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MINDIN as a mediator of tumor-bone interaction: involvement of integrins in prostate cancer metastatic progression

Luis Álvarez-Carrión^{1,3}, Irene Gutiérrez-Rojas¹, Juan A. Ardura^{2,3}, Verónica Alonso^{1,3}

¹Bone Cancer and Metastasis Laboratory (Metastasis Unit). Department of Basic Medical Sciences. Faculty of Medicine. Universidad CEU San Pablo. CEU Universities. Boadilla del Monte, Madrid. Spain. ²Bone Pathophysiology Laboratory. Instituto de Medicina Molecular Aplicada (IMMA). Faculty of Medicine. Universidad San Pablo-CEU. Boadilla del Monte, Madrid. Spain. ³Department of Basic Medical Sciences. Faculty of Medicine. Universidad San Pablo-CEU. Boadilla del Monte, Madrid. Spain

Abstract

Bone is a dynamic organ subjected to constant remodeling, a process that depends on the coordinated activity of osteoblasts, osteoclasts, and osteocytes. Bone is also a common site of metastasis for solid tumors, as occurs in prostate cancer, whose bone metastasis is associated with a poor prognosis. Furthermore, it has been proposed that, before the establishment of metastases, tumors induce changes in distant organs, promoting the formation of premetastatic niches that favor tumor implantation. MINDIN, an extracellular matrix protein, has been identified as a potential biomarker of prostate cancer and is highly expressed in patients with bone metastases. This protein can modify both tumor cells and the bone environment, and it has been suggested that it may promote tumor growth and metastatic dissemination. MINDIN may also act as a ligand for integrins, a family of transmembrane receptors that play a key role in the regulation of tumor cell adhesion, signaling, and migration. Based on these observations, we hypothesized that MINDIN may promote the formation of the bone premetastatic niche through the modification of specific integrin expression in tumor and bone cells. A murine tumor model was used to study the influence of MINDIN on integrin expression and its possible involvement in the formation of the bone premetastatic niche, together with in vitro experimental models employing osteoblastic (MC3T3-E1), osteocytic (MLO-Y4), and prostate tumor (TRAMP-C1) cell lines. In the experimental animal model previously developed by our group, in which a primary tumor was established in the absence of overt bone metastases, an increase in *integrin β6* gene expression was observed in both the prostates and tibias of tumor-bearing mice, an effect that was reversed by MINDIN silencing. These data were confirmed in our in vitro experimental model, where TRAMP-C1 cells stimulated with MINDIN showed increased *integrin β6* expression, whereas MINDIN silencing significantly reduced its expression. In osteoblastic cells (MC3T3-E1), MINDIN also induced overexpression of integrin β6. In contrast, in osteocytic cells (MLO-Y4), MINDIN did not induce β6 overexpression, but instead increased *integrin α2* expression. These results suggest possible cell-specificity in the interaction between MINDIN and integrins, where MINDIN may favor the creation of premetastatic niches in bone through the overexpression of *integrin β6*.

Keywords:
MINDIN. Integrins.
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Bone metastases.

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Correspondence:

Verónica Alonso Rodríguez. Instituto de Medicina Molecular Aplicada (IMMA). Facultad de Medicina. Universidad San Pablo-CEU. 28925 Alcorcón, Madrid. Spain
e-mail: veronica.alonsoarodriguez@ceu.es

INTRODUCTION

Bone is a metabolically active organ that undergoes continuous remodeling throughout life. The bone remodeling process, in which bone resorption and formation are coupled, serves to adjust bone architecture to the mechanical demands placed on bone. In addition, bone is the principal storage organ for calcium, phosphorus, and magnesium. Plasma concentrations of these minerals depend on the balance between bone formation and resorption, regulated by hormones such as parathyroid hormone (PTH), calcitonin, and 1,25-dihydroxyvitamin D (1). This process is mediated by osteoblasts and osteoclasts, which carry out bone formation and resorption in a coordinated manner, respectively. Furthermore, a third type of bone cell, osteocytes, acts as a sensor of mechanical stimulation and is capable of communicating with osteoblasts and osteoclasts by modulating their activity (2,3).

Although not all solid tumors metastasize to bone, bone represents a common target organ in the metastatic dissemination of cancers such as prostate, breast, lung, kidney, and thyroid cancer, with the development of metastatic disease constituting a serious threat to the survival rate of patients who develop these types of tumors (4,5). Bone metastasis is a frequent complication in advanced stages of patients with prostate cancer, one of the tumors associated with the highest mortality and morbidity in developed countries (6). Tumors cause two different (although not mutually exclusive) types of skeletal lesions. The most common form, represented for example by breast cancer, is the osteolytic lesion, associated with altered bone remodeling caused by increased osteoclastic activity and the consequent osteolysis (7-10). On the other hand, there is a second type of lesion known as osteoblastic lesions, characteristic for example of prostate cancer, characterized by increased osteoblastic activity, increased osteoid, and an increased mineralization rate (11,12). Nevertheless, the existence of a resorptive component mediated by osteoclasts as a prior step for the establishment of osteoblastic lesions is currently recognized (11,12).

Recent studies have described prometastatic changes in organs where metastases subsequently develop (13). Such changes induce the formation of premetastatic niches that favor the implantation of tumor cells in specific target organs (13). The cellular complexity of bone, together with its continuous regulation of metabolism and bone remodeling, raises the possibility that the formation of the bone premetastatic niche is the consequence of a complex network of combined or sequential modifications in bone cell functions (14). Preventing the different stages required for tumor cells to leave the primary tumor, migrate, and establish themselves within the bone microenvironment is one of the main strategies to prevent the dissemination of bone metastases (14). The invasion of primary tumor

cells into skeletal niches is associated with the activation of bone cells that release growth factors and cytokines, which in turn promote tumor growth within metastases (4). As a result, the so-called "vicious cycle" of bone metastases is generated, altering bone physiology and disrupting bone remodeling (4).

MINDIN, also known as spondin-2, is a secreted extracellular matrix protein belonging to the class of molecules containing thrombospondin type 1 repeats. MINDIN has recently been suggested to act as a specific diagnostic biomarker for prostate cancer (15,16), with higher concentrations of this protein being found in the serum of patients with prostate cancer and bone metastases (17). Furthermore, MINDIN has been shown to be highly expressed in other tumors, such as lung cancer (18), liver cancer (19,20), colorectal cancer (CRC) (21), gastric cancer (22), clear cell renal carcinoma (23), ovarian cancer (24), breast cancer (25), pancreatic cancer (26), and Barrett adenocarcinoma (27). Although MINDIN overexpression is significantly associated with the progression of colorectal, prostate, and ovarian tumors, and is used as a predictor of poor survival prognosis, the role of MINDIN in other tumors remains controversial due to the lack of studies and available information (19,20,22,24-32). Of note, MINDIN is capable of inducing proliferation, migration, and the acquisition of bone-like molecular characteristics in prostate tumor cells, a process known as osteomimicry (33,34). Furthermore, MINDIN is capable of modifying the expression of osteoblastic and osteoclastic markers within the bone environment, with increased gene expression of tartrate-resistant acid phosphatase (TRAP) and osterix, as well as increased TRAP protein expression. These changes have suggested that MINDIN may favor tumor progression and the formation of bone metastases (33,34).

Integrins are heterodimeric transmembrane receptors composed of alpha and beta subunits that function as anchoring and cellular signaling molecules (35). There are 18 alpha subunits and 8 beta subunits, giving rise to at least 24 known distinct integrin heterodimers with different functions and ligand-binding activities. Integrins act as anchoring molecules by mediating adhesion between the cellular cytoskeleton and the extracellular matrix (ECM). They also function as bidirectional signaling molecules by mediating signaling from the outside to the inside of the cell and vice versa, thereby controlling a series of vital cellular functions such as adhesion, polarity, differentiation, migration, and cell division (36-38). In pathological states, defective integrin signaling may alter these functions and lead to abnormal cell division, migration, and adhesion, which are characteristic features of cancer and metastasis (39-43). It has been observed that tumor processes are associated with dysregulation of integrin expression, in some cases showing overexpression ($\beta 1$, $\beta 3$, $\beta 6$) and in others decreased expression ($\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$) (39-44) (Table I). Former studies have demon-

Table 1. Summary of integrin subunits involved in tumor development and metastasis

Integrin α/β subunit	Dysregulated expression	References
$\alpha 2$	Decreased expression in prostate adenocarcinoma and increased expression in bone metastases	47-50
$\alpha 3, \alpha 4, \alpha 5, \alpha 7$	Decreased expression in prostate adenocarcinomas	47,48
$\alpha 6$	Overexpressed in bone metastases	48,51,52
$\beta 1$	Increased expression in prostate adenocarcinoma	53,54
$\beta 3$	Expressed in prostate adenocarcinoma and metastatic lesions; absent in normal cells	53,54
$\beta 6$	Expressed in prostate adenocarcinoma and bone metastatic lesions; absent in normal cells	55,56

stated that the MINDIN protein can interact with integrins in neutrophils and that this MINDIN-integrin interaction is essential for the recruitment of inflammatory cells in certain *in vivo* models (45,46). However, it remains unknown whether MINDIN can alter the integrin expression profile in tumor and bone cells to promote tumor progression and bone metastasis processes (45,46).

Based on all these observations, we hypothesized that MINDIN may modify the integrin expression profile in bone and prostate tumor cells to potentially favor the development of bone metastases. To this end, in the present study we investigated the effects of the protumoral protein MINDIN on the gene expression profile in bone cells (osteoblasts and osteocytes) and prostate tumor cells of those integrins that are key in the development of bone metastases.

MATERIALS AND METHODS

ANIMAL MODEL

Three-month-old male C57BL/6 mice (Charles River, Wilmington, MA, United States) were used and maintained in cages under standard conditions: room temperature of $20 \pm 0.5^\circ\text{C}$, relative humidity of $55 \pm 5\%$, and a 12 h/12 h light/dark cycle. Animals remained unrestricted in movement and received a standard pellet diet (Teklad Global 18 % Protein Rodent Diet, Envigo, Madison, WI) and tap water *ad libitum*.

Surgical intervention was performed under aseptic conditions with mice anesthetized by isoflurane in-

halation and administration of xylazine (10 mg/kg) and ketamine (25 mg/kg). Through a lower midline abdominal incision, 50 μL of vehicle (PBS), 5×10^5 TRAMP-C1 cells transfected with scrambled siRNAs, or 5×10^5 TRAMP-C1 cells transfected with three siRNAs directed against MINDIN (s97640, s97638, s87252; Life Technologies, Paisley, United Kingdom) were injected into the right posterior prostatic lobe. An injection was considered technically correct when the bleb was localized within the prostatic lobe. After the intervention, the abdominal cavity, muscle, and skin were closed with surgical staples.

One month later, primary prostate tumors and tibias were extracted and stored in Trizol (Thermo Scientific) for subsequent RNA extraction and analysis by quantitative real-time PCR. All procedures were approved by the Institutional Animal Care and Use Committee of Universidad San Pablo CEU (Madrid, Spain).

CELL CULTURES

For the experiments of the present study, the following cell lines were cultured at 37°C with 5 % CO_2 and 95 % humidity in media supplemented with 1 % streptomycin/penicillin: mouse mesenchymal preosteoblastic cells (MC3T3-E1 subclone 4, ATCC, CRL-2593). These cells were cultured in alpha MEM medium without ascorbic acid ("minimum essential medium", Thermofisher, A1049001) supplemented with 10 % FBS (fetal bovine serum) and 1 % L-glutamine. Murine osteocytic MLO-Y4 cells (generously provided by Lynda Bonewald) were cultured in alpha MEM medium (Thermofisher, 22571038) supplemented with 2.5 % FBS and 2.5 % calf serum (CS; Thermofisher, 16010159). We used the murine prostate adenocarcinoma cell line TRAMP-C1 (ATCC® CRL-2730 TM), cultured in DMEM medium ("Dulbecco's Modified Eagle Medium", Thermofisher, 11965092) supplemented with 10 % FBS and 1 % L-glutamine.

Cells were seeded in 6-well culture plates and, after 24 hours, stimulated with MINDIN (5 ng/mL) for 6 or 24 hours. Following stimulation, each sample was collected in Trizol for subsequent total RNA extraction and quantitative real-time PCR analysis.

CELL SILENCING

TRAMP-C1 cells were silenced with a mixture of three siRNAs (20 nM each) directed against different coding sequences of murine *MINDIN* (s97640; s97638; s87252) (Life Technologies, Paisley, United Kingdom) using Lipofectamine RNAiMax (Life Technologies) and following the manufacturer's instructions. A scrambled

silencer not directed against any specific genomic sequence (control siRNA-A, Santa Cruz Biotechnology, Dallas, TX) was used as a negative control to verify the specificity of the MINDIN siRNA-dependent changes in the different evaluated parameters. The efficacy of MINDIN silencing was evaluated by real-time PCR: at 24 hours it was 0.05 ± 0.0060 vs 1 ± 0.03 ; $p < 0.0001$; at 48 hours, 0.15 ± 0.1 vs 1 ± 0.07 ; $p < 0.001$; and finally, after 15 days, 0.4 ± 0.13 vs 1 ± 0.01 ; $p < 0.001$.

REAL-TIME POLYMERASE CHAIN REACTION (PCR)

Total RNA was isolated using a standard procedure (Trizol, Life Technologies), and 2 µg of RNA from each sample were reverse-transcribed using a high-capacity cDNA reverse transcription kit ("High Capacity RNA to cDNA Kit", ThermoFisher, 4387406) following the manufacturer's instructions.

Real-time PCR expression analysis was performed based on fluorescence emission from the fluorochrome SYBR Green premix ex Taq (Takara, Otsu, Japan), using an ABI PRISM 7500 system (Applied Biosystems). The number of mRNA copies was calculated for each sample using the threshold cycle (Ct) value. The *18S* or β -*ACTIN* genes (used as constitutive expression controls) were amplified in parallel as control genes, allowing the calculation of the number of amplification cycles required to reach an arbitrary Ct intensity. Relative gene expression was defined as relative expression compared with the control, calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\text{treatment}} - \Delta Ct_{\text{control}}$ and $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{control gene}}$ (*18S*/ β -*ACTIN*). Gene expression from animal samples was represented as individual data points by calculating $2^{-\Delta Ct}$, as previously described (57). The specificity of each amplicon was confirmed by the presence of a single peak in the dissociation curve for each qPCR reaction.

STATISTICAL ANALYSIS

Data are expressed as means $\pm$ standard error. Data distribution was analyzed and, since they did not fit a normal distribution, differences between experimental groups were evaluated using non-parametric analysis of variance (Kruskal-Wallis). Determination of possible differences between experimental groups was performed using Dunn's or Mann-Whitney tests. GraphPad Prism software was used for statistical analyses and outlier detection. Outlier exclusion followed standard criteria based on standard deviation according to software analysis. A value of $p < 0.05$ was considered statistically significant.

RESULTS

MINDIN EXPRESSION IN PRIMARY PROSTATE TUMORS MODIFIES INTEGRIN GENE EXPRESSION IN THE PROSTATES AND TIBIAS OF MICE

In the present study, we characterized the gene expression patterns of integrins that could favor the metastasis of prostate tumor cells within the bone microenvironment. We used a C57BL/6 mouse model that recapitulates the progression of prostate cancer from hyperplasia to prostatic intraepithelial neoplasm, potentially metastasizing to lymph nodes, lungs, and bone. In this previously developed model, 1 month after orthotopic injection of TRAMP-C1 prostate cancer cells, overexpression of the tumor marker *PSCA* (1966 ± 834 vs 1 ± 0.4 ; $p = 0.024$) and *MINDIN* (714.25 ± 348.32 vs 1 ± 0.3 ; $p < 0.0001$) (34) was observed in the tumor group compared with the control group, indicating establishment of the primary tumor at this time point. Prostate tumors generated by TRAMP-C1 cells silenced for MINDIN showed a drastic decrease in *MINDIN* expression (28.57 ± 16.6 vs 714.25 ± 348.32 ; $p = 0.0012$) without affecting expression of the tumor burden marker *PSCA* (1136.3 ± 315.3 vs 1966 ± 834 ; $p < 0.43$), compared with the tumor group expressing *MINDIN* (33,34). In this model, since no changes in *PSCA* or *MINDIN* expression were observed in the tibias, we can conclude that tumor metastasis to bone had not occurred at the studied time point (33).

However, we had previously observed changes in the bones of this model, reflected by an increase in gene expression of the bone resorption marker TRAP and modification of bone microarchitecture; these changes were reversed when *MINDIN* was silenced in the primary tumor (33).

Since changes to integrin levels have been described during tumor progression, suggesting that they may be molecules through which MINDIN exerts its actions, we performed an integrin profiling study. In this model, among the different key integrins involved in bone metastases that were studied (Figs. 1A-E and 2A-E), establishment of prostate tumors with *MINDIN* overexpression induced only an increase in *integrin $\beta 6$* gene expression in primary prostate tumors (Fig. 1E) and mouse tibias (Fig. 2E). The observed changes were reversed when MINDIN was silenced in the primary prostate tumor (Figs. 1E and 2E). These results indicate that MINDIN is capable of modulating *integrin $\beta 6$* expression both in the primary tumor and in bone, where the tumor does not yet appear to be established. This could suggest that modification of the integrin profile may be considered a change that promotes formation of the bone premetastatic niche.

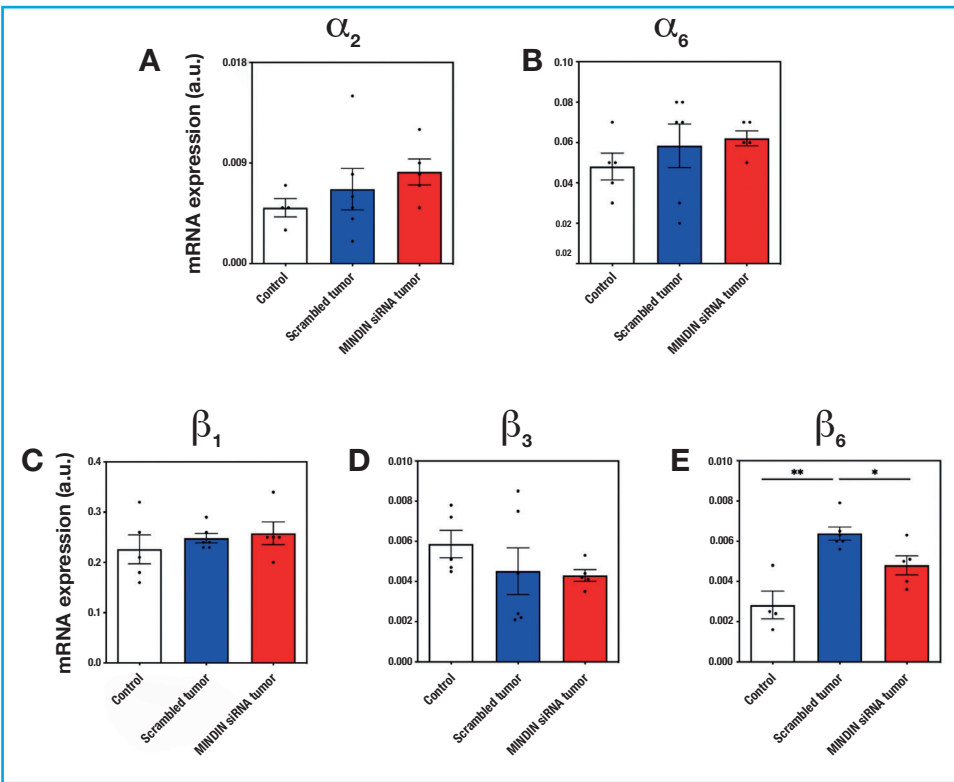


Figure 1. MINDIN induces integrin β_6 overexpression in prostate tumors in an in vivo mouse model. mRNA expression of integrins (A) α_2 , (B) α_6 , (C) β_1 , (D) β_3 , and (E) β_6 in mouse prostates evaluated by real-time PCR. Data are presented as mean $\pm$ SEM ($n = 4-6$ per group). $p < 0.05$; ** $p < 0.01$. The samples used were obtained from a previously developed and published animal model (33,34). The *18S* gene was used as the endogenous control.

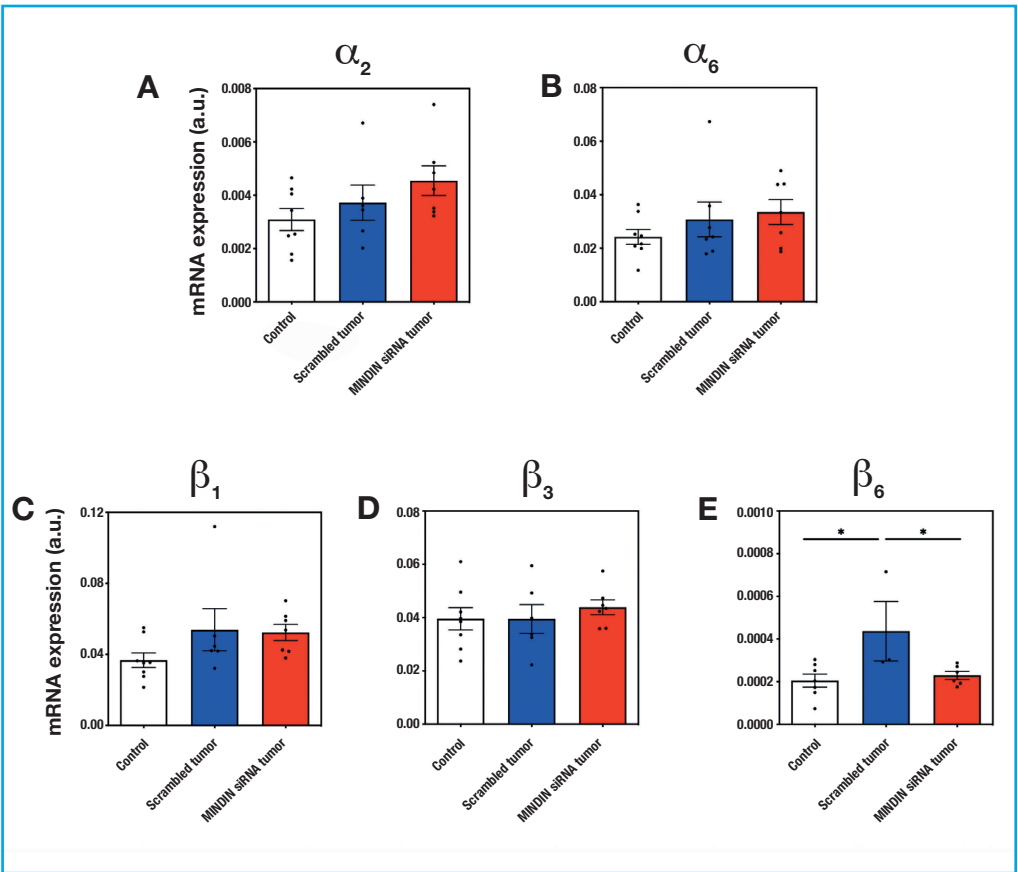


Figure 2. MINDIN induces integrin β_6 overexpression in tibias of mice with prostate tumors. mRNA expression of integrins (A) α_2 , (B) α_6 , (C) β_1 , (D) β_3 , and (E) β_6 in mouse tibias evaluated by real-time PCR. Data are presented as mean $\pm$ SEM ($n = 3-7$ per group). $p < 0.05$; ** $p < 0.01$. The samples used were obtained from a previously developed and published animal model (33,34). The *18S* gene was used as the endogenous control (33,34).

MINDIN INDUCES MODIFICATIONS IN INTEGRIN GENE EXPRESSION IN PROSTATE TUMOR CELLS

MINDIN has been reported to increase adhesion of different cell types to the extracellular matrix, including neutrophils, macrophages, lymphocytes, and prostate tumor cells (46,58). Therefore, since integrins are key transmembrane proteins involved in cell adhesion to the matrix, and considering the observed modification in *integrin $\beta 6$* gene expression in the primary tumor, we investigated whether MINDIN secretion by prostate tumors could modify integrin expression to increase adhesion of prostate adenocarcinoma cells to bone surfaces. To this end, we used TRAMP-C1 prostate tumor cells stimulated with MINDIN. An increase in *integrin $\beta 6$* expression was observed following stimulation with MINDIN for 6 or 24 hours (Figs. 3A-B). Furthermore, MINDIN silencing for 24 hours in TRAMP-C1 tumor cells caused a significant decrease in *integrin $\beta 6$* expression (Fig. 3C). Although an increase in *integrin $\alpha 2$* expression levels was observed after 24 hours of stimulation with MINDIN, this increase did not reach statistical significance ($p = 0.117$).

MINDIN INDUCES CHANGES TO INTEGRIN GENE EXPRESSION IN OSTEOBLASTIC AND OSTEOCYTIC CELLS

To determine whether MINDIN could also modify the integrin expression profile in bone cells, we analyzed

the effects of MINDIN in different bone cell types, including MC3T3-E1 osteoblastic cells and MLO-Y4 osteocytic cells. In MC3T3 osteoblastic cells, an increase in *integrin $\beta 6$* gene expression was observed after both 6 and 24 hours of stimulation with MINDIN (Figs. 4A-B). However, in MLO-Y4 cells, no increase in *integrin $\beta 6$* expression was observed ($p = 0.1$) after 6 hours of stimulation with MINDIN; instead, an increase in *integrin $\alpha 2$* expression was detected. These data suggest that, depending on the target cell type, MINDIN may modify the expression of different integrins (Figs. 4B-C).

Taken together, the data from the present study indicate that MINDIN can modify the transcriptional profile of several integrins associated with tumor progression and the establishment of metastases in both bone and tumor cells.

DISCUSSION

Bone metastases are painful, difficult to cure, and associated with a poor survival prognosis. Primary tumors create premetastatic niches favorable to tumor cells in secondary organs that subsequently lead to the development of metastases (59). Furthermore, during the initial phase of premetastatic niche formation, primary tumor cells produce various soluble factors to trigger the formation of an immature premetastatic niche (59). Therefore, identifying the factors that regulate tumor-bone interactions and increasing our

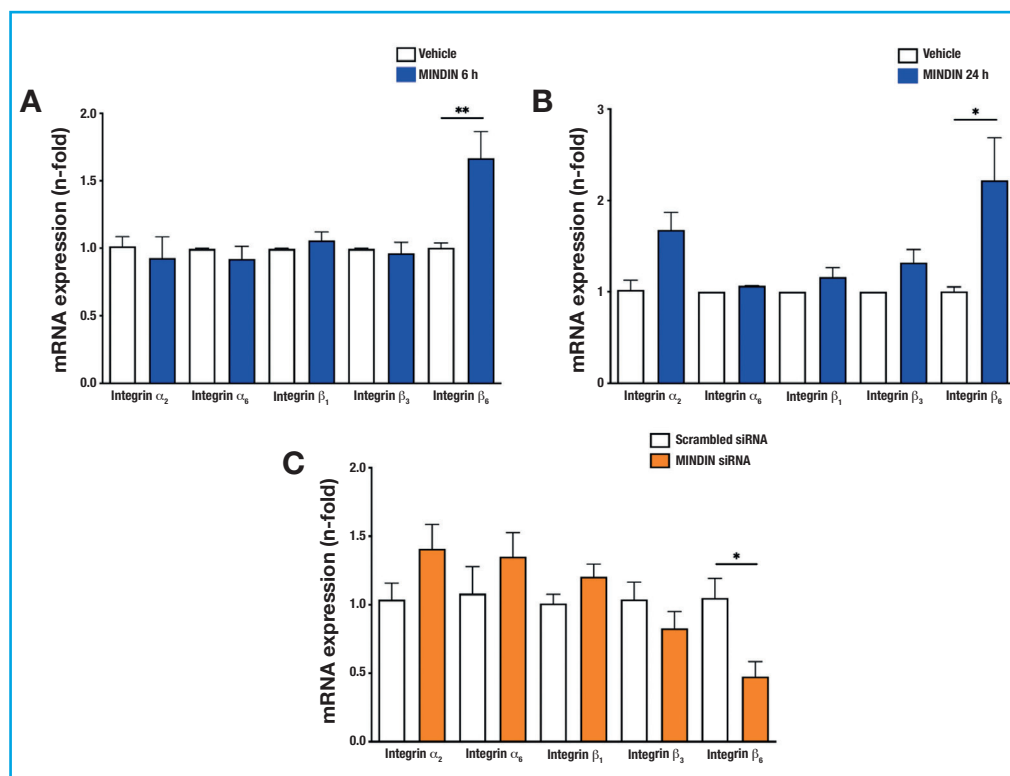


Figure 3. MINDIN induces *integrin $\beta 6$* overexpression in prostate tumor cells. mRNA expression of integrins $\alpha 2$, $\alpha 6$, $\beta 1$, $\beta 3$, and $\beta 6$ in TRAMP-C1 prostate tumor cells stimulated with MINDIN for (A) 6 h, (B) 24 h, or (C) with MINDIN silenced, evaluated by real-time PCR. Data are expressed as mean $\pm$ SEM of 3 independent experiments performed in triplicate. $p < 0.05$ vs control; ** $p < 0.01$ vs control. The β -ACTIN gene was used as the endogenous control.

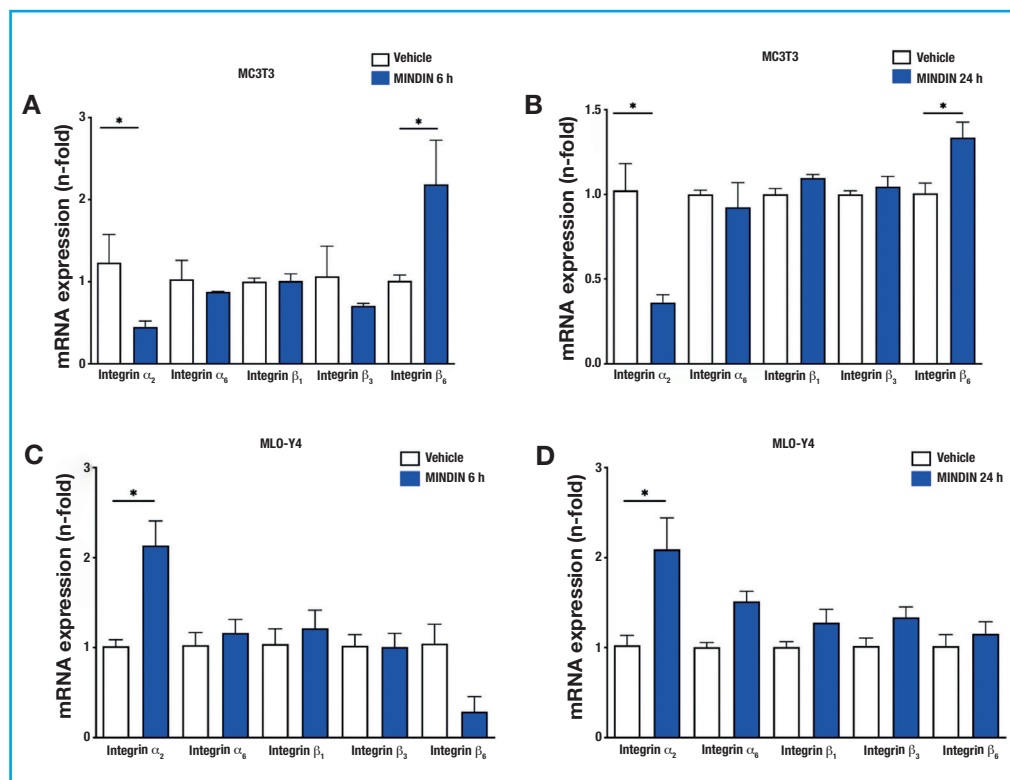


Figure 4. MINDIN induces *integrin β_6* overexpression in osteoblasts and *integrin α_2* overexpression in osteocytes. mRNA expression of integrins α_2 , α_6 , β_1 , β_3 , and β_6 in MC3T3 osteoblasts stimulated with MINDIN for (A) 6 h or (B) 24 h, and osteocytes stimulated with MINDIN for (C) 6 h or (D) 24 h, evaluated by real-time PCR. Data are presented as mean $\pm$ SEM of three independent experiments performed in triplicate. $p < 0.05$. The β -ACTIN gene was used as the endogenous control.

knowledge of the mechanisms controlling them are key to developing new therapeutic approaches. In the present study, we identified MINDIN, an extracellular matrix protein secreted by prostate tumors, as a factor capable of inducing and modulating integrin gene expression in both the primary tumor and bone. These changes in integrin expression could increase tumor cell adhesion to bone and favor the development of bone metastases, making them potential therapeutic targets against prostate cancer progression.

Dysregulation of *MINDIN* gene expression has been documented in several human tumors, such as prostate cancer (15,16), gastric cancer (60), and ovarian cancer (61). *MINDIN* expression has been described as being higher in samples from patients with more aggressive prostate cancer and poorer prognosis, and even higher in those with bone metastases (16,17). Evaluation of MINDIN by immunostaining in tumors with different Gleason scores revealed that MINDIN levels were highest in individuals with prostate cancer and bone metastases, followed by individuals without bone metastases, hyperplasia, and controls (16,17). MINDIN has also been shown to be a ligand for integrins, with MINDIN-integrin interactions being critical for the recruitment of neutrophils, macrophages, and T lymphocytes in inflammatory *in vivo* models (46,58). Furthermore, MINDIN has been observed to regulate Rho GTPase expression after interacting with $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins, contributing to tumor development in colon carcinoma and hepatocellular carcinoma (58).

In male C57BL/6 mice from a previously published animal model (33,34), we observed that tumors induced by TRAMP-C1 prostate tumor cells caused changes in integrin gene expression, producing an increase in MINDIN-dependent *integrin β_6* expression in primary prostate tumors. Although no changes in *MINDIN* or *PSCA* expression were observed in bone, indicating the absence of detectable bone metastases, an increase in *integrin β_6* expression was observed in the tibias of tumor-bearing mice compared with controls. These changes could be related to modifications in *TRAP* gene expression and bone microarchitecture that had been previously observed (33). These results suggest that changes in integrin expression could be associated with the formation of bone premetastatic niches. However, further studies are required to confirm whether this increase is dependent on MINDIN.

Former data from our group revealed that MINDIN could be a key factor promoting increased adhesion of prostate adenocarcinoma cells to bone surfaces (34). Furthermore, MINDIN has been shown to induce activation of the ERK kinase pathway (34) in prostate adenocarcinoma cells. Increased adhesion generally requires increased expression of matrix-binding proteins such as integrins (62). It has also been documented that the MINDIN protein is capable of interacting with different integrins in immune and tumor cells (60,63). Therefore, it is possible that MINDIN binds to specific integrins in prostate cancer cells to enhance their adhesion to the bone extracellular matrix. In this

regard, a relationship between integrins and the Fak and Src kinases in cell adhesion has been observed (64). Former data show the suppressive action of Fak and Src kinase inhibitors on the ability of bone cells to promote adhesion of TRAMP-C1 cells, suggesting an interaction between these kinases and integrins in the adhesion of prostate cancer cells to bone (33). However, it remains unknown which integrins may be involved in the interaction between tumor cells and MINDIN to promote cell adhesion (33). In the present study, we demonstrated that stimulation of prostate tumor cells with MINDIN induces an increase in *integrin* $\beta 6$ expression, an increase that is reversed when *MINDIN* is silenced in tumor cells, indicating a possible dependence of $\beta 6$ expression on *MINDIN*. It should be emphasized that several studies have shown that integrin $\alpha \beta 6$ actively participates in TGF- β activation (56,65), extracellular matrix remodeling through regulation of *MMP2* expression (56), and facilitation of endothelial adhesion (66), which would favor colonization of the bone environment. However, our data do not necessarily imply that *MINDIN* binds directly to integrin $\beta 6$.

Progression to invasive and metastatic carcinoma involves profound changes to the epithelial structure and function, as well as bone alterations to accommodate metastatic development. Integrins are essential mediators of tumorigenesis, adhesion, and migration. In particular, high $\beta 6$ expression in primary colon carcinomas, squamous cell carcinomas, pancreatic tumors, and breast tumors has been shown to act as a prognostic marker of aggressive disease and increased mortality in these patients (67-70). It has been observed that the *integrin* $\beta 6$ expression pattern is highly heterogeneous in primary prostate tumors; its expression is increased in cases of metastatic bone prostate cancer, whereas its expression is undetectable in neuroendocrine prostate tumors (55,56,71,72).

On the other hand, binding of *MINDIN* to integrins on osteoblasts and osteocytes could trigger signaling in these bone cells to facilitate adhesion of prostate tumor cells. The effects of *MINDIN* on bone are not restricted solely to modification of integrin expression, but also include actions of this protein on osteoblast proliferation, differentiation, and stimulation of the β -catenin signaling pathway, as well as modulation of different genes involved in bone formation and remodeling (for example, *Runx2*, *osteocalcin*, *osterix*, *OPG*, and *RANK-L*) (33). In the present study, we observed that stimulation with *MINDIN* induced an increase in *integrin* $\beta 6$ expression in osteoblasts and *integrin* $\alpha 2$ expression in osteocytes, as well as a decrease in $\alpha 2$ expression in osteoblasts, suggesting that, depending on the cell type, *MINDIN* may modify the expression of different integrins. Although not widely described, integrin $\alpha 2 \beta 1$ is a principal receptor for type I collagen, the predominant component of bone, and it has been demonstrated

that tumor cells with high $\alpha 2 \beta 1$ expression exhibit greater affinity for bone tissue due to their ability to adhere to collagen (73).

However, in the animal model, only modifications in *integrin* $\beta 6$ were observed, despite osteocytes being the predominant cell type in bone. These changes observed in mice with prostate tumors induced by direct implantation of TRAMP-C1 cells into the prostatic lobe could be due to osteoblasts being more active cells (particularly in terms of collagen production and bone formation) and possessing a cellular machinery different from that of osteocytes, which may influence their ability to respond differently to proteins transported through the serum, such as *MINDIN*. In addition, because an early premetastatic model was used (1 month of nonmetastatic primary tumor formation), it is likely that the concentrations of *MINDIN* that may have entered the matrix interior, where osteocytes are located, were still too low to observe modifications in the integrins expressed by osteocytes.

A more detailed understanding of the molecular changes involved in the formation of the bone premetastatic niche, as well as the secretome factors that induce it, could provide new therapeutic targets or intervention protocols, thereby improving prognosis. The present data show that *MINDIN* expression in primary prostate tumors promotes changes in integrin expression associated with modifications of the bone microenvironment and gene expression in prostate tumor cells. Our data may suggest that *MINDIN* favors tumor progression and the creation of premetastatic niches in bone through overexpression of *integrin* $\beta 6$ in tumor and osteoblastic cells. However, further studies (including immunohistochemical analyses) are required to elucidate the changes occurring in the different cell types, as well as studies aimed at clarifying how *MINDIN* may influence *integrin* $\beta 6$ overexpression and promote tumor growth.

Although our study provides new insights into the role of *MINDIN* in integrin regulation and the possible formation of bone premetastatic niches in prostate cancer, it presents several limitations. First, the experimental models used, both *in vitro* (osteoblastic, osteocytic, and tumor cell lines) and *in vivo* (murine model), may not fully reproduce the complexity of the tumor and bone microenvironment in humans. Furthermore, the murine model employed did not develop established bone metastases, which limits direct evaluation of metastatic progression under real clinical conditions. Another aspect to consider is that the sample size in the animal experiments was limited. It should be emphasized that current data point toward a direct effect of *MINDIN* on integrin $\beta 6$ expression, although an individualized correlation study would be a valuable approach for future investigations. Finally, although differential modulation of integrins by *MINDIN* was demonstrated in different cell types, additional studies

are required to confirm the molecular mechanisms involved and their relevance in human contexts. Future investigations using more representative models and larger sample sizes will be necessary to validate and expand these findings.

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