

Biphasic $\alpha 2\beta 1$ and Breast Cancer

Subjects: Oncology

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Integrins participate in the pathogenesis and progression of tumors at many stages during the metastatic cascade. However, current evidence for the role of integrins in breast cancer progression is contradictory and seems to be dependent on tumor stage, differentiation status, and microenvironmental influences. While some studies suggest that loss of $\alpha 2\beta 1$ enhances cancer metastasis, other studies suggest that this integrin is pro-tumorigenic. However, few studies have looked at $\alpha 2\beta 1$ in the context of bone metastasis. In this study, we aimed to understand the role of $\alpha 2\beta 1$ integrin in breast cancer metastasis to bone. To address this, we utilized in vivo models of breast cancer metastasis to bone using MDA-MB-231 cells transfected with an $\alpha 2$ expression plasmid (MDA-OE $\alpha 2$). MDA cells overexpressing the $\alpha 2$ integrin subunit had increased primary tumor growth and dissemination to bone but had no change in tumor establishment and bone destruction. Further in vitro analysis revealed that tumors in the bone have decreased $\alpha 2\beta 1$ expression and increased osteolytic signaling compared to primary tumors. Taken together, these data suggest an inverse correlation between $\alpha 2\beta 1$ expression and bone-metastatic potential. Inhibiting $\alpha 2\beta 1$ expression may be beneficial to limit the expansion of primary tumors but could be harmful once tumors have established in bone.

Keywords: tumor-induced bone disease ; breast cancer ; bone metastasis ; integrin $\alpha 2\beta 1$

1. Introduction

Breast cancer is the most common cancer among women and the second leading cause of cancer-related deaths [1]. Despite advances in early detection and therapeutic options for patients, metastatic disease remains the leading cause of patient mortality. Metastatic breast cancer cells have a high preference for bone. It is estimated that 70% of patients with metastatic disease will have bone involvement [2][3], resulting in increased fracture risk, hypercalcemia, increased morbidity and decreased quality of life [4]. Despite the high prevalence of bone metastases, the pathology and risk factors of breast cancer metastasis to bone are not fully understood.

Recent studies have revealed that the expression profile of primary tumors and composition of the surrounding extracellular matrix (ECM) are important factors contributing to tumor progression and metastasis [5][6][7]. Specifically, the expression of cell surface adhesion receptors, such as integrins, have been shown to be prognostic [8][9][10]. Integrins are $\alpha\beta$ heterodimeric transmembrane receptors that support cell adhesion to the extracellular matrix (ECM) and trigger intracellular signaling that can modify cellular behavior [11][12]. Alterations in integrin expression are commonly found in cancer and have been linked to increased tumor proliferation, invasion, and secondary site colonization, as well as decreased patient survival [13][14].

$\alpha 2\beta 1$ integrin has been implicated as an important target in cancer progression due to its critical role in a variety of cancers [15]. Studies have shown that $\alpha 2\beta 1$ integrin is a marker of malignant progression in prostate cancer [16][17][18], liver cancer [19][20], gastric cancer [21][22][23], and melanoma [24]. However, in breast cancer, there is conflicting evidence for the role of $\alpha 2\beta 1$ integrin. While some studies suggest that the loss of $\alpha 2\beta 1$ promotes breast cancer metastasis [25][26], other studies suggest that high $\alpha 2\beta 1$ expression correlates with a metastatic phenotype [19][27][28]. It is believed that $\alpha 2\beta 1$ integrin may also play an important role in bone metastasis due to its high affinity to bind collagen type 1 (which is the main component of the organic part of bone) [29]. While $\alpha 2\beta 1$ has been shown to promote skeletal metastases in prostate cancer [18][30], very few studies have looked at this integrin in the context of breast cancer metastasis to bone.

Thus, this study aimed to investigate the role of $\alpha 2\beta 1$ integrin in breast cancer metastasis to bone. We hypothesized that $\alpha 2\beta 1$ integrin promotes a bone-metastatic potential of breast cancer cells. To test this hypothesis, we used in vivo mouse models of cancer metastasis to bone (orthotopic: mammary fat pad—MFP, metastasis and colonization: intracardiac—IC, and establishment in bone: intratibial—IT). In order to investigate tumor progression and metastasis with respect to $\alpha 2\beta 1$ expression, we developed high expressing MDA-MB-231 breast cancer cells by transfecting cells with an $\alpha 2$ DNA plasmid (OE- $\alpha 2$). In this study, we demonstrate that $\alpha 2\beta 1$ integrin promotes tumor development at the primary site and metastasis to bone but has no effect on bone destruction once tumors have established in bone.

2. $\alpha 2\beta 1$ Expression Correlates with an Invasive and Migratory Phenotype

Integrin $\alpha 2\beta 1$ expression is often upregulated in metastatic cancer cells [8][31][32]. Whole exome sequencing of tumor biopsies from patients collected under the Metastatic Breast Cancer Project revealed that metastatic primary tumors have higher ITGA2 and ITGB1 copy number compared to non-metastatic primary tumors (Figure 1A). In order to study the effect of elevated $\alpha 2\beta 1$ expression on breast tumor behavior, we generated a model of MDA-MB-231 breast cancer cells with high $\alpha 2\beta 1$ by stably transfecting a bone-derived clone of MDA-MB-231 (MDA-Bone) with an expression plasmid for $\alpha 2$ (MDA-OE $\alpha 2$) or an empty vector control (MDA-Ctrl). Manipulation of integrin expression and signaling was confirmed by qPCR and western blot analysis (Figure 1B,C). Although we only introduced an $\alpha 2$ expression plasmid into the cells, we were able to achieve significantly higher mRNA and protein expression for both $\alpha 2$ and $\beta 1$ subunits compared to Ctrl cells. Downstream integrin signaling was also shown to be activated in MDA-OE $\alpha 2$ cells (Figure 1C).

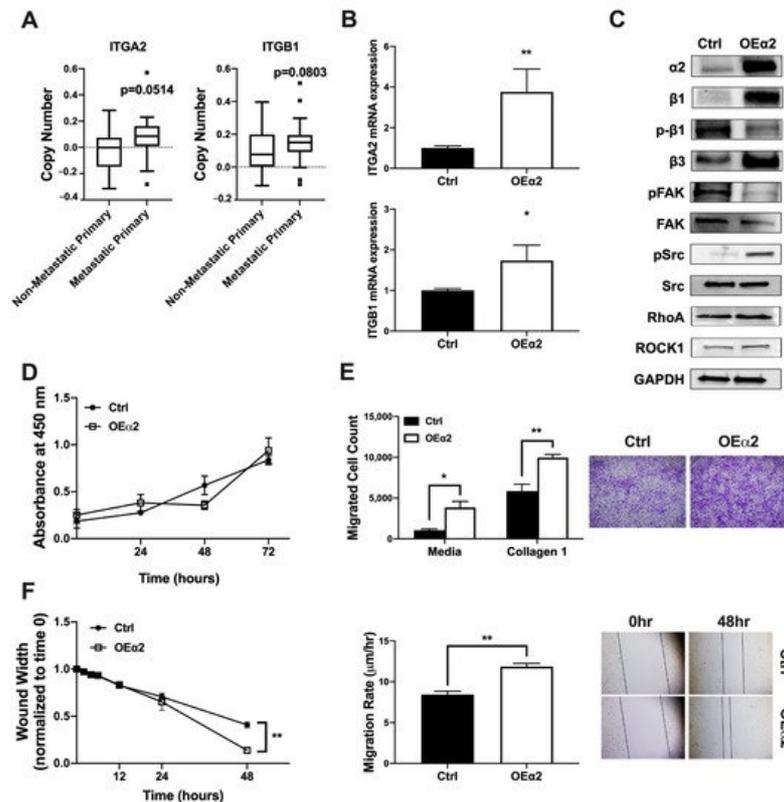


Figure 1. (A) Whole exome sequencing of tumor biopsies from patients collected under the Metastatic Breast Cancer Project was analyzed for copy number alterations in ITGA2 and ITGB1. N = 14 non-metastatic primary, N = 42 metastatic primary tumors. Mann–Whitney test. (B) qPCR and (C) western blot analysis confirmed that cells overexpressing $\alpha 2$ (OE $\alpha 2$) had increased ITGA2 and ITGB1 mRNA expression as well as increased $\alpha 2$ and $\beta 1$ subunit protein expression and activated integrin signaling. (D) An MTS proliferation assay showed no significant difference in cell growth at 24, 72, or 120 h in cells expressing high $\alpha 2$ compared to control. (E) A significantly higher number of MDA-OE $\alpha 2$ tumor cells compared to MDA-Ctrl cells migrated in a Transwell Invasion Assay using either complete media or media + Collagen 1 as a chemoattractant. (F) MDA-OE $\alpha 2$ cells migrated at a faster rate compared to MDA-Ctrl cells in a scratch assay as measured by changes in wound width over time. N = 3 biological replicates. Data presented as fold change over control (Ctrl). Student's t-test * p < 0.05, ** p < 0.01.

We further wanted to analyze these cells for changes in proliferation and invasive or migratory phenotype in response to enhanced integrin expression. While there was no change in tumor proliferation, we found that tumor cells with high $\alpha 2\beta 1$ expression were more invasive and migratory (Figure 1D,F). A higher number of MDA-OE $\alpha 2$ cells migrated in a transwell invasion assay compared to MDA-Ctrl cells. Furthermore, a scratch assay revealed an increased migration rate in MDA-OE $\alpha 2$ cells.

3. $\alpha 2\beta 1$ Integrin Promotes Primary Tumor Growth and Dissemination to Bone

Current evidence suggests that $\alpha 2\beta 1$ integrin can act as both a tumor suppressor [25][26][33] and a tumor promoter [19][27][28] in breast cancer and seems to be dependent on tumor status [34]. While most of these studies have looked at invasion and dissemination to soft tissue sites, few studies have elucidated the role of $\alpha 2\beta 1$ integrin in breast cancer dissemination to the bone. Here, we used an in vivo mammary fat pad model of human breast cancer to investigate the effect of elevated

$\alpha 2\beta 1$ expression on primary tumor growth, flow cytometry analysis to determine changes in circulating tumor cells (CTCs) and dissemination to bone, and histology analysis to investigate metastases to the lung or bone.

Tumor growth analysis revealed that breast cancer cells expressing high levels of $\alpha 2\beta 1$ have increased growth in vivo (**Figure 2A**) and larger tumors at sacrifice (**Figure 2B,C**) compared to control tumors. Immunohistochemical analysis for the $\alpha 2$ integrin subunit confirmed higher expression in MDA-OE $\alpha 2$ tumors. Interestingly, we found that $\alpha 2$ expression in the control tumors was higher at the periphery of the tumor, while $\alpha 2$ overexpressing tumors had high expression throughout the tumor (**Supplemental Figure S1**). These data support our hypothesis that $\alpha 2\beta 1$ is needed for migration and invasion from the primary site.

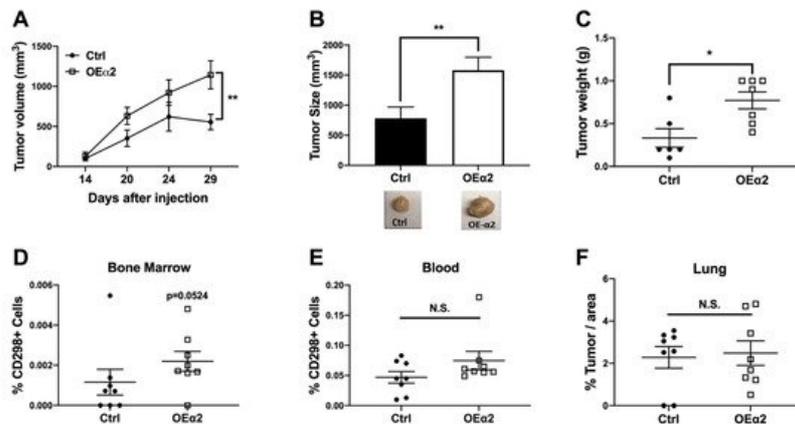


Figure 2. Four–six-week-old athymic nude mice were injected with 5×10^5 MDA-Ctrl or MDA-OE $\alpha 2$ cells into the mammary fat pad. Mice injected with MDA cells overexpressing $\alpha 2$ had increased tumor growth (**A**), larger tumors at sacrifice (**B**), and increased tumor weight (**C**) compared to mice injected with control MDAs. (**D,E**) Flow cytometry analysis was performed on bone marrow and blood using a novel protocol to detect the presence of human tumor cells using the marker CD298 (ATP1B3). Mice injected with cells overexpressing the integrin $\alpha 2$ subunit had an increased % CD298⁺ cells in the bone marrow (**D**), and a trending (n.s.) increase in the blood (**E**) compared to mice injected with control cells. (**F**) Lung metastases were quantified by histological analysis, and no difference was observed between OE $\alpha 2$ and Ctrl cells. N = 8 per group. Mice were sacrificed 30 days post tumor inoculation. Two-way ANOVA and Mann–Whitney test. * p < 0.05, ** p < 0.01.

Using a novel flow cytometry technique for detecting disseminated tumor cells in models of low tumor burden using the human cell marker CD298 [35], we analyzed plasma for the presence of CTCs and bone marrow for the presence of disseminated tumor cells (DTCs) (gating scheme can be found in **Supplemental Figure S2**). Consistent with the tumor growth analysis, we found that mice injected with MDA-OE $\alpha 2$ cells had an increase in the number of disseminated tumor cells in the bone marrow compared to mice injected with MDA-Ctrl cells (**Figure 2D**). The presence of tumor cells in the bone marrow was confirmed by histomorphometry analysis with H&E staining (**Supplemental Figure S3**). Although not statistically significant, there was a trending increase in the presence of CTCs for mice given MDA-OE $\alpha 2$ cells (**Figure 2E**). While there was an observed increase in bone metastases (**Supplemental Figure S3**), histological analysis revealed no significant difference in lung metastases (**Figure 2F**). Taken together, these data reveal that high $\alpha 2$ expression in tumors at the primary site results in increased tumor growth and increased dissemination to the bone. Higher $\alpha 2$ expression at the periphery of the tumor and the presence of CTCs also suggest that $\alpha 2\beta 1$ integrin may be creating a more invasive and metastatic phenotype in these breast cancer cells.

4. Breast Cancer Cells with High $\alpha 2\beta 1$ Expression Have Increased Colonization in the Bone, but Have No Effect on Bone Destruction

Integrins have been shown to play an important role at many stages of the metastatic cascade [13]. Specifically, $\beta 1$ integrins have been implicated in extravasation from the vasculature and colonization into secondary sites [13][32][36]. To study the role of $\alpha 2\beta 1$ integrin in tumor cell colonization of the bone, we used an in vivo metastasis model where tumor cells are introduced directly into the vasculature via intracardiac (IC) injection. Four–six-week-old female athymic nude mice were injected with MDA-OE $\alpha 2$ or -Ctrl cells. Histological analysis revealed that high expression of $\alpha 2\beta 1$ integrin on the surface of tumor cells increased the amount of tumor cells that colonized the bone but had no effect on subsequent bone destruction (**Figure 3A–C**). There was significantly higher % tumor area in the tibias of mice given MDA-OE $\alpha 2$ cells compared to mice given MDA-Ctrl cells, but no significant differences were found in bone volume (%BV/TV) by μ CT or

lesion area by X-ray. These data support our findings in the MFP model that $\alpha 2\beta 1$ expression correlates with an increase in breast tumor dissemination to bone.

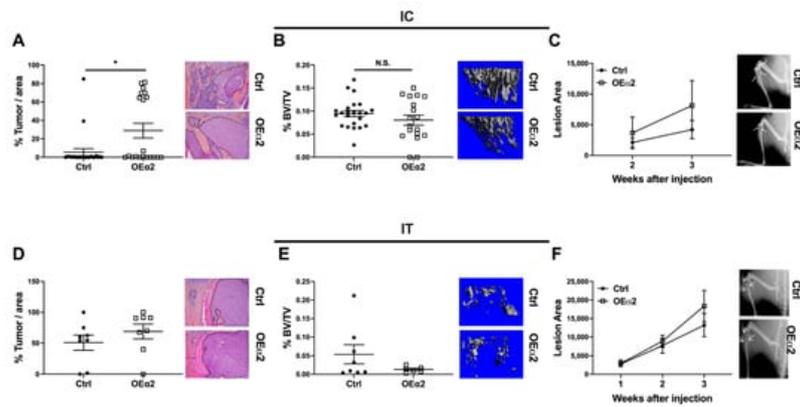


Figure 3. (A–C) Four–six-week-old athymic nude mice were injected via intracardiac (IC) injection with 1×10^5 MDA-Ctrl or MDA-OE $\alpha 2$ cells. (A) H&E staining revealed increased percentage of tumor cells in the tibias of mice injected with MDA-OE $\alpha 2$ cells compared to mice injected with MDA-Ctrl cells. (B) μ CT analysis and (C) X-ray analysis show no change in bone volume and lesion area. N = 12 mice per group, 2 bones analyzed per mouse. IC mice were sacrificed 30 days post tumor inoculation. Mann–Whitney test. * $p < 0.05$. (D–F) Four–six-week-old athymic nude mice were injected via intratibial (IT) injection with 1×10^5 MDA-Ctrl or MDA-OE $\alpha 2$ cells. (D) Histomorphometry reveals no difference in tumor area between MDA-OE $\alpha 2$ and MDA-Ctrl injected mice. (E) μ CT analysis shows no difference in bone volume, and (F) X-ray analysis shows no difference in the lesion area. N = 8 per group. IT mice were sacrificed 21 days post tumor inoculation. Mann–Whitney test.

To study the effect of $\alpha 2\beta 1$ expression on tumors that have already established in bone, we used an in vivo model of tumor growth in bone. MDA-OE $\alpha 2$ or MDA-Ctrl cells were injected in the right tibia (IT injection) of 4–6-week-old athymic nude mice. PBS was injected into the contralateral limb for a non-tumor control. Interestingly, high $\alpha 2$ expression in established bone metastases had no effect on overall tumor burden and bone destruction (Figure 3D–F). Substantial bone destruction was observed by X-ray and μ CT analysis for mice given MDA-Ctrl cells and for mice given MDA-OE $\alpha 2$ cells. H&E staining showed significant tumor burden in the tibias of both sets of mice, with no significant difference in % tumor area between OE $\alpha 2$ and Ctrl cells.

5. Osteolytic Breast Tumor Cells Have Decreased $\alpha 2\beta 1$ Expression

The in vivo data reveals $\alpha 2\beta 1$ to be a tumor promoter at earlier stages of metastasis, such as invasion and extravasation, but seem to have no effect on tumors already established in bone. To further understand the phenotype, we wanted to evaluate differences in gene expression for tumors that metastasize to bone and cause bone destruction versus primary tumors. We analyzed the mRNA and protein expression profiles of our bone-metastatic clone of MDA-MB-231 cells (MDA-Bone) and the parental MDA-MB-231 cells from ATCC (MDA-Parental) and found that bone-metastatic cells have decreased integrin signaling (Figure 4A,B). MDA-Bone cells have decreased expression of $\alpha 2$ and $\beta 1$ subunits and decreased protein expression of the downstream signaling factors SRC, RhoGTP, and ROCK. Due to its critical role in bone metastases [37], the integrin subunit $\beta 3$ was also evaluated; however, there was no significant difference in $\beta 3$ mRNA or protein expression, suggesting that these changes in integrin signaling are driven primarily by $\alpha 2\beta 1$.

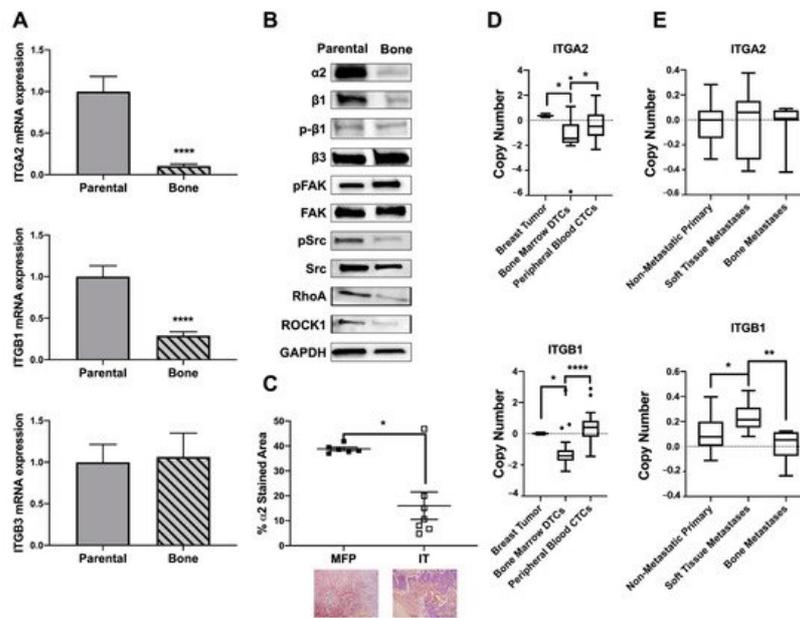


Figure 4. (A,B) A bone metastatic clone of MDA-MB-231 (Bone) and the parental MDA-MB-231 cells (Parental) were analyzed for integrin expression by (A) qPCR and (B) western blot analysis. Bone metastatic cells have less expression of the integrin subunits $\alpha 2$ and $\beta 1$ and downstream integrin signaling compared to parental cells. Data presented as fold change over parental. N = 3 biological replicates. Student's t-test. **** p < 0.0001. (C) $\alpha 2$ expression was analyzed in vivo by immunohistochemistry revealing that tumors in the bone (IT, intratibial injection) have less tumor expression of $\alpha 2$ compared to tumors in the primary site (MFP, mammary fat pad injection). N = 8 mice per group. Mann–Whitney test. * p < 0.05. (D) Microarray database analysis collected from the NCBI gene expression omnibus GEO accession GSE27574 revealed that disseminated tumor cells (DTCs) in the bone marrow have fewer copy numbers of ITGA2 and ITGB1 compared to primary breast tumors and circulating tumor cells (CTCs). N = 3 breast tumors, N = 24 DTCs, N = 28 CTCs. Kruskal–Wallis test. * p < 0.05, **** p < 0.0001. (E) Whole exome sequencing of tumor biopsies from patients collected under the Metastatic Breast Cancer Project was analyzed for copy number alterations in ITGA2 and ITGB1. Soft tissue biopsies had higher ITGB1 than non-metastatic primary tumors and tumor biopsies from bone metastases had fewer copy numbers of ITGB1 compared to soft tissue metastases. No significant difference was observed for ITGA2. N = 14 non-metastatic primary, N = 42 metastatic primary tumors, N = 8 bone metastases, N = 10 soft tissue metastases. Kruskal–Wallis test. * p < 0.05, ** p < 0.01.

This decrease in $\alpha 2\beta 1$ integrin expression in bone metastases was also observed in vivo. Immunohistochemical analysis revealed that tumors in the bone have significantly lower expression of $\alpha 2$ compared to tumors in the mammary fat pad (Figure 4C). Publicly available genome expression datasets of metastatic breast cancer patients were analyzed to confirm the clinical relevance of our findings. Single-cell microarray analysis of circulating tumor cells (CTCs) isolated from peripheral blood and disseminated tumor cells (DTCs) isolated from bone marrow aspirates of breast cancer patients [38] (GEO accession GSE27574) reveals that DTCs have a decrease in copy number of ITGA2 and ITGB1 compared to primary breast tumor samples and CTCs (Figure 4D). Whole exome sequencing from the Metastatic Breast Cancer Project [39][40] was analyzed for putative copy number alterations of ITGA2 and ITGB1 in biopsies of primary tumors with no evidence of metastatic disease (non-metastatic primary), biopsies of bone metastases, and biopsies of soft tissue metastases (Figure 4E). While no significant difference was observed for ITGA2, ITGB1 was significantly increased in soft tissue metastases, compared to primary tumors, and bone metastases had lower ITGB1 than soft tissue metastases.

6. $\alpha 2\beta 1$ Integrin Expression Is Inversely Correlated with Osteolytic Gene Expression

It is well documented that once tumors metastasize to bone, they can respond to stimuli from the bone microenvironment to adapt a bone-destructive phenotype [41][42]. Once in the bone, breast tumors begin to secrete parathyroid hormone-related protein (PTHrP) to stimulate osteoclastogenesis and bone destruction [43][44][45]. This increased bone destruction causes the release of matrix-derived proteins such as transformation growth factor β (TGF- β), which then feeds back on the tumor cells to promote further production of PTHrP [46][47], which is regulated by the transcription factor Gli2 [48][49]. To evaluate the expression patterns of genes involved in tumor-induced osteolysis with respect to $\alpha 2\beta 1$ integrin, we performed qPCR and western blot analysis for PTHrP and Gli2 (Figure 5A,B) and TGF β RII and RUNX2 (Supplemental Figure S4) in MDA-Ctrl or MDA-OE $\alpha 2$ cells, and MDA-Parental or MDA-Bone cells. MDA-MB-231 cells overexpressing $\alpha 2$ integrin had decreased PTHrP and Gli2 expression compared to bone-metastatic cells and Ctrl cells, while no significant

change was observed for TGF β RII and RUNX2. Comprehensive RNA sequencing data collected as a part of the MET500 cohort [50] was analyzed for gene expression of Gli2, PTHLH, ITGB1, and ITGA2 in metastatic breast cancer samples. Spearman correlation analysis of gene signatures in metastatic biopsies of breast cancer reveal a significant ($p < 0.001$) negative correlation between PTHLH and ITGA2 ($p < 0.001$), PTHLH and ITGB1 ($p < 0.01$), Gli2 and ITGA2 ($p < 0.001$), and Gli2 and ITGB1 ($p < 0.0001$) (Figure 5C,D).

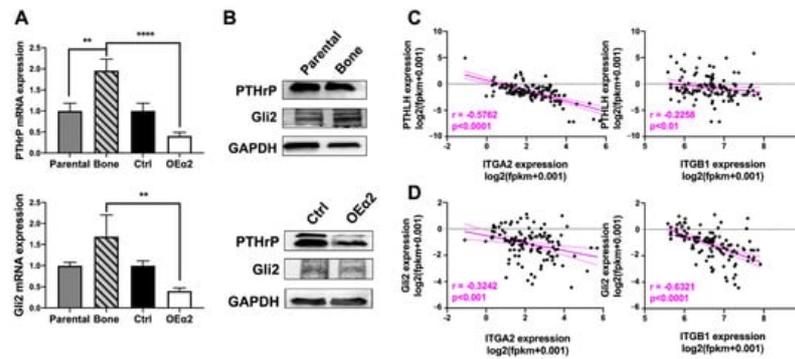


Figure 5. MDA-Parental, MDA-Bone, MDA-Ctrl, and MDA-OEc2 cells were analyzed for osteolytic gene expression by (A) qPCR and (B) western blot analysis. Tumor cells overexpressing the $\alpha 2$ integrin subunit had decreased PTHrP and Gli2 expression compared to bone and control cells (each set at 1). $N = 3$ biological replicates. Student's t-test. ** $p < 0.01$, **** $p < 0.0001$. (C,D) RNA sequencing analysis from metastatic breast cancer biopsies from the MET500 cohort was analyzed for correlation between (C) PTHLH, ITGA1, and ITGB1, and (D) Gli2, ITGA2, and ITGB1 gene signatures. Spearman correlation analysis reveal a significant negative correlation between integrin $\alpha 2\beta 1$ and osteolytic genes. $N = 120$ tumor samples.

Taken together, these data support the hypothesis that once tumors metastasize to the bone microenvironment, they undergo genetic changes and adapt a bone destructive phenotype. While expression of $\alpha 2\beta 1$ integrin plays an important role in tumor invasion, extravasation, and dissemination, once tumors establish in bone, they turn off the expression of $\alpha 2\beta 1$ and turn on expression of genes important for growth and survival in bone and bone destruction.

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