT assay

Cell viability was assessed using MTT assay. Cells were seeded in 96-well plates at a density of 5×10^3 cells/well in 100 µL complete medium and allowed to adhere overnight. For 2D vs. 3D comparison, 3D spheroids were transferred to flat-bottom 96-well plates on day 6 post-seeding.

At the indicated time points, 10 µL of MTT solution (5 mg/mL in PBS, Sigma-Aldrich Cat# M2128) was added to each well, and plates were incubated at 37 °C in 5% CO_2 for exactly 4 hours. Following incubation, the medium was carefully removed, and formazan crystals were dissolved in 100 µL DMSO. Optical density (OD) was measured at 570 nm with a reference wavelength of 630 nm using a Multiskan™ FC microplate reader (Thermo Scientific).

Blank wells containing medium only (no cells) were included in every plate and used for background subtraction. Absorbance values were calculated as: ODnet = OD570 – OD630 – ODblank (mean of 6 blank wells). Cell viability

was expressed as percentage of untreated control (4T1T cells in 2D culture). All experiments were performed in three independent biological replicates, with six technical replicates per condition in each experiment (total n = 18 per group). Data are presented as mean ± SD

Wound Healing (Migration) Assay

Cell migration was quantified using a standardized wound healing assay. After generating a scratch with a sterile 200-µL pipette tip, images were captured at 0 h, 24 h, and 48 h using an inverted microscope. For each well, five non-overlapping fields along the wound were imaged. Wound width was measured using ImageJ software (NIH) with the "Wound Healing Tool" plugin.After 24 hours, the medium was replaced with serum-free DMEM for 16–24 hours. A sterile 200-µl pipette tip was used to create a scratch (wound) in the monolayer, and wells were gently washed with PBS to remove detached cells. Cells were then cultured in DMEM containing 10% FBS for 24 hours. Images were captured at 0 and 24 hours using an inverted microscope (CK40, Olympus, Japan) to monitor wound closure. Analysis was performed by an investigator blinded to the cell-line identity. All experiments were performed with three independent biological replicates, each containing three technical replicates.

Quantitative Real Time PCR (RT-qPCR)

To evaluate Parva and Parvb gene expression, total RNA was extracted from 4T1T, 4T1B, and 4T1L tumor cells seeded in 24-well plates (1×10^4 cells/well) using an RNA extraction kit (Pars Tous, Iran). RNA quality was assessed via Nanodrop spectrophotometry and agarose gel electrophoresis. cDNA was synthesized by the Parstous cDNA synthesis Kit. RT-qPCR was performed with SYBR Green Master Mix (Amplicon A/S, Denmark) using a Bio-Rad Real-Time PCR system. The thermal profile included an initial denaturation at 95°C for 15 minutes, followed by 40 cycles of 95°C for 30 seconds, 56.7°C for 30 seconds, and 72°C for 30 seconds. Primer specificity was validated by the presence of a single peak in the melting curve analysis, and no amplification was observed in no-template controls. PCR efficiency for all primer pairs ranged between 90–110%, calculated from standard dilution curves. Each reaction was performed with three biological replicates, each containing three technical replicates.

Quantification Method: Relative gene expression levels were calculated using the **2^-ΔCT method**, with GAPDH using as the internal control. Expression levels were calculated using the 1/ΔCT method, with GAPDH using as the internal control. we use of the geNorm algorithm to assess GAPDH stability across all experimental conditions (primary and metastatic cell lines). The M-value for GAPDH was 0.32(<1.5) confirming its suitability as a stable internal control ΔCT=CT target−CTGAPDH

This method provides normalized expression values relative to the internal control gene GAPDH. Mean CT values ± SD for all genes (target and reference) are provided in the Supplementary Table. All PCR products were confirmed to represent single amplicons (single melting-curve peak), and no genomic contamination was detected.

Primer sequences used are as follows:

Genes	Accession number	FW (5' - 3)	Rev (5' - 3)	Amplicon size (bp)	PCR eff(%)
Parvin-B	NM_133167.3	GTG TAC CTA GTT CTACTTCTGG	AAG TTC ACC ACA TCTTCAGG	136	98.7
Parvin-A	NM_020606.6	CAT CAA CCT GCC TCTTAGC	ATG TCT CAG TCC TGTCTATATG	112	96.4
GAPDH	NM_001289726.2	CCTGGAGAAACC TGCCAAGTA	GGCATCGAAG GTGGAAGAGT	123	101.2

Sample Type	RNA yield (µg)	A260/280
3T3	190	1.89
4T1T	248	2.01
4T1B	207	1.91
4T1L	289	2.035

Statistical analysis

All results are presented as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA, USA). one-way

ANOVA was followed by Tukey's Honestly Significant Difference (HSD) test to identify specific group differences when ANOVA indicated significance ($p < 0.05$). We also clarified that all experiments were performed with three biological replicates, each with three technical replicates (n = 9 total measurements per group).

Results

2D and 3D Culture of Metastatic(4T1B,4T1L) and Primary(4T1T) Tumor Cells

4T1T and 4T1B,4T1L were successfully isolated from tumor-bearing mouse tissues. Due to their rapid proliferation, the tumor cells were purified following three consecutive passages. 4T1T, 4T1B and 4T1L were cultured under two-dimensional (2D) conditions (Figure 1). For three-dimensional (3D) spheroid culture, cells were seeded in non-adherent 96-well U-bottom plates. As illustrated in Figure 1, compact spheroid structures were formed within six days of incubation, indicating the self-aggregation capability of both primary and metastatic cells in 3D culture.

Viability of Tumor Cells in 2D and 3D Cultures

Cellular viability was assessed using the MTT assay in both 2D and 3D cultures of tumor cells (Figure 2). No significant differences in cell viability were observed between primary (4T1T) and metastatic (4T1B and 4T1L) cells in either 2D or 3D culture conditions (one-way ANOVA: $F(5,48) = 1.27$, $p = 0.291$). Mean viability values ranged from 94.3 ± 6.8% to 102.1 ± 8.2% of control (n = 9 per group).

Enhanced Migration Capacity of Metastatic Tumor Cells

The wound healing assay was performed under two-dimensional culture conditions to assess the migratory potential of tumor cells. This method evaluates the capacity of viable cells to move into a cell-free gap generated by a scratch, thereby mimicking processes of tissue repair and invasion. Metastatic cells exhibited significantly higher migratory capacity than primary cells (one-way ANOVA: $F(2,24) = 68.4$, $p < 0.0001$). Post-hoc Tukey's test revealed that 4T1B cells closed 40.2 ± 4.1% of the wound at 24 h and 92.6 ± 5.3% at 48 h, compared with 14.8 ± 3.2% and 38.5 ± 4.9% for 4T1T cells ($p < 0.001$ for both time points). 4T1L cells showed intermediate migration ($p < 0.01$ vs. 4T1T, $p < 0.01$ vs. 4T1B).(Figure 3).

Up-Regulation of PARVB, PARVA in 4T1B, 4T1L

Parva and Parvb mRNA levels were significantly upregulated in metastatic cells (one-way ANOVA: Parva $F(3,32) = 42.6$, $p < 0.0001$; Parvb $F(3,32) = 36.8$, $p < 0.0001$). Tukey's post-hoc test showed: • Parva: 4T1B 3.41 ± 0.38-fold ($p < 0.001$), 4T1L 2.64 ± 0.31-fold ($p < 0.01$) vs. 4T1T • Parvb: 4T1B 2.97 ± 0.34-fold ($p < 0.001$), 4T1L 2.41 ± 0.29-fold ($p < 0.01$) vs. 4T1T

(Figure 4). To establish a reference, the mouse fibroblast cell line NIH-3T3 was included as a control. Notably, metastatic cells exhibited markedly higher expression of both genes relative to NIH-3T3, whereas primary 4T1T cells displayed reduced expression compared with both the metastatic lines and the fibroblast control. These findings suggest a metastasis-specific role for Parva and Parvb, implicating them as potential drivers of the invasive and progressive phenotype observed in metastatic breast cancer cells.

Discussion

In the present study, we observed significant upregulation of Parva and Parvb mRNA in highly metastatic 4T1-derived breast tumor cells (4T1B and 4T1L) compared with their primary counterparts (4T1T). This upregulation correlated with markedly increased migratory capacity in the wound-healing assay, whereas cell viability remained comparable across sublines in both 2D and 3D culture conditions.

Our findings are consistent with several recent reports linking elevated Parvin expression to aggressive phenotypes in breast cancer. For instance, Sun et al. (2020) reported that α-parvin promotes migration and lung metastasis in triple-negative breast cancer via G3BP2/TWIST1 signaling (18). Similarly, PARVA overexpression has been associated with enhanced invasion and poor prognosis in multiple cohorts (15). The observed correlation between Parvb levels and migration in our model aligns with earlier evidence that β-parvin modulates ILK activity and cytoskeletal dynamics during dissemination (19). Collectively, these observations strongly implicate Parva and Parvb as molecular drivers of tumor aggressiveness across diverse malignancies.

The contribution of Parvins to tumor progression may lie in their role at the crossroads of integrin

signaling and actin cytoskeleton remodeling. In metastatic tumor cells, ILK phosphorylates FAK in response to extracellular stimuli such as growth factors and chemokines, subsequently activating downstream cascades that promote cellular motility and invasive capacity (20). Upregulated expression of Parva and Parvb may potentiate integrin-mediated signaling, thereby reinforcing the integrin–actin cytoskeletal linkage and facilitating the dynamic reorganization required for metastatic dissemination.

A significant aspect of our study was the differential expression pattern of Parvins in malignant compared to non-malignant contexts. While primary breast tumor cells expressed lower levels of Parva and Parvb compared to NIH-3T3 fibroblasts, metastatic breast cancer cells exhibited significantly higher levels than both. This suggests that Parvins function in a metastasis-specific manner, augmenting invasive traits rather than broadly contributing to cellular proliferation or maintenance. This selective upregulation underscores the potential of Parva and Parvb as molecular biomarkers capable of distinguishing aggressive breast tumors from their less invasive counterparts.

In the present study, we employed freshly isolated primary and metastatic breast tumor cells from a murine model, which strengthens the physiological relevance of our findings. By avoiding long-term cell culture adaptation, which can alter gene expression profiles, our study provides insight into early molecular events underpinning metastatic progression. To the best of our knowledge, this is the first report demonstrating pronounced upregulation of Parva and Parvb specifically in metastatic breast carcinoma cells compared to their primary tumor counterparts.

Despite these promising results, several limitations warrant consideration. The exact mechanistic role of Parvins within the integrin signaling network remains incompletely defined. Furthermore, while our findings implicate Parvins in metastatic breast cancer, validation across larger patient-derived datasets and clinical samples is essential to confirm their translational potential.

Future investigation needs to focus on dissecting the downstream effectors of Parva and Parvb, potentially through proteomic approaches, and gene-silencing strategies such as siRNA or CRISPR-mediated knockout. Functional assays combined with in vivo metastasis models could clarify their role in tumor dissemination and colonization of distant organs. Moreover, exploring whether targeting Parvin-mediated pathways can attenuate metastatic progression may open new insights for therapeutic intervention in metastatic breast cancer.

Conclusion

Our findings indicate that upregulation of Parva and Parvb in metastatic breast cancer cells (4T1B and 4T1L) compared to primary cells (4T1T) is associated with enhanced migratory capacity, suggesting a potential role in breast cancer metastasis. These results provide preliminary evidence of Parvins' involvement in metastatic processes, possibly through their regulation of cell motility and cytoskeletal dynamics. However, further functional studies, including gene knockdown or overexpression experiments, and validation in human clinical samples are necessary to establish Parvins as reliable biomarkers or therapeutic targets for breast cancer metastasis.

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Authors' Contributions

Sadegh Dylami: Conducted Real-Time PCR experiments, contributed to methodology design, and prepared the original draft.

Mohammad Kamalabadi Farahani: Conceptualization, Methodology, Performing the Experiments (Animal Study, Cell Culture), Supervised the Experimentators

Vahid Kia Performing the Experiments (Real Time PCR), Supervised the Experimentators

Maryam Kazemi: Performing the Experiments (Primer design)

All authors reviewed and confirmed the final version of the manuscript.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shahroud University of Medical Sciences (Ethics code: IR.SHMU.REC.1400.89). All procedures were conducted in accordance with the ARRIVE guidelines (https://arriveguidelines.org) and complied with relevant institutional and national regulations for animal experimentation.

Consent to publication

Not applicable.

Competing interests

The author declares that they have no competing interests.

Limitations of the Study

This study has several limitations. First, Parva and Parvb expression was assessed only by RT-qPCR, without protein-level validation (e.g., Western blotting) due to limited tumor samples, antibody availability, and funding. Second, the integrin/ILK/FAK pathway was discussed based on prior literature, as we did not perform experiments to study phosphorylation or protein interactions. Third, siRNA/shRNA knockdown experiments to confirm the causal role of Parvins were not conducted due to technical constraints. Fourth, 3D spheroid assays were limited to viability assessment, and invasion or drug resistance were not evaluated due to resource limitations.

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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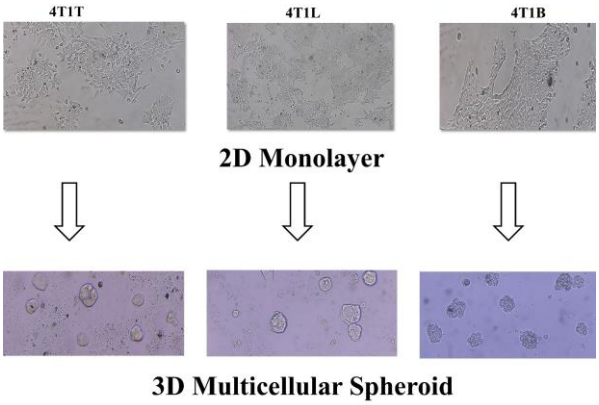

Figure 1. Two- and three-dimensional culture of primary and metastatic breast tumor cells. Primary tumor cells (4T1T) and their metastatic counterparts (4T1B and 4T1L) were cultured under conventional 2D conditions. For 3D culture, spheroids were generated in spheroid-forming medium, with well-defined structures observed after six days of incubation.

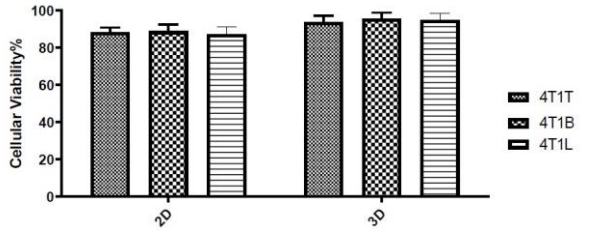

Figure 2. Cell Viability Assessment with MTT: The MTT assay compared viability of primary (4T1T) and metastatic (4T1B and 4T1L) breast tumor cells in 2D and 3D cultures. No significant differences were observed between culture conditions. Data represent mean ± SD from three biological replicates (n = 9).

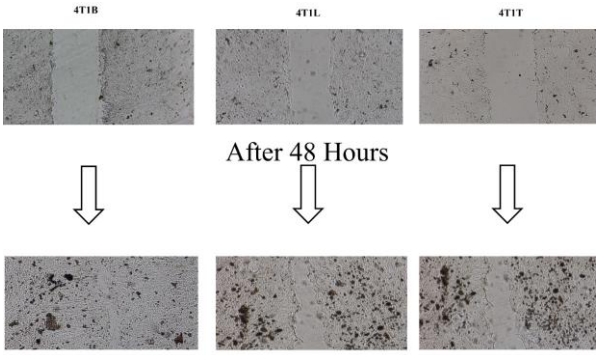

Figure3. Wound Scratch Assay: Compared with other group, in brain metastatic tumor cells (4T1B), cell migration was very dynamic. In these cells, the wound achieving a value of 40% after only 24 h and after 48 h there was narrow signs of the wound. One-way ANOVA $F(2,24) = 68.4$, $p < 0.0001$; Tukey's test: ***$p < 0.001$ vs. 4T1T. Data are mean ± SD (n = 9).

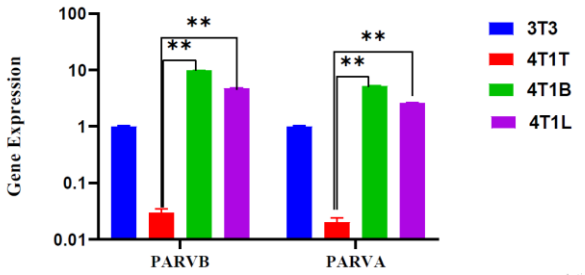

Figure 4: Upregulation of PARVA and PARVB in Metastatic Breast Cancer Cells. Relative expression of PARVA and PARVB in primary (4T1T) and metastatic (4T1B and 4T1L) breast tumor cells was measured by RT-qPCR, using 3T3 fibroblasts as a control. Both genes were significantly upregulated in 4T1B and 4T1L compared with 4T1T ($p < 0.05$, one-way ANOVA with Tukey's HSD test). Data represent mean ± SD from three biological replicates (n = 9).