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# Total Knockdown of LMW-PTP in MDA-MB-231 Cells Reduces Osteoclastogenesis

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**Abstract.** *Background: Low molecular weight protein tyrosine phosphatase (LMW-PTP) has been related to tumorigenesis, having both oncogenic and anti-oncogenic roles. The differential roles of its main active isoforms (fast and slow) may account for these discrepancies. The fast isoform has been described to be involved in the bone-metastatic process, although knockdown of the slow isoform was recently reported to reduce osteoclastogenesis. We aimed to study the influence of LMW-PTP isoforms on osteoclast differentiation. Materials and Methods: Osteoclast precursors (RAW 264.7) were cultured with conditioned medium from MDA-MB-231 breast cancer cells with total knockdown of LMW-PTP and with knockdown of the slow isoform of LMW-PTP. Tartarate-resistant acid phosphatase (TRAP) staining and quantification were performed to assess osteoclast differentiation. Results: Total knockdown of LMW-PTP, but not of slow LMW-PTP significantly reduced osteoclast differentiation of RAW 264.7 cells. Conclusion: We suggest that total LMW-PTP increases osteoclastic differentiation, albeit not at the expense of the slow isoform.*

Breast cancer is the leading cause of cancer death among females worldwide (1). Importantly, bone metastases are responsible for decreased survival and quality of life among these patients (2).

Several factors have been identified as mediating bone colonization. Once in bone, breast cancer cells indirectly stimulate osteoclastogenesis through paracrine mutual interactions between tumor cells and different bone cell types. This model constitutes the vicious cycle of bone metastasis and gives rise to predominantly lytic bone lesions (3).

Tyrosine phosphorylation is relevant in numerous physiological processes and aberrant tyrosine signaling is a remarkable feature in cancer biology, including the metastatic process (4). This recognition led to an extensive study of protein tyrosine kinases as cancer therapeutic targets and some of them are already used in clinical practice. More recently, protein tyrosine phosphatases (PTPs) have been recognized as being important in the control of the phosphorylation status, regulating many physiological and pathological processes (5).

Low molecular weight-PTP (LMW-PTP) has drawn particular attention because it has been associated with both breast cancer (6) and bone metabolism, either by regulating osteoblast differentiation and adhesion or through the modulation of the sarcoma (SRC) kinase activity (7-9).

LMW-PTP is encoded by the acid phosphatase 1 (*ACP1*) gene (2p25.3) in the human genome and through a process of alternative splicing produces two main functional isoforms, named fast and slow based on their electrophoretic mobility (10, 11).

Overexpression of LMW-PTP has been demonstrated in a variety of human cancer types, including breast cancer, but its role in tumor biology is still unclear and controversial (12). Both oncogenic and anti-oncogenic properties have been reported, depending on the association with different substrates and activation status, finely regulated by cysteine oxidation and tyrosine phosphorylation (6, 13, 14).

In a conciliatory view arising from different studies (15-19), Alho *et al.* proposed that the two functional isoforms may act differentially and in opposite directions, the fast isoform having a more important role in a later stage of tumor development and the slow isoform being more relevant in an earlier stage, as the former is mainly involved in migration (through a Ras homolog gene family, member A [RhoA]-dependent mechanism) and the latter in cellular growth arrest (15, 18).

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Challenging these conclusions, it was recently documented that the knockdown of the slow isoform reduced osteoclastic differentiation, possibly *via* regulation of the SRC kinase activity and interleukin (IL)-8 secretion, which suggests a role for this isoform in breast cancer-derived bone metastases (20).

Such contradictory results prompted us to design a study that is methodologically similar to the one reporting these findings. We aimed to clarify the role of LMW-PTP isoforms derived from breast cancer cells in modulating osteoclastogenesis, using a different cell line, in order to address the validity of these conclusions across different models of breast neoplasms.

## Materials and Methods

*MDA-MB-231 cell culture*. The MDA-MB-231 breast adenocarcinoma cell line, derived from a metastatic pleural effusion, was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Foster City, CA, USA), supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin and 10 μg/ml streptomycin (ThermoFisher Scientific, Waltham, MA, USA), in a humidified atmosphere of 5% $CO_2$ at 37˚C.

*Knockdown of LMW-PTP isoforms*. Five different siRNA sequences were designed to specifically totally knockdown expression of LMW-PTP (GenBank NC_000002.11) (CI#1 5'-GCA AGA CAG ATT ACC AAA GAA-3'; CII#2 5'-GCC TGT TGS GAC TTA GAT AAT-3'; CIII#3 5'CTA TGT ATG GAT GAA AGC AAT3'; CV#5 5'-GAA CTA CTT GGG AGC TAT GAT-3') and knockdown that of the slow isoform (GenBank NM_007099.3) (CIV#4 5'- GCC CAT AAA GCA AGA CAG ATT-3'). One scramble non-targeted siRNA was used as control. Since the lentiviral vectors were part of a pre-existing library that did not include siRNAs for specific knockdown of the fast isoform, we only used the existing siRNAs.

Infection was performed after plating $3.5\times10^4$ cells per well in 96-well plates, followed by incubation with the lentiviral vectors for 1 h 30 min at 2,200 rpm at 37°C. MDA-MB-231 cells with positive knockdown were selected with puromycin after determining the optimal concentration of 0.3 μg/ml. Efficiency of infection was determined by real-time reverse transcription polymerase chain reaction (RT-PCR) using TaqMan® with primers/probe specific for each of the isoforms and using the Human glyceraldehyde-3-phosphate dehydrogenase (GAPD) Endogenous Control (Applied Biosystems, Foster City, CA, USA).

*RNA isolation and RT-PCR*. Cellular mRNA was isolated with the ISOLATE II RNA Mini Kit® (Bioline, London, UK). RNA was quantified by absorbance at 260 nm and purity was determined by absorbance at 280 and 310 nm (NanoDrop; ThermoFisher Scientific, Waltham, MA, USA).

RNA (1 μg) was converted to cDNA using the Tetro cDNA Synthesis Kit® (Bioline). cDNA (20 ng) was then amplified in a LightCycler® 480 (Roche) real-time RT-PCR using the following TaqMan® Gene Expression Assays (Applied Biosystems): acid phosphatase 1, soluble (ACP1 Hs00962877 g1), acid phosphatase 1 fast isoform, soluble (ACP1 fast isoform, Hs00964348 g1), acid phosphatase 1 slow isoform (ACP1 slow isoform, Hs00246642 m1). Results were normalized to real-time RT-PCR of glyceraldehyde 3-phosphate dehydrogenase (GAPD) using the Human GAPD Endogenous Control (4333764F; Applied Biosystems) and are expressed using the ddCt method.

*RAW 264.7 culture with conditioned medium*. The RAW 264.7 mouse monocytic cell line for osteoclast differentiation was maintained in DMEM, with 10% heat inactivated FBS and 1% penicillin/streptomycin.

For osteoclast differentiation, RAW 264.7 were plated at 960 cells per well in 96-well plates and supplemented with differentiation medium, according to the supplier's specifications: α-Minimum Essential Medium (ThermoFisher Scientific, Waltham, MA. USA) with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Receptor activator of nuclear factor kappa-B ligand (RANKL) (100 ng/ml) was then added to all wells except those of the negative control.

On day three, cultures were supplemented with 60% of conditioned medium from each selected knockdown clone plus 40% of differentiation medium with RANKL (100 ng/ml), as optimized by Alho *et al*. (20). Controls were only supplemented with differentiation medium containing 100 ng/ml of RANKL, except for the negative control to which no RANKL was added. On day five, samples were fixed and stained for osteoclast-derived tartarate-resistant acid phosphatase (TRAP) and supernatants were collected for TRAP quantification. All experiments were performed in sextuplicates.

*TRAP staining*. Cells were stained for TRAP using the commercial Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich, St. Louis, MO, USA) without the final step with acid hematoxylin. Osteoclasts were identified as TRAP-positive cells with more than three nuclei.

*TRAP quantification*. Supernatants were collected and TRACP5b (TRAP isoform 5b) was later quantified by Mouse TRAP® Assay (Immunodiagnostic Systems Ltd., Boldon, UK), a solid-phase immunofixed enzyme activity assay for the determination of osteoclast-derived TRACP5b in mouse serum (limit of detection 0.3 U/l, limit of quantification 10.0 U/l).

Figure 1 is a schematic representation of the methods described above.

*Statistical analysis*. All data are expressed as mean±standard deviation. Significance was established by Student's *t*-test. Differences were considered significant for $p<0.05$ (two-sided).

## Results

*LMW-PTP knockdown*. In order to study the role of LMW-PTP on bone metastasis, we examined the influence of these phosphatase isoforms on osteoclast differentiation, since the development of osteolytic bone lesions largely relies on the ability of tumor cells to indirectly stimulate the formation of bone-resorbing osteoclasts (21). To achieve this aim, we produced cells with total knockdown of LMW-PTP, and cells with knockdown of its slow isoform using siRNA.

Since we specifically wanted to address bone metastasis from breast cancer, we selected the MDA-MB-231 cell line as a model of metastatic breast tumor.

The efficiency of the knockdown was confirmed by evaluation of mRNA expression as shown in Figure 2. Protein quantification by western blot analysis was not performed due to the lack of antibodies targeting LMW-PTP isoforms differentially.

The clone CI was excluded from post analysis because cells infected with lentivirus containing this siRNA sequence exhibited erratic cellular behavior, which corroborates previous observations from our group using the same viral vector and siRNA in MDA-MB-435 cells (18).

Efficient total knockdown of LMW-PTP was achieved in the three clones infected with siRNA targeting the total enzyme (CII, CIII, and CV). Decreases in the mRNA expression of both fast and slow isoforms were observed in these clones. In clone IV, where only the slow isoform was silenced, both total and slow LMW-PTP were statistically significantly reduced in expression. The expression of the fast isoform increased in this clone, although not reaching statistical significance.

We selected two clones for further studies: CV as having the most effective total knockdown of LMW-PTP and CIV as having the most representative knockdown of slow LMW-PTP.

*Culture of RAW 264.7 cells with conditioned media*. TRAP staining: RAW 264.7 cells (Figure 3A) form osteoclasts only when treated with RANKL (100 ng/ml) for 5 days (Figure 3B). In order to study the influence of LMW-PTP expression on osteoclast differentiation, we cultured RAW 264.7 cells with medium from the previously selected knockdown MDA-MB-231 clones (CV and CIV, KD LMW-PTP total and KD LMW-PTP slow respectively) and from the MDA-MB-231 with scramble siRNA, given expression levels were not different from those of the parental cell line.

Osteoclasts obtained either under influence of MDA-MB-231 cells with knockdown (Figure 3D and E) or from the positive control (Figure 3B) were smaller and less numerous than those obtained under scramble siRNA influence (Figure 3C).

*TRAP quantification*. To quantify these findings, we measured the TRACP5b level in cell culture supernatants (Figure 4) and observed that both medium from the positive control and from cells with total knockdown of LMW-PTP had statistically significant lower TRACP5b levels than the medium from cells with scramble siRNA. The decrease of the TRACP5b level shown in medium from cells with knockdown of slow LMW-PTP did not reach statistical significance.

## Discussion

Although recognizably undervalued and misused in practice, reproducibility remains the cornerstone of science (22). This work is not a direct reproducibility study but rather intended to contribute new data to further explore some recent contradictory conclusions on the role of LMW-PTP in the progression of bone metastases from breast cancer (20).

In this study, we selected the MDA-MB-231 cell line as an *in vitro* model of an invasive breast adenocarcinoma because it has been widely used as a model of breast cancer (23) and was previously characterized alongside MDA-MB-435 (17), which additionally facilitates the comparison of our results with those obtained by Alho *et al*. (20). Furthermore, MDA-MB-231 cells have been demonstrated to stimulate osteoclast formation by the production of unknown soluble factors in the absence of supporting cells (21). Accordingly, we observed that RAW 264.7 cells differentiated more into osteoclasts under the influence of RANKL and medium of MDA-MB-231 without altered gene expression (scramble siRNA) than under the influence of RANKL alone (positive control).

Our results for the confirmation of knockdown efficiency through mRNA expression analysis showed that the knockdown of the slow isoform was accompanied by a non-significant increase in the expression of the fast isoform. A possible explanation for this might be the cells' need to compensate for the loss of the slow isoform by overexpressing the fast counterpart. This fine regulation is in accordance with the remarkable conservative nature of the protein throughout evolution, strongly suggesting its involvement in vital cellular functions (5, 13). We also observed reductions in the mRNA expression of both fast and slow isoforms in clones where total LMW-PTP was silenced. This is not unexpected since the total LMW-PTP expression represents the sum of all its isoforms.

Regarding the topic of osteoclast differentiation, our results suggest that total knockdown of LMW-PTP significantly reduced RAW 264.7 osteoclastic differentiation, while the reduction of osteoclastogenesis for knockdown of slow LMW-PTP did not reach statistical significance. These data suggest that total LMW-PTP might increase osteoclastogenesis, probably stimulating the vicious cycle of osteolytic metastases, but not at the expense of the slow isoform.

It may be hypothesized that this increase in osteoclastogenesis would largely be due to the fast isoform, since fast and slow are the only known functional isozymes. This notion finds solid theoretical support, starting with the recognition of p190RhoGAP as a substrate of the fast/cytoskeleton-associated fraction of LMW-PTP. The dephosphorylation of p190RhoGAP by the fast LMW-PTP isoform is able to up-regulate Rho-GTP levels and consequently increase RhoA activity, reducing cell–cell adhesion and influencing the migratory potential of cells, a significant feature of metastasis (14, 24). In agreement with this, elevated expression of RhoA has been correlated with tumor stage and enhanced metastasis in several cancer types, including of the breast (6).

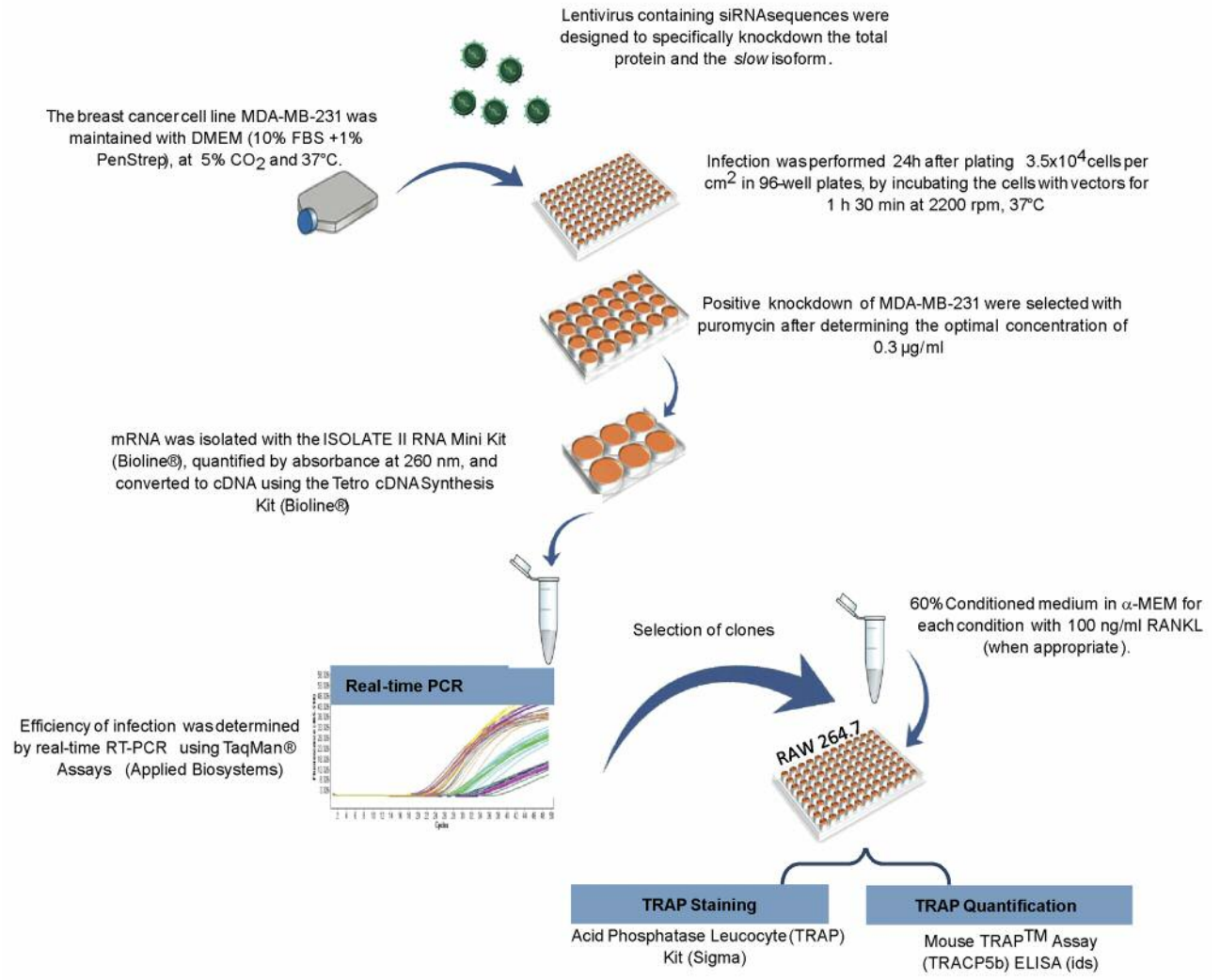


Figure 1. *Schematic representation of the methods used in this work. α-MEM: α-Minimum essential medium; DMEM: Dulbecco's modified Eagle's medium; ELISA: enzyme-linked immunosorbent assay; FBS: fetal bovine serum; penStrep: penicillin/streptomycin; RANKL: receptor activator of nuclear factor kappa-B ligand; RT-PCR: reverse transcription polymerase chain Reaction; TRAP: tartarate-resistant acid phosphatase; TRACP5b: TRAP isoform 5b.*

The expression profiles of total LMW-PTP, and its fast and slow isoforms in human samples of normal breast, primary breast cancer and bone metastatic breast cancer tissues have equally been addressed by our group. Total LMW-PTP was found to be expressed at higher levels in primary breast cancer tissue and expression of the fast isoform was prominent in metastatic tissue, corroborating our observations (15).

Recently, Alho *et al*. reported that knockdown of the slow isoform reduced osteoclastic differentiation (20). We speculate that the differences observed between these and our results may be due to the following reasons:

i) The two cell lines used are genotypically distinct. MDA-MB-435 used by Alho *et al*. had an AA genotype for the ACP1 gene while the MDA-MB-231 used by us had an AB genotype (17). Importantly, the B allele is reported to produce as much as twice the fast isoform as the A allele (11), which might account for the discrepancies. In a study analyzing the differential expression of the isoforms in a panel of breast cancer cell lines, MDA-MB-435 exhibited a much higher expression of the slow isoform than MDA-MB-231, while expression of the fast isoform was comparable (17).

ii) The conclusions might be applicable only to a particular tissue. Tissue specificity assumes particular relevance since MDA-MB-435 has now been recognized to be a melanoma cell line misidentified as breast cancer (25-

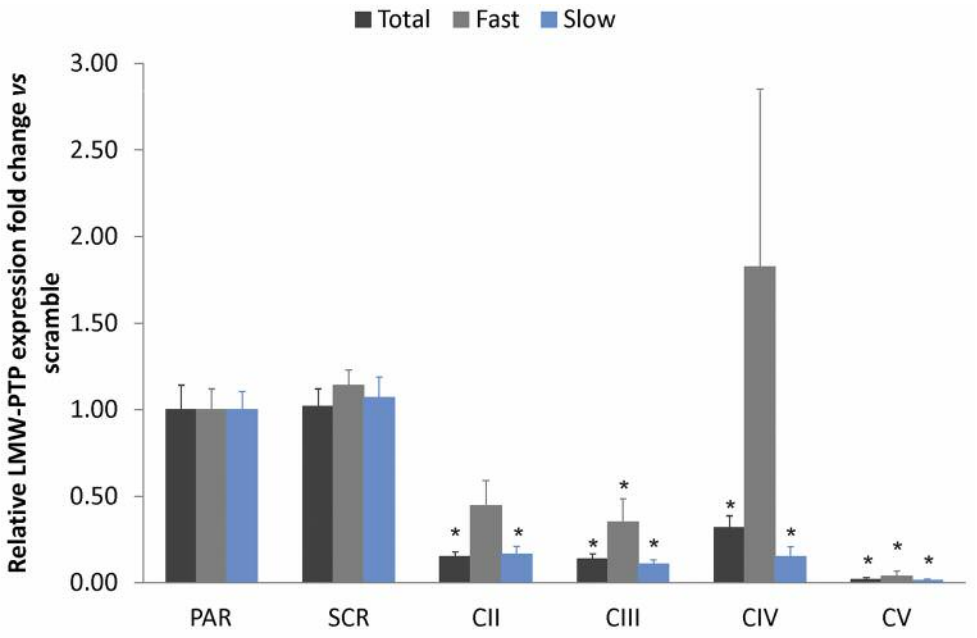


Figure 2. *Relative expression of low molecular weight protein tyrosine phosphatase (LMW-PTP) and its fast and slow isoforms in the MDA-MB-231 cell line (PAR) and in clones with knockdown. Data are means. Error bars represent standard deviation (n=3 independent experiments). SCR refers to cells with non-targeted scramble siRNA sequences (control); CII, CIII and CV represent cells with total knockdown of LMW-PTP; CIV represent cells with knockdown of slow LMW-PTP. *Significantly different at p<0.05 compared to cells with scramble siRNA.*

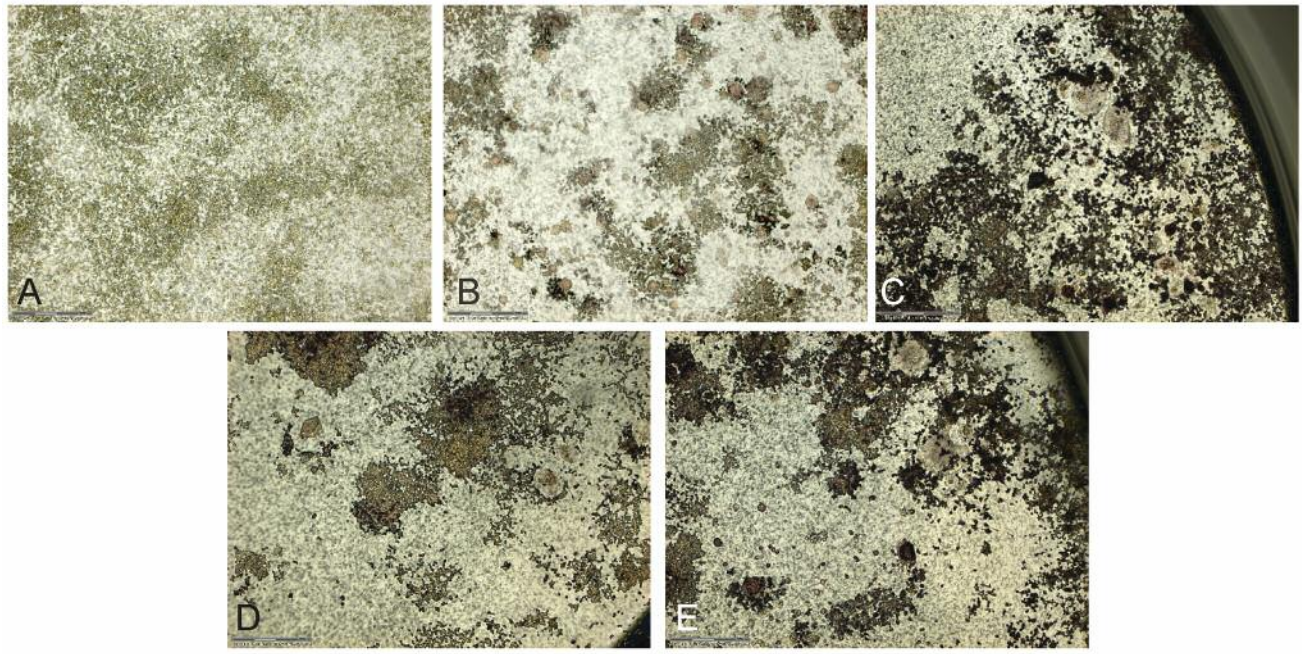


Figure 3. *Osteoclast differentiation depending on MDA-MB-231 conditioned medium. RAW 264.7 monocytic cells were stimulated with receptor activator of nuclear factor kappa-B ligand (RANKL) (100 ng/ml) and stained for tartarate-resistant acid phosphatase (TRAP) as described in the Materials and Methods section. A: Negative control: differentiation medium without RANKL. B: Positive control: differentiation medium with 100 ng/ml RANKL; C-E: Osteoclast differentiation with conditioned medium derived from supernatant of MDA-MB-231 cells with scramble siRNA (C), total knockdown of low molecular weight protein tyrosine phosphatase (LMW-PTP) (D) and knockdown of slow LMW-PTP (E). Osteoclasts were identified as multinucleated (more than three nuclei) TRAP-positive cells.*

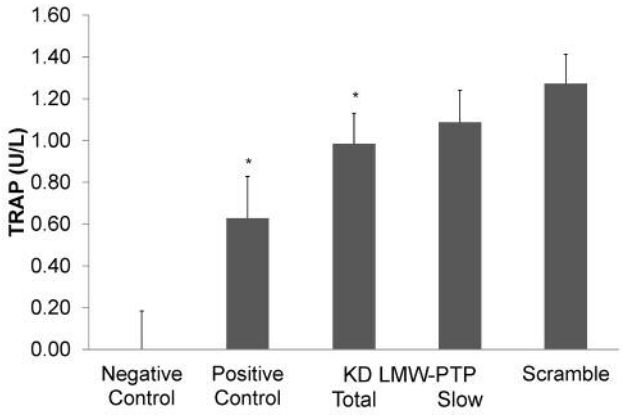


Figure 4. *Tartarate-resistant acid phosphatase isoform 5b (TRACP5b) quantification in cell culture supernatants. KD LMW-PTP refers to knockdowns of low molecular weight protein tyrosine phosphatase. Data are means. Error bars represent standard deviation (n=6 independent experiments). *Significantly different at p<0.05 compared to cells differentiated with conditioned medium from MDA-MB-231 cells with scramble siRNA.*

28). The debate around the origin of these cells started with the recognition that MDA-MB-435 cells express a cluster of genes highly expressed in melanoma-derived lines (29). Later, Rae *et al*. concluded that all the available stocks of MDA-MB-435 cells were indeed derived from the M14 melanoma cell line (30). These conclusions are not unanimously accepted, as some authors propose that the two cell lines represent a poorly differentiated breast tumor line, with expression of both epithelial and melanocytic markers (31, 32). However, even considering the two cell lines as being of breast origin, MDA-MB-435 does not seem to behave as most breast cancer cell lines and thus the results obtained by studying this line are difficult to interpret and extrapolate.

iii) The two cell lines can also diverge in the expression of LMW-PTP substrate molecules. Since the fast and slow isoforms have distinct substrates (6), these differences may favor the pathways elicited by one isoform or the other, and ultimately contribute to the different experimental results.

As an exploratory study, we did not entirely mimic the tumor microenvironment nor did we address the pathway mediating the proposed influence of the LMW-PTP fast isoform on osteoclastogenesis. The only possible assumption is, therefore, the ability of soluble tumor-derived factors to interfere with the metastatic potential of neoplasms, which confirms the existing evidence (20).

Finally, the conclusions drawn here may perhaps be a first step towards the development of isoform-specific inhibitors that could be beneficial in different stages of tumor development, targeting the slow isoform in an early phase and the fast in the metastatic stage of disease. Another potential exploitation of the differential role of LMW-PTP isoforms in clinical practice might be the use of LMW-PTP fast-specific immunohistochemical staining to identify neoplasms with increased risk of developing bone metastases. This could eventually help to identify patients benefiting from early therapy by analyzing biopsy samples alone.

In conclusion, our results show a significant decrease in osteoclastic differentiation of RAW 264.7 cells when exposed to conditioned medium from MDA-MB-231 cells with total knockdown of LMW-PTP, while this did not happen with the knockdown of the slow LMW-PTP isoform. Considering that total LMW-PTP although not its slow isoform would increase osteoclastogenesis, and further recognizing that fast and slow are the only functional isozymes, we suggest that the fast LMW-PTP isoform is a key mediator of breast cancer-derived bone metastasis. These findings have promising clinical applications, either in drug development or as a tool in the context of patient selection for anti-resorptive therapy. Being aware that these are relevant conclusions, animal and clinical studies are necessary for a more definitive elucidation.

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## Conflicts of Interest

The Authors declare that they have no conflict of interest regarding this paper.

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