Breast Cancer Res Treat
DOI 10.1007/s10549-010-0848-5

PRECLINICAL STUDY

Enhanced serine production by bone metastatic breast cancer cells stimulates osteoclastogenesis

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Received: 20 October 2009 / Accepted: 13 March 2010

Abstract Since bone metastatic breast cancer is an incurable disease, causing significant morbidity and mortality, an understanding of the underlying molecular mechanisms would be highly valuable. Here, we describe in vitro and in vivo evidences for the importance of serine biosynthesis in the metastasis of breast cancer to bone. We first characterized the bone metastatic propensity of the MDA-MB-231(SA) cell line variant as compared to the parental MDA-MB-231 cells by radiographic and histological observations in the inoculated mice. Genome-wide gene expression profiling of this isogenic cell line pair revealed that all the three genes involved in the L-serine biosynthesis pathway, phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), and phosphoserine phosphatase (PSPH) were upregulated in the highly metastatic variant. This pathway is the primary endogenous source for L-serine in mammalian tissues. Consistently, we observed that the proliferation of MDA-MB-231(SA) cells in serine-free conditions was dependent on PSAT1 expression. In addition, we observed that L-serine is essential for the formation of bone resorbing human osteoclasts and may thus contribute to the vicious cycle of osteolytic bone metastasis. High expression of PHGDH and PSAT1 in primary breast cancer was significantly associated with decreased relapse-free and overall survival of patients and malignant phenotypic features of breast cancer. In conclusion, high expression of serine biosynthesis genes in metastatic breast cancer cells and the stimulating effect of L-serine on osteoclastogenesis and cancer cell proliferation indicate a functionally critical role for serine biosynthesis in bone metastatic breast cancer and thereby an opportunity for targeted therapeutic interventions.

Keywords Breast cancer · Bone metastasis · Osteoclast · L-serine

Electronic supplementary material The online version of this article (doi:10.1007/s10549-010-0848-5) contains supplementary material, which is available to authorized users.

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Introduction

Metastasis to bone is the most frequent cause of breast cancer morbidity and mortality. Currently available therapies are able to limit disease progression and alleviate painful symptoms but bone metastatic cancer remains incurable. In the metastatic process, tumor cells detach from the primary site, invade into blood vessels, and extravasate into the metastatic site. In order to form metastatic lesions, tumor cells need to adapt to the bone

Published online: 30 March 2010

microenvironment and interact with the host cells. The majority of the skeletal lesions in breast cancer are osteolytic, characterized by enhanced osteoclast activity and bone destruction. Cancer cells adapt to the bone microenvironment and start a vicious cycle causing bone destruction. Several cancer cell-derived factors, such as parathyroid hormone-related peptide (PTHrP), have been shown to contribute to this process [1, 2]. Bone is a rich source of growth factors, including transforming growth factor (TGF) α and β, insulin-like growth factors (IGFs), and fibroblast growth factors (FGFs), which are released as a consequence of osteoclastic bone resorption and can stimulate cancer cell survival and production of more osteolytic factors [3]. However, in order to develop more effective treatment strategies, a comprehensive understanding of the critical molecular mechanisms involved in the bone metastatic process is needed.

In vivo modeling of bone metastatic breast cancer is limited by the fact that spontaneous bone metastases are rare in animals. The MDA-MB-231 breast cancer cell line is commonly used to model breast cancer bone metastasis in vivo. This cell line was derived from the pleural effusion of an estrogen receptor negative, late-stage breast cancer patient. When MDA-MB-231 cells were inoculated into the blood circulation of immunodeficient mice by intracardiac inoculation, osteolytic lesions were formed [4]. MDA-MB-231 variants with enhanced bone metastatic abilities have been created, compared, and used for studying molecular mechanisms of bone metastasis [5, 6]. The MDA-MB-231(SA) variant has been used in several studies [7, 8] and is known to be highly bone metastatic. However, comparative molecular studies on this variant and the parental MDA-MB-231 cell line have not been published. Here, we compared the in vivo bone metastatic properties between the MDA-MB-231(SA) variant and parental MDA-MB-231 cells and then used genome-wide gene expression profiling to identify molecular mechanisms that could explain the different metastatic properties of the two cell types.

Materials and methods

Breast cancer cell lines and culture conditions

The parental MDA-MB-231 cancer cell line was obtained from the American Type Culture Collection (ATCC). MDA-MB-231(SA) cells were spontaneously derived from these cells during a long in vitro culture. Both cell types were maintained in low-glucose DMEM with 10% inactivated fetal bovine serum (FBS), 1% nonessential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Mouse experiments

The animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center in San Antonio and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. 100,000 MDA-MB-231(SA) or parental MDA-MB-231 cells were inoculated into the left cardiac ventricle of anesthetized (30% ketamine and 20% xylazine in 0.9% NaCl) 5-week-old female athymic nude mice (Balb/c nu/nu, Harlan, Indianapolis, IN) ($n = 7$ mice/cell line) as described previously [2, 4]. Body weight of the mice was monitored during the experiment, and the mice were killed when they became cachectic, or paraplegia was observed. The development of bone metastases was monitored weekly or bi-weekly by X-ray radiography. Animals were anaesthetized with Ketamine–Xylazine cocktail and X-rayed in a prone position using a 43855A X-ray system (Faxitron, Lincolnshire, IL, USA), as described previously [2]. The total area and number of lesions in hind (left and right tibia and femur) and fore (left and right humerus) limbs per mouse were quantified from the images using image analysis software (Java, Jandel Video Analysis; Jandel Scientific, Corte Madera, CA, USA). All of the radiographs were evaluated without knowledge of the treatment groups. Heart, lungs, spleen, kidneys, liver, and adrenal glands were collected when killed for histology to determine possible soft tissue metastasis in addition to the bone metastasis. Tissue sections (4 µm) were cut and stained with hematoxylin and eosin (H&E), orange G and phloxine B using standard protocols.

Genome-wide comparative genomic hybridization

Array-based CGH was performed using Agilent 244K oligonucleotide microarrays according to the direct method of the June 2006, version 4 protocol (Agilent Technologies). Female genomic DNA (Promega) was used as reference in both hybridizations. Labeling of 3 µg of digested and purified tumor and reference DNA was performed with Cy5-dUTP and Cy3-dUTP (Perkin-Elmer), respectively, according to the protocol. Labeled tumor and reference samples were pooled and hybridized onto an array. The arrays were washed and scanned with a laser confocal scanner (Agilent Technologies). Signal intensities were extracted using the Feature Extraction software (Agilent Technologies). CGH Analytics (Agilent Technologies) was used for initial data analysis and visualization.

Gene expression profiling

Gene expression levels were measured using the Affymetrix GeneChip U133 Plus 2 system (Affymetrix). RNA was extracted from the parental MDA-MB-231 and MDA-MB-231(SA) cells using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Sample processing and labeling were performed according to the protocol provided by Affymetrix. We used 3 μg of total RNA for the initial cDNA synthesis. Chips were scanned using the GeneChip Scanner 3000 (Affymetrix). The raw cel files were processed separately using the R language [9] and the RMA method implemented in the Bioconductor [10] package affy [11]. Alternative CDF files mapping Affymetrix probes directly to Ensembl gene IDs were used in the preprocessing [12]. The microarray data were deposited in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo, accession number GSE20085).

Western blot analyses

Cells were lysed in SDS buffer (containing 30% glycerol, 3% SDS, 3% β-mercaptoethanol, 0.015% bromophenol blue, and 0.19 M Tris–HCl (pH 6.7)). Lysates were incubated at 95°C for 5 min and then loaded into an SDS-PAGE gel. Separated proteins were transferred to a nitrocellulose membrane and immunoblotted using chicken anti-human PSAT1 antibody (US Biological, Swampscott, MA, USA), rabbit anti-human PSAT1 antibody (Strategic Diagnostics Inc., Newark, DE, USA), mouse anti-human PHGDH antibody (Abnova, Taipei, Taiwan), mouse anti-β-actin (Sigma) or rat anti-Hsc70 antibody (Stressgen, Canada). Secondary antibodies: HRP-conjugated donkey anti-chicken IgY (Jackson ImmunoResearch, West Grove, PA, USA), IRDye 800-conjugated anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA), Alexa Fluor 680 goat anti-mouse IgG (Invitrogen), HRP-conjugated goat anti-rat IgG (GE Healthcare). The Amersham enhanced chemiluminescence system (GE Healthcare) was used for chemiluminescent imaging, and Odyssey Licor device for fluorescence imaging of proteins.

Quantitative real-time RT-PCR

Total RNA was isolated from cells using RNeasy Mini kit (Qiagen). Total RNA weighing 300 ng was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The primers and probes specific for each target gene or the endogenous control ACTB (β-actin) were designed by the the ProbeFinder software (Roche Applied Biosciences, Basel, Switzerland) and are listed in the Online Resource 1 in Electronic supplementary material. The cDNAs were diluted 1/10, and TaqMan quantitative real-time reverse transcription PCR (RT-PCR) was performed using the Applied Biosystems 7900HT instrument. The results were analyzed using the SDS 2.3 and RQ manager softwares (Applied Biosystems, Foster City, CA, USA), and the expression of target mRNA was determined by relative quantitation using β-actin as an endogenous control.

Generation of shRNA expressing cells

MDA-MB-231(SA) cells were transduced with lentiviral particles containing an shRNA sequence targeting PSAT1 (Sigma) or a scrambled shRNA sequence not targeting any human gene (Sigma). Transduction was performed in the presence of 8 μg/ml hexadimethrine bromide. Transduced cells were selected with 4 μg/ml puromycin. PSAT1 knockdown was confirmed using quantitative RT-PCR and Western blotting.

Cell proliferation assay

In order to remove the amino acids, inactivated FBS was dialyzed against PBS using a dialysis cassette with a molecular weight cut-off of 2,000 at 4°C for 29 h. Cells were plated in 96-well plates in Minimum Essential Medium (MEM) (Invitrogen) with 10% dialyzed FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. L-serine (Sigma) was added to the wells at specific concentrations indicated in the figure legends. Each condition was tested in three replicates. The cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cell viability was measured using the CellTiter-Blue Reagent (Promega) according to the manufacturer's instructions.

Osteoclast culture

In order to remove the amino acids, FBS (Osteoclast Medium BulletKit, Lonza) was dialyzed against PBS using a dialysis cassette with a molecular weight cut-off of 2,000 at 4°C for 27 h. A total of 10,000 human osteoclast precursor cells (Lonza) were seeded onto bovine bone slices (Nordic Bioscience Diagnostics, Herlev, Denmark) in 96-well plates and cultured in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The culture conditions for the first 7 days of culture were as follows: MEM (Invitrogen) with 9% dialyzed FBS and following supplements from the Osteoclast Medium BulletKit (Lonza): 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 26.6 ng/ml M-CSF, and 53.2 ng/ml soluble RANKL. The pH of the medium was adjusted to 7.4. L-serine (Sigma) or O-phospho-DL-serine (Sigma) was added to the culture wells at specific concentrations indicated in the figure legends for each experiment. After 7

days of culture, the medium was collected and replaced with MEM with 9% dialyzed FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 33 ng/ml recombinant human M-CSF (R&D Systems), and 66 ng/ml recombinant human soluble RANKL (PeproTech EC, London, UK). The pH of the medium was adjusted to 6.9. L-serine or O-phospho-DL-serine was added to the culture wells at specific concentrations indicated in the figure legends for each experiment. Each condition was tested in at least four replicates. After 11 days of culture, medium samples were collected. The cells were fixed with 3% PFA and stained for tartrate-resistant acid phosphatase (TRACP) using the Acid Phosphatase Leukocyte Kit (Sigma) according to the manufacturer's instructions.

Immunoassays

Tartrate-resistant acid phosphatase (TRACP) 5b activity was measured from the medium samples using a specific immunoassay (BoneTRAP® Assay, Immunodiagnostic Systems Ltd, Boldon, UK) after 7 days of culture. Bone resorption was quantified by measuring the concentration of the degradation products of C-terminal telopeptides of type I collagen (CTX) in the conditioned medium using the CrossLaps® for Culture ELISA kit (Nordic Bioscience Diagnostics) after 11 days of culture.

Analysis of gene expression in clinical breast cancer samples

Association of PHGDH, PSAT1, PSPH, and SLC1A4 expression with time to relapse in the Affymetrix gene expression studies GSE6532 and GSE12276 ($n = 368$) and with overall survival in the Affymetrix gene expression studies GSE3494 and GSE1456 ($n = 393$) was assessed by Kaplan–Meier analysis. Correlation of differential expression of PHGDH, PSAT1, PSPH, and SLC1A4 with clinical features was analyzed using a data set of 251 breast tumor samples hybridized to Affymetrix U133A and U133B human GeneChips (GSE3494) [13]. U133A and U133B chips were preprocessed separately using the R language [9] and the RMA method implemented in the Bioconductor [10] package affy [11]. Alternative CDF files mapping Affymetrix probes directly to Ensembl gene ids were used in preprocessing [12]. Data from U133A and U133B were combined by calculating the medians of genes appearing on both array types. TP53 status is based on the classifier developed by Miller et al. [13]. Breast cancer subtypes were assigned to samples based on expression data [14]. Ki67, PCNA, and ERBB2 status was likewise estimated from the expression data itself.

Statistical analysis

The results are reported as mean ± SD. Comparison between the groups in the in vivo experiment was done using the one way repeated measures ANOVA (body weight, lesion area, lesion number) or by a log-rank test (survival). Statistical significance of the immunoassay and cell proliferation data were analyzed using an unpaired Student's t test. Statistical significance of survival differences for breast cancer patients was determined by a log-rank test between the highest quartile and the remaining three quartiles with respect to the gene expression values.

Results

Bone metastatic activity of MDA-MB-231(SA) and parental MDA-MB-231 cells in vivo

Owing to the tumor-induced cachexia and paraplegia, all mice inoculated with MDA-MB-231(SA) cells were killed on or before day 26 after inoculation (survival 23 ± 4.2 days, mean ± SD). The mean body weight of the MDA-MB-231(SA) group was significantly smaller already on day 21 when compared to the parental MDA-MB-231 group (Fig. 1a). The first mouse in the parental MDA-MB-231 group was killed on day 58, and the mean survival time in this group was 76.5 ± 19.1 days (mean ± SD) (Fig. 1b). None of the mice in this group was paraplegic when killed. Clear osteolytic lesions were observed in six out of seven mice in the MDA-MB-231(SA) 2 weeks after inoculation and the lesions were severe when killed (Fig. 1c, Online Resource 2 in Electronic supplementary material). Histology revealed no soft tissue metastasis. Only one mouse in the parental MDA-MB-231 group had a clear osteolytic lesion, which was visible in X-ray on day 56. However, five mice in this group had soft tissue tumors according to autopsy and histology.

Genes involved in L-serine biosynthesis or transport are overexpressed in the highly bone metastatic MDA-MB-231(SA) cells

We used array-based comparative genomic hybridization (CGH) to compare the highly metastatic MDA-MB-231(SA) variant and the parental cell line. This analysis showed that the genetic aberrations were highly similar in the two cell lines (Online Resource 3 in Electronic supplementary material). We then compared the genome-wide gene expression profiles of the highly metastatic and parental MDA-MB-231 cells. Only 1.7% (315 genes) of all genes were over 2.5-fold upregulated and 1.1% (198 genes) over 2.5-fold downregulated in the highly metastatic

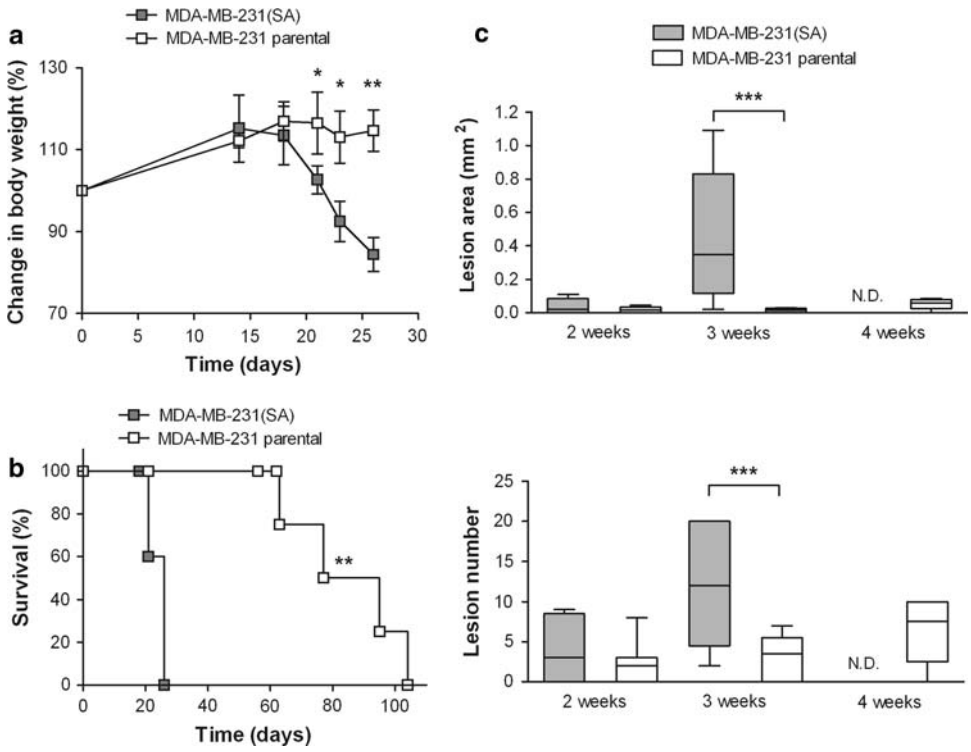

Fig. 1 Bone metastatic activity of MDA-MB-231(SA) and parental MDA-MB-231 cells in mice. **a** Change in body weight of the mice as a function of time after inoculation. **b** Survival of the mice inoculated with MDA-MB-231(SA) or parental MDA-MB-231 cells. **c** Radiographic analysis of the osteolytic lesions. *N.D.* not determined because the mice inoculated with MDA-MB-231(SA) cells had already been killed before this time point. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from the mice inoculated with parental MDA-MB-231 cells

variant. We used the DAVID functional annotation tool [15] and WebGestalt gene set analysis toolkit [16] to assess enrichment of gene ontology (GO) and pathway associations for the upregulated genes. These analyses indicated highest enrichment for organic acid metabolic process ($P = 1.3 \times 10^{-6}$), amino acid and derivative metabolic process ($P = 1.7 \times 10^{-6}$), amine metabolic process ($P = 2.6 \times 10^{-5}$), and nitrogen compound biosynthetic process ($P = 5.1 \times 10^{-4}$) (Online Resource 4 in Electronic supplementary material). The most significantly enriched KEGG pathway was Glycine, serine, and threonine metabolism ($P = 0.0079$) (Online Resource 5 in Electronic supplementary material). Three genes encoding the enzymes that catalyze the steps of the phosphorylated pathway of L-serine biosynthesis (Fig. 2), phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), and phosphoserine phosphatase (PSPH) are involved in all of the above mentioned enriched GO categories and in the most significantly enriched pathway, and upregulated (5.1-, 5.8-, and 2.6-fold, respectively) in the highly metastatic MDA-MB-231(SA) cells. Furthermore, a transporter for serine, alanine, cysteine, and threonine, SLC1A4 (solute carrier family 1 (glutamate/neutral amino acid transporter), member 4) was 3.4-fold upregulated in the MDA-MB-231(SA) cells. Overexpression of PHGDH, PSAT1, PSPH, and SLC1A4 mRNA was confirmed by quantitative RT-PCR and overexpression of PHGDH and PSAT1 at protein level by Western blotting (Fig. 3).

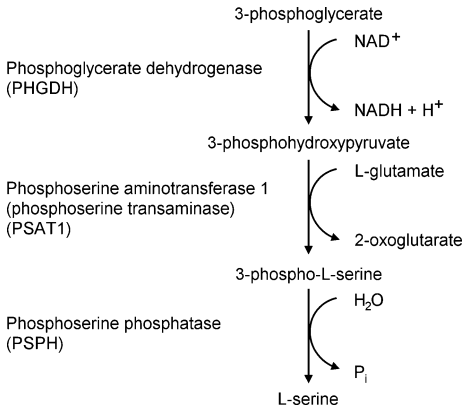

Fig. 2 Phosphorylated pathway of L-serine biosynthesis

Expression of genes involved in L-serine biosynthesis or transport in in vivo selected bone metastatic MDA-MB-231 variants

Kang et al. [5] profiled gene expression (Affymetrix U133A) in 12 in vivo selected MDA-MB-231 subpopulations with different bone metastatic abilities and in the parental MDA-MB-231 cells. We used the data provided by Kang et al. and compared the expression of PHGDH, PSAT1, PSPH, and SLC1A4 between the strongly ($n = 6$) and weakly ($n = 6$) metastatic cells. Because the gene expression data were not publicly available in raw cel format and therefore not applicable to the normalization method described above, we used the data at probe set level

a

Gene symbol	Gene name	Expression fold change in MDA-MB-231(SA) vs. parental MDA-MB-231: Affymetrix profiling	Expression fold change in MDA-MB-231(SA) vs. parental MDA-MB-231: Quantitative RT-PCR
PHGDH	Phosphoglycerate dehydrogenase	5.09	648
PSAT1	Phosphoserine aminotransferase 1	5.81	11.1
PSPH	Phosphoserine phosphatase	2.61	2.75
SLC1A4	Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	3.45	7.45

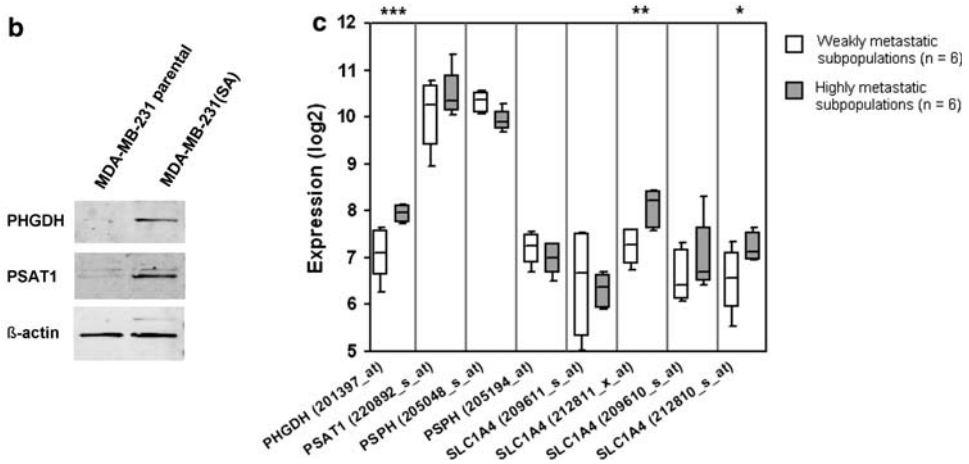

Fig. 3 Expression of genes involved in L-serine biosynthesis or transport in MDA-MB-231 cell line variants. **a** Expression of PHGDH, PSAT1, PSPH, and SLC1A4 mRNA in MDA-MB-231(SA) as compared to parental MDA-MB-231 cells based on Affymetrix profiling and quantitative RT-PCR analysis. **b** PHGDH and PSAT1 protein expression in parental MDA-MB-231 and MDA-MB-231(SA) cells determined by Western blot. **c** Expression of PHGDH, PSAT1, PSPH, and SLC1A4 mRNA in MDA-MB-231 subpopulations with different bone metastatic abilities based on the gene expression profiling (Affymetrix U133A) data published by Kang et al. [5]. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different between the weakly and highly metastatic groups

as given in the supplementary table 1 in Kang et al. [5]. PHGDH expression was significantly higher ($P < 0.001$) in the strongly versus weakly metastatic cells, and the same was true for SLC1A4, based on two of the four probe sets (212811_x_at, $P < 0.01$; 212810_s_at, $P < 0.05$). The difference in PSAT1 expression between the weakly and strongly metastatic cells was not significant but PSAT1 expression was the highest in the strongly bone metastatic subpopulation 1833. PSPH expression did not correlate with metastatic ability in these MDA-MB-231 subpopulations (Fig. 3c).

Knockdown of serine biosynthesis inhibits MDA-MB-231(SA) cell proliferation in serine-free conditions

In order to study whether the phosphorylated pathway of L-serine biosynthesis is critical for the proliferation of the MDA-MB-231(SA) cells, we aimed to inhibit one step in the pathway as efficiently as possible. The best available shRNA (short hairpin RNA) construct caused 97% reduction in the PSAT1 mRNA levels when stably expressed in the MDA-MB-231(SA) cells. PSAT1 silencing was also confirmed at protein level (Online Resource 6 in Electronic supplementary material). As a control, we generated MDA-MB-231(SA) cells stably expressing a scrambled shRNA. We cultured PSAT1 knockdown and scrambled control cells as well as parental MDA-MB-231 cells in serine-free conditions and in varying concentrations of L-serine for 96 h. Cell proliferation rates of all cell types were significantly lower in the absence of serine than in L-serine containing medium. However, proliferation of the parental MDA-MB-231 cells as well as MDA-MB-231(SA) cells with PSAT1 knockdown was significantly more dependent on L-serine than that of the MDA-MB-231(SA) scrambled control cells. 1 μg/ml (9.5 μM) L-serine, which is 1/10–1/42 of the L-serine concentration in the most commonly used cell culture media and 1/10 of the reported physiological serum concentration [17], was sufficient to compensate for the PSAT1 knockdown (Fig. 4).

Effect of L-serine on osteoclastogenesis

Since it was suggested by Ogawa et al. [18] that L-serine is essential for the osteoclastogenesis of the RAW264 mouse monocyte/macrophage cell line, we studied whether L-serine is also needed for the formation of human osteoclasts capable of resorbing bone and/or for their bone resorption activity. We cultured primary human osteoclast precursors on bone slices in the absence and in two different concentrations of L-serine or phospho-L-serine, and quantified the extent of osteoclastogenesis, i.e., the amount of mature osteoclasts, after 7 days of culture by measuring the activity of an osteoclast specific enzyme, tartrate-resistant acid phosphatase (TRACP) 5b, in the culture medium. TRACP 5b activity detected by the immunoassay has been shown to correlate with the number of osteoclasts [19]. After 11 days of culture, the cells were also fixed and

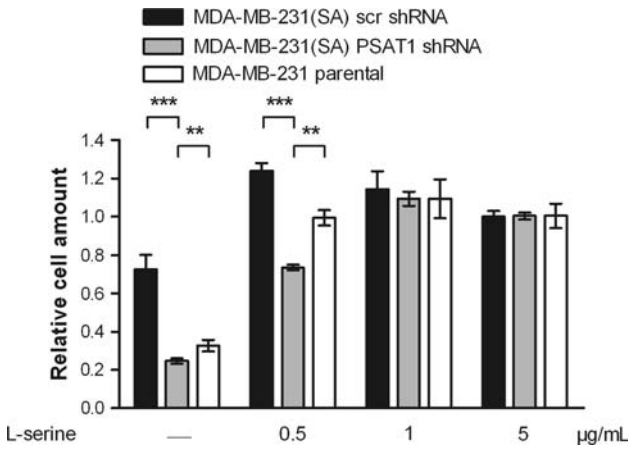

Fig. 4 Proliferation of the MDA-MB-231(SA) and parental MDA-MB-231 cells in the absence and presence of L-serine and the effect of PSAT1 knockdown. Cell viability was measured 96 h after cell plating using the CellTiter-Blue reagent. Results are shown relative to the cell viability in the presence of 5 µg/ml L-serine. ** $P < 0.01$, *** $P < 0.001$

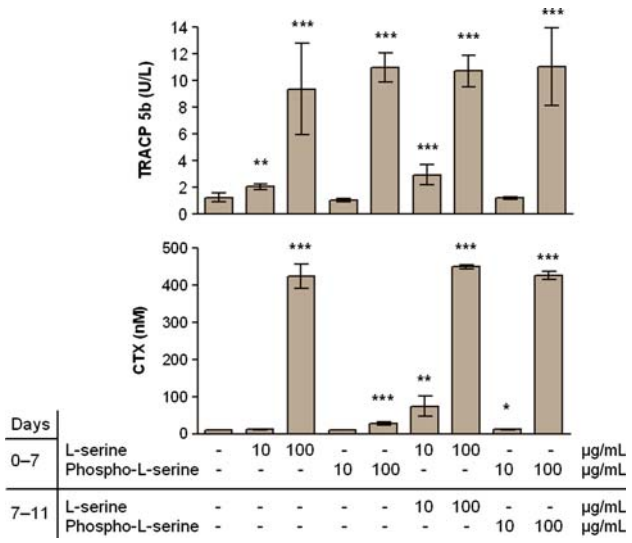

Fig. 5 Effect of L-serine and phospho-L-serine on human osteoclastogenesis and bone resorption. Human osteoclast precursors were cultured in the presence or absence of L-serine or phospho-L-serine. Medium was changed after 7 days of culture. The activity of an osteoclast specific enzyme TRACP 5b in the culture medium, as measured by an immunoassay, indicates the number of differentiated osteoclasts. Concentration of the degradation products of C-terminal telopeptides of type I collagen (CTX) was measured to quantify the bone resorption activity of the differentiated osteoclasts. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from the control (medium without serine and phosphoserine)

stained with TRACP. In the absence of L-serine and phospho-L-serine, the TRACP 5b activity was very low (Fig. 5) and the cells remained mononuclear (data not shown). Addition of 10 µg/ml (95 µM) L-serine significantly increased osteoclastogenesis ($P < 0.01$). The effect of 100 µg/ml L-serine, as well as 100 µg/ml phospho-L-serine, was more dramatic and significant ($P < 0.001$) (Fig. 5). In addition, we quantified bone resorption by measuring the concentration of the degradation products of C-terminal telopeptides of type I collagen (CTX) in the medium after 11 days of culture. Bone resorption activity was in line with the number of osteoclasts with the following exceptions: 10 µg/ml L-serine or 100 µg/ml phospho-L-serine in medium during the first 7 days of culture did not significantly increase bone resorption (Fig. 5).

Expression of genes involved in serine biosynthesis or transport in clinical breast cancer samples

We used previously published gene expression data on breast cancer samples to assess clinical significance of PHGDH, PSAT1, PSPH, and SLC1A4. First, we analyzed the correlation of PHGDH, PSAT1, PSPH, and SLC1A4 expression to time to relapse in 368 breast cancers and to overall survival time in 393 breast cancers. Our analysis demonstrated a significant association between high PHGDH expression and shorter time to relapse ($P < 0.001$) as well as shorter overall survival time ($P = 0.002$). Also, high PSAT1 expression was associated with shorter relapse-free ($P < 0.001$) and overall survival ($P = 0.028$) (Fig. 6a, b). High expression of PSPH correlated significantly with overall survival ($P = 0.003$) but not with time to relapse. SLC1A4 expression did not correlate with time to relapse or overall survival in these data sets (data not shown). Second, we looked at whether differential expression of PHGDH, PSAT1, PSPH, or SLC1A4 correlated with clinically relevant features in a set of 251 breast cancer samples. High expression of PHGDH and PSAT1 was associated with several features typical of poor outcome: estrogen and progesterone receptor negative status, mutated p53, high versus low grade, high expression of the cell proliferation markers PCNA (proliferating cell nuclear antigen) and Ki-67, and high ERBB2 expression (Fig. 6c). A similar but not as strong and significant association was seen between these clinical parameters and high PSPH expression. SLC1A4 expression did not correlate with these parameters (data not shown).

Discussion

We demonstrated the remarkably enhanced bone metastatic ability of the MDA-MB-231(SA) variant as compared to the parental MDA-MB-231 cell line. The results from both array-CGH and gene expression profiling indicated that the MDA-MB-231(SA) variant was closely related to the parental cell line suggesting that a limited number of gene copy number and expression changes were sufficient to cause a major difference in the metastatic propensity. The

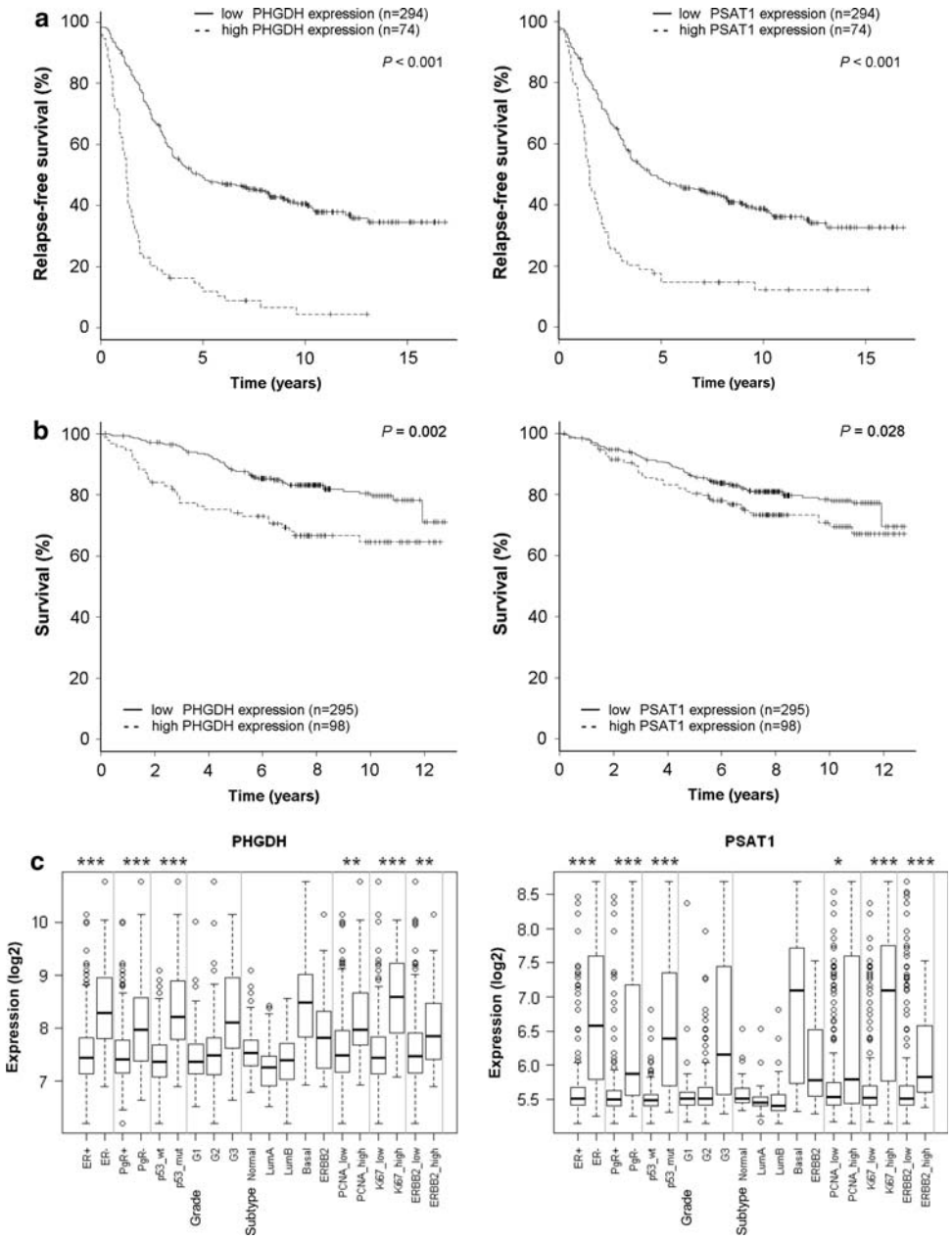

Fig. 6 Expression of PHGDH and PSAT1 in clinical breast cancer samples. **a** Kaplan–Meier analysis of time to relapse showing a difference between patients with high versus low PHGDH or PSAT1 expression. **b** Kaplan–Meier analysis of survival showing a difference between patients with high versus low PHGDH or PSAT1 expression. **c** PHGDH and PSAT1 expression in 251 breast cancer samples in relation to clinical parameters. *ER* estrogen receptor, *PgR* progesterone receptor, *wt* wild type, *mut* mutated, *Lum* luminal, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

most significantly enriched pathway among the upregulated genes was L-serine metabolism, and all the three enzymes involved in the phosphorylated pathway of L-serine biosynthesis, PHGDH, PSAT1, and PSPH, were upregulated in the MDA-MB-231(SA) cells. Especially, there was a very strong, 648-fold induction of PHGDH expression. Therefore, this metabolic pathway has a potentially critical functional role in the causation of the metastatic activity of these cells. This was further supported by the gene expression profiling data on in vivo selected MDA-MB-231 subpopulations with different bone metastatic abilities: PHGDH expression was significantly higher in the strongly versus weakly metastatic cells, and PSAT1 expression was highest in one of the most highly bone metastatic subpopulations.

As the first step of the phosphorylated pathway of L-serine biosynthesis, PHGDH catalyzes the oxidation of the glycolytic intermediate 3-phosphoglycerate to 3-phosphohydroxypyruvate. PSAT1 then transaminates 3-phosphohydroxypyruvate with glutamate to yield 3-phosphoserine, and finally, dephosphorylation of 3-phosphoserine by PSPH produces L-serine. This pathway is the primary source of L-serine in mammalian tissues [20]. Consistently, our data indicated that proliferation of MDA-MB-231(SA) cells in serine-free conditions was strongly inhibited by PSAT1 knockdown. Serine is transported in and out of cells via amino acid transporters: the Na^+-dependent transporters ASCT1 (SLC1A4) and ASCT2 (SLC1A5) [21, 22], the system A transporters SA1 (SLC38A1) and SA2 (SLC38A2)

[23], and the Na^+-independent alanine–serine–cysteine transporter 1 (Asc-1 (SLC7A10)) [24]. One of these transporters, SLC1A4, was upregulated in the MDA-MB-231(SA) variant.

Our analysis of gene expression data on breast cancer patient samples provided evidence for clinical relevance of the L-serine biosynthesis pathway: high expression of PHGDH and PSAT1 was associated with shorter time to relapse and overall survival as well as with several features typical of poor outcome in breast cancer. This extends previous observations suggesting that L-serine biosynthesis is important for cell proliferation [25] and an observation by Martens et al. [26] who showed that high PSAT1 expression was associated with poor response to tamoxifen therapy in patients with recurrent breast cancer.

Upon PSAT1 knockdown, proliferation of MDA-MB-231(SA) cells was severely affected in serine-free medium. However, even lower than physiological serum concentrations of L-serine were able to compensate for the PSAT1 knockdown. L-serine concentrations may be rate-limiting in the microenvironment of the breast cancer cells in vivo, such as in the bone marrow or other phases of the metastatic process, and hence enhanced expression of serine biosynthesis genes in metastatic cell clones may provide a selective advantage for cancer progression.

In addition to cell proliferation, our data suggest that enhanced serine biosynthesis may promote other aspects in the metastatic cascade. Bone destruction in osteolytic lesions is mediated by osteoclasts, and breast cancer cells can stimulate osteoclastogenesis [27]. As suggested by Ogawa et al. [18], L-serine is essential for the RANKL-induced osteoclastogenesis of the RAW264 mouse monocyte/macrophage cell line. This cell line is commonly used as a model for studying osteoclast differentiation but the resulting multinuclear TRACP positive cells do not resorb bone. We used primary human osteoclast precursors and demonstrated that L-serine also stimulated the formation of human osteoclasts that actively resorbed bone. Enhanced L-serine production by metastatic breast cancer cells may thereby contribute to bone destruction. In conclusion, because L-serine biosynthesis genes are highly expressed in bone metastatic breast cancer cells and L-serine stimulates osteoclastogenesis and cancer cell proliferation, interfering with serine production locally at the bone microenvironment may provide a therapeutic opportunity for metastatic bone disease.

Acknowledgements This study was supported by the Academy of Finland Center of Excellence grant (Center of Excellence in Translational Genome-Scale Biology 2006–2011), TIME (Disseminated Tumour Cells as Targets for Inhibiting Metastasis of Epithelial Tumours) project, Drug Discovery Graduate School, NIH grant R01 CA69158 from the National Cancer Institute, and by grants from the Finnish Cancer Organisations, the Sigrid Jusélius Foundation, and the Finnish Cultural Foundation. We thank Barry G. Grubbs and Rami Käkönen for excellent technical assistance in the mouse studies, Pharmatest Services Ltd (http://www.pharmatest.fi/) for technical help and discussions regarding the osteoclast cultures, and John Patrick Mpindi for help in bioinformatic analyses.

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