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Targeting heat shock protein 27 (HspB1) interferes with bone metastasis and tumour formation *in vivo*

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BACKGROUND: The small stress heat shock protein 27 (Hsp27) has recently turned as a promising target for cancer treatment. Hsp27 upregulation is associated with tumour growth and resistance to chemo- and radio-therapeutic treatments, and several ongoing drugs inhibiting Hsp27 expression are under clinical trial. Hsp27 is now well described to counteract apoptosis and its elevated expression is associated with increased aggressiveness of several primary tumours. However, its role in the later stage of tumour progression and, more specifically, in the later and most deadly stage of tumour metastasis is still unclear.

METHODS/RESULTS: In the present study, we showed by qRT–PCR that *Hsp27* gene is overexpressed in a large fraction of the metastatic breast cancer area in 53 patients. We further analysed the role of this protein in mice during bone metastasis invasion and establishment by using Hsp27 genetically depleted MDA-MB231/B02 human breast cancer cell line as a model. We demonstrate that Hsp27 silencing led to reduced cell migration and invasion *in vitro* and that *in vivo* it correlated with a decreased ability of breast cancer cells to metastasise and grow in the skeleton.

CONCLUSION: Altogether, these data characterised Hsp27 as a potent therapeutic target in breast cancer bone metastasis and skeletal tumour growth.

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Heat shock protein 27 (Hsp27), also called HspB1, is a member of the human small heat shock protein family characterised by a highly conserved α-crystalline domain. This protein has a complex structural organisation that modulates its chaperone activity (Paul *et al*, 2010). Thus, like other small heat shock proteins, Hsp27 can sequester damaged proteins and prevent their aggregation (Wyttenbach *et al*, 2002; Arrigo, 2011). This property of molecular sponge leads to increased cell survival, and is correlated with complex patterns of Hsp27 oligomerisation and phosphorylation on three serine residues (Jaya *et al*, 2009; Paul *et al*, 2010).

Hsp27 is also well characterised to counteract apoptotic cell death induced by different inducers (Mehlen *et al*, 1996; Paul *et al*, 2010). It modulates cell death negatively by blocking the apoptotic cascade at different levels. For example, Hsp27 binds and inactivates cytochrome-*c* released from mitochondria during apoptotic cell death (Bruey *et al*, 2000), as well as the pro-domain of procaspase-3, and inhibits the activation process of this caspase, two crucial events for apoptosis execution (Voss *et al*, 2007). Moreover, Hsp27 is able to directly stabilise actin filaments to protect cell integrity in response to heat, reactive oxygen species, cytotoxic agents (Venkatakrishnan *et al*, 2006; Pivovarova *et al*, 2007) and some apoptotic inducers, as for example staurosporine (Paul *et al*, 2002, 2010). Hsp27 is also implicated in radio- and chemo-resistance and can negatively modulate cell death induced by anticancerous cytotoxic agents like cisplatin, adriamycin, etoposide or γ-rays (Zhang and Shen, 2007; Aloy *et al*, 2008). γ-Ray resistance may be related to the ability of this protein to counteract oxidative stress (Arrigo, 1998).

In murine *vivo* models, a direct demonstration of the tumourigenic and metastatic potential of this protein has been done (Garrido *et al*, 1998; Gibert *et al*, 2011). Of interest, Hsp27 is highly expressed in many types of human tumours, particularly in those of carcinoma origin, including ovarian, breast, head and neck cancer. Hsp27 overexpression is considered a bad prognostic for patient survival (Ciocca and Calderwood, 2005). This increase in Hsp27 level is probably due to the pro-survival effect of the protein allowing the proliferative potential of cancerous cells in adverse *in vivo* conditions. All these faculties implicate Hsp27 as a major therapeutic target in cancer (Arrigo *et al*, 2007; Gibert *et al*, 2011).

The role and clinical outcome of Hsp27 in primary tumours has been well studied and documented (Ciocca and Calderwood, 2005; Calderwood *et al*, 2006; Tsuruta *et al*, 2008; Huang *et al*, 2010). However, its function in metastasis invasion has been less studied, even though *Hsp27* gene has been shown by proteomic analysis to be overexpressed in tumour cells of patients that are able to metastasise (Song *et al*, 2006; Tian *et al*, 2007). It is also interesting to note that surface expression of murine Hsp27 stimulates tumour growth and metastasis of the highly metastatic murine 4T1 breast

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adenocarcinoma cells, whereas silencing of this protein eliminates migration capability (Bausero *et al*, 2004; Kaur *et al*, 2011). In the present study, we showed that Hsp27 is overexpressed in tumours that are able to metastasise, in 53 human breast cancer patients. We further investigated the role of human Hsp27 during bone metastasis formation and tumour growth in mice. Bone metastasis invasion is very often a dramatic stage of tumour progression, which frequently occurs and generally leads to patient death. Bone metastases in breast cancer patients are also associated with bone destruction that correlates with hypercalcaemia and fractures.

We used the breast MDA-MB231/B02 metastatic cell line as a model to analyse the effect of Hsp27 silencing (Pecheur *et al*, 2002; Peyruchaud *et al*, 2003). This cell line displays a unique tropism for bone dissemination and constitutively expresses a high level of Hsp27. The aim of this work was to investigate the role of Hsp27 in bone colonisation by breast cancer cells. We provide here experimental evidence for a role of Hsp27 in migration and invasion of bone tissue by metastatic breast cancer cells.

MATERIALS AND METHODS

Cell culture and transfections

The MDA-MB-231/B02 luciferase human breast cancer cell line is a subpopulation of the MDA-MB-231 cancer cell line that was selected for its high efficiency to metastasise to bones after intravenous inoculation (Pecheur *et al*, 2002; Peyruchaud *et al*, 2003).

All cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. MDA-MD231/B02 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. Cells were transfected according to the jet prime reagent procedure (Poly plus transfection, Pontoise, France). For transient transfection assays, DNA vector was left on cells for 6 h; thereafter, cells were washed with phosphate-buffered saline before being further incubated in fresh medium.

Gel electrophoresis and immunoblotting

Cultured cells were immediately rinsed twice in ice-cold phosphate-buffered saline and scraped off the dish. At this point, aliquots were withdrawn for determination of protein concentration. Thereafter, cells were directly lysed and denatured in SDS buffer (62.5 mM Tris-HCl, pH 6.8; 0.1 M dithioerythritol; 1% SDS; 0.001% bromophenol blue; and 10% glycerol). Cell lysates were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) performed as previously described (Javouhey *et al*, 2008). Immunoblots probed with different specific antibodies were revealed with the ECL system (Amersham Life Science, Pantin, France). Autoradiographs were recorded on X-Omat LS films (Eastman Kodak Co, Rochester, NY, USA).

ShRNA construction

The pCIstricke plasmid (Promega, Charbonnières, France) was used for DNA vector-based shRNA construction. The Hsp27 targeting sequence of the designed oligonucleotides was 5′-GCTGCAAAATCCGATGAG-3′. After annealing, ligation and transformation of the resulting DNA vector into competent DH5α bacteria (Invitrogen, Cergy Pontoise, France), antibiotic resistance was used to select the positive bacterial colonies. The correct sequences of the final DNA preparations for pCIstrike-ShRNA27 (Sh27) were confirmed by sequencing (GenomExpress, Meylan, France). pCIstrike-MsRNA27 (Ms27) vectors were designed as a degenerated control from the above sequence of pCIstrike-ShRNA27.

Generation of stable MDA-MB231/B02 cells containing reduced levels of Hsp27

Four cell culture dishes were seeded at 1×10^6 cells per 78 cm^2. Transfection of MDA-MB231/B02 luciferase (B02) cells was performed with pCIstrike-ShRNA27 or pCIstrike-MsRNA27 DNA vector (Pecheur *et al*, 2002; Peyruchaud *et al*, 2003). One day later, B02 cells were incubated in the presence of neomycin at a concentration of 1 mg ml^{-1}. Neomycin-resistant clones were tested for their Hsp27 levels by western blot. Three independent clones expressing minimal endogenous Hsp27 (named: B02-Sh27) and three independent control clones expressing normal levels of Hsp27 (named: B02-Ms27) were selected, propagated and further analysed.

Determination of cellular proliferation

Cell proliferation was determined using the WST-1 test (Promega, Charbonnières, France). The B02 cells were seeded in 96-well plates (7.5×10^3 per well), incubated with 10 μl per well for 4 h in tetrazolium WST-1 salt (4-[3-(4-iodophenyl)-2-(4-bonitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate) and followed by absorbance measurement at 450 nm with a Wallac 1420 Multilabel Counter (PerkinElmer, Courtabœuf, France). The percentage of cellular proliferation was calculated based on a control absorbance.

Chemotaxis assay

Cell migration and invasion assays were performed in 24-well cell culture chambers with 8-μm-diameter pore-size inserts (Becton Dickinson, Pont de Claix, France), as previously described (Zhao *et al*, 2007). Inserts were coated with 100 μl basement membrane Matrigel (3 μg ml^{-1}; Becton Dickinson) for 90 min at 37°C in order to perform cell invasion experiments. B02 cancer cells (1.5×10^5 cells per ml) were re-suspended in Dulbecco's modified Eagle's medium containing 0.1% (w/v) bovine serum albumin, and 300 μl of this cell suspension was loaded into each insert (upper chamber). The chemo-attractant (10% (v/v) fetal calf serum) was placed in the lower chamber (750 μl per well). The plates were incubated for 6 h at 37°C in a 5% CO_2 incubator. After incubation, the inserts were collected carefully, the non-migrating cells were removed, and the migrating cells on the under-surface of the inserts were fixed and stained with crystal violet. The membranes were mounted on glass slides and cells were counted under microscope.

Animal studies

Animal studies were performed according to ethics recommendations and animal welfare (Workman *et al*, 2010). Bone metastasis experiments were conducted in Nude mice, as described previously (Pecheur *et al*, 2002; Peyruchaud *et al*, 2003; Zhao *et al*, 2007). MDA-MB-231/B02 cells that had been stably transfected with shRNAs directed against Hsp27 or control mismatches (5×10^5 in 100 μl of phosphate-buffered saline) were injected into the tail vein of nude mice anaesthetised with 130 mg/kg ketamin and 8.8 mg kg^{-1} xylazin. Radiographs of anaesthetised animals were taken weekly with the use of MIN-R2000 films (Eastman Kodak Co) in an MX-20 cabinet X-ray system (Faxitron X-ray Corporation, Wheeling, IL, USA). Osteolytic lesions were identified on radiographs as radiolucent lesions in the bone. The area of osteolytic lesions was measured using a Visiolab 2000 computerised image analysis system (Explora Nova, La Rochelle, France), and the extent of bone destruction per leg was expressed in square millimetres. Anaesthetised mice were killed by cervical dislocation after radiography on day 28 (Workman *et al*, 2010).

Intra-osseous tumour xenograft experiments were conducted in nude mice, as described previously (Zhao *et al*, 2007). Briefly,

a small hole was drilled with a 30-gauge sterile needle through the tibial plateau with the knee flexed. Using a new sterile needle fitted to a 50-μl sterile Hamilton syringe (Hamilton Co., Reno, NV, USA), a single-cell suspension (1×10^5 cells in 30 μl of phosphate-buffered saline) was injected into the bone marrow cavity. The progression of osteolytic lesions was monitored by radiography as described above (Zhao *et al*, 2007). Anaesthetised animals were killed by cervical dislocation 7 weeks after tumour cell inoculation.

Bone histology and histomorphometry

Bone histology and histomorphometry analysis of bone lesions were performed as previously described (Pecheur *et al*, 2002; Peyruchaud *et al*, 2003; Zhao *et al*, 2007). Following killing of metastatic animals, both hind limbs from each animal were dissected, fixed in 80% (v/v) alcohol, dehydrated and embedded in methylmethacrylate. A microtome (Microm, Micron Microtech, Francheville, France, HM350S) was used to cut 7–9-μm thick sections of undecalcified long bones, and the sections were stained with Goldner's trichrome. Histologic and histomorphometric analyses were performed on Goldner's trichrome-stained longitudinal medial sections of the tibial metaphysis using a computerised image analysis system (Visiolab 2000, Explora Nova). Histomorphometric measurements (i.e., bone volume/tissue volume (BV/TV) and tumour burden/soft tissue volume (TB/ STV) ratios) were performed in a standard zone of the tibial metaphysis, situated at 0.5 mm distance from the growth plate, including cortical and trabecular bone. The BV/TV ratio represents the percentage of bone tissue. The TV/STV ratio represents the percentage of tumour tissue.

Statistics

The statistical significance of differences between groups was evaluated by the Mann–Whitney *U*-test. All statistical tests were two-sided, and *P* values <0.05 were considered to be statistically significant: $^*P<0.05$, $^{**}P<0.01$.

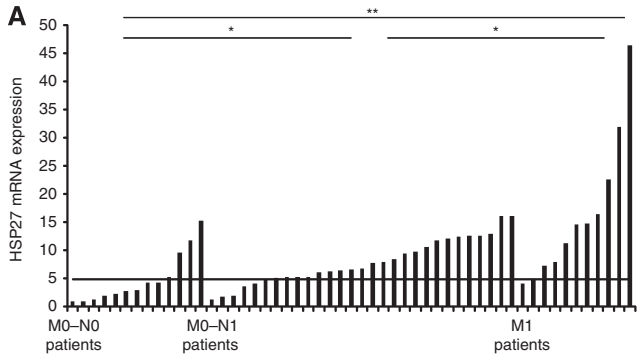
Human breast tumour samples, quantitative PCR

Fifty-three human breast cancer samples were provided by the tumour bank of the Centre Léon Bérard after obtaining patients' consent. Fresh tissue of the tumour was obtained during breast surgery before any systemic therapy and snap-frozen in liquid nitrogen. To assay *Hsp27* expression, total RNA was extracted from samples with the Nucleospin RNAII kit (Macherey-Nagel, Düren, Germany) and 1 μg of RNA was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time qRT–PCR was performed on a LightCycler 2.0 using the LightCycler TaqMan Master kit (Roche Applied Science, Basel, Switzerland) according to the manufacturer's instructions. The ubiquitously expressed *TBP* gene was used as internal control and the same results have been confirmed with *HPRT* and *HMBS* genes (de Kok *et al*, 2005). Primers and probe were given by Universal Probe Library Assay Design Center website and are available upon request (Roche Applied Science). All patient tissue specimens were used according to French laws and regulations.

RESULTS

Hsp27 is a marker for human metastatic breast cancer

We first analysed Hsp27 expression by quantitative-PCR in a panel of 53 human breast primary tumours (Figure 1A). These tumours were from lymph node-negative patients (M0–N0, 13 patients), lymph node-positive patients (M0–N1, 29 patients) or metastatic patients at the time of diagnosis (M1, 11 patients). Hsp27 is significantly more expressed in M0–N1 tumours than in M0–N0 tumours (median 2.95 *vs* 6.85; $P=0.0119$). An even more striking



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	M0 N0	M0 N1	M1
Hsp27 median expression	1	2.57	2.95
Hsp27 overexpression	30.80%	79.30%	90.90%

Figure 1 Hsp27 relative expression in metastatic breast tumours. (**A**) Expression profile of Hsp27 examined by quantitative real-time PCR by using mRNA extracted from 53 tumour biopsy specimens were obtained from patients with tumours localised to the breast (M0–N0), with only node involvement (M0–N1), and with distant metastases at diagnosis (M1) ($^*P\leqslant0.05$; $^{**}P\leqslant0.01$). *TBP* gene (TATAA-box-binding protein) was used as a reference here because it shows a weak variability in the breast (de Kok *et al*, 2005). Identical results were obtained with HBMS and HPRT housekeeping genes. (**B**) Hsp27 expression is given as the ratio between Hsp27 expression in each sample and the average of Hsp27 expression in the M0–N0 samples, shown by a horizontal bar in (**A**). Hsp27 overexpression: percentages of M0–N0, M0–N1 and M1 of samples showing a superior average as compared with the M0–N0 group average.

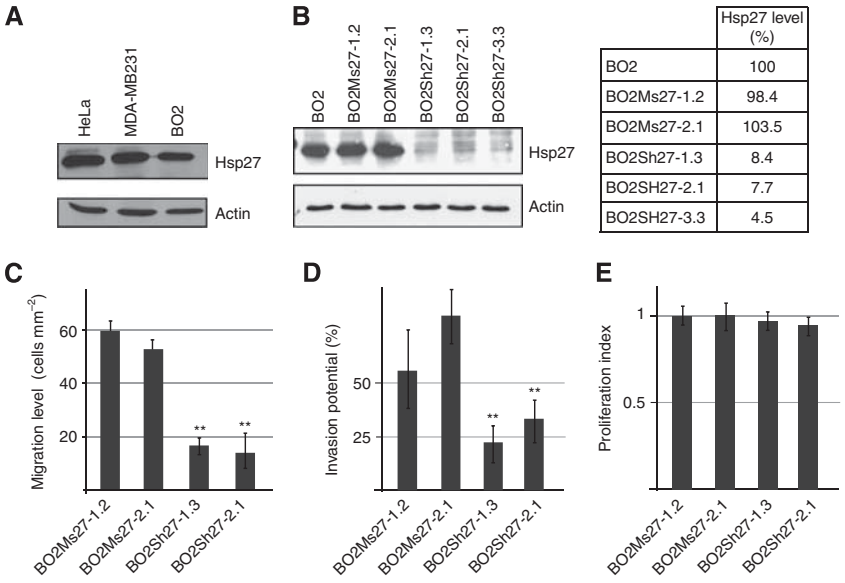
difference is observed when comparing Hsp27 expression in M0–N0 with that in M1 tumours (median 2.95 *vs* 14.58; $P=0.0019$). A significant difference exists between M0–N1 and M1 (median 6.85 *vs* 14.58; $P=0.026$). As shown in Figure 1B, Hsp27 is overexpressed in 79.30% of patients in the M0–N1 and 90.90% in the M1 group (based on the average of the M0–N0 group). Altogether, these data prove that Hsp27 is a marker of the aggressive forms of human breast tumours, especially the metastatic ones.

Establishment of Hsp27-depleted clones

The human breast cancer cell line MDA-MB231 is a well-characterised model of metastasis invasion *in vivo*. To assess the role of Hsp27 in breast cancer metastasis, we used a sub-clone of this cell line, called MDA-MB231/B02 (B02), that has been characterised to specifically metastasise in the bone marrow and not in other tissues when injected in mice (Pecheur *et al*, 2002; Peyruchaud *et al*, 2003). This cell line can be efficiently tracked *in vivo* and its metastatic aggressiveness could be quantified easily by radiographies. We first compared Hsp27 levels in B02 and parental MDA-MB231. These cells have rather similar levels of Hsp27. Using HeLa cells as control (about 4 ng μg^{-1} of total proteins; Paul *et al*, 2010), their Hsp27 level was estimated at about 3 ng μg^{-1}. B02 cells expressed almost the same Hsp27 level as parental cells (Figure 2A). The fact that Hsp27 expression level is similar between MDA-MB231 and B02 cells suggests that Hsp27 expression is not related to B02 cells' osteotropism.

To investigate the pro-oncogenic role of Hsp27 during metastasis process, we established independent clones of B02 cells transfected with shRNA directed against Hsp27 or with mismatches of shRNA. Assessment of Hsp27 levels in B02 cells, by western blotting analysis, revealed a 90% inhibition of Hsp27 protein expression in hsp27 shRNA-transfected clones (Figure 2B).

Translational Therapeutics



	Hsp27 level (%)
BO2	100
BO2Ms27-1.2	98.4
BO2Ms27-2.1	103.5
BO2Sh27-1.3	8.4
BO2SH27-2.1	7.7
BO2SH27-3.3	4.5

Figure 2 Characterisation of Hsp27-depleted B02 clones. (**A**) HeLa, MDA-MB231 and MDA-MB231 B02 (B02) cells were cultured as described in Materials and Methods. Samples were collected 48 h after transfection and Hsp27 level was analysed by western blot. Level of actin was used as a loading control. (**B**) pCIstrike-MsRNA27 (Ms27) and pCIstrike-ShRNA27 (Sh27) vectors were constitutively expressed in B02 cells. Immunoblots were revealed by the corresponding antibodies. (**C**) Hsp27 depletion reduces migration and invasion potential but not proliferation of B02 cells. The modified Boyden chamber technique was used for cell migration assessment. B02 cells were cultured without serum in the insert chambers with pore inserts. Cells able to migrate through the membrane were stained with crystal violet and counted (**$P \leqslant 0.01$). (**D**) Invasiveness of cells was studied by addition of a Matrigel layer in the upper chambers, and like in the migration assay, clones were quantified as described in Materials and Methods (**$P \leqslant 0.01$). (**E**) WST-1 tests were performed every 12 h during 5 days. Proliferation index was determined and reported for each clone.

Two shRNA clones, as well as two negative control mismatch clones, were selected for further studies.

Hsp27 silencing inhibits both cellular migration and invasion but not proliferation

Metastatic tumour cells usually show enhanced migratory capacity and this is often associated with a decreased adhesion. In order to determine whether Hsp27 downregulation could impact this mechanism, we tested the cellular migration of the genetically modified B02 cell clones by performing chemotaxis assays in cell culture chambers with pore inserts. As shown in Figure 2C, migration of Hsp27-depleted cells was dramatically reduced down to 70% as compared with control scramble cells ($P \leqslant 0.01$).

Invasion of tumour cells into surrounding tissues is also known as a crucial event leading to development of distant metastases. Therefore, we evaluated the invasion potential using inserts that were coated with Matrigel basement membrane to evaluate whether matrix degradation was occurring. Matrix invasion by Hsp27-depleted clones was significantly decreased compared with control cells (Figure 2D), but in the same proportions as in the migration assay performed without Matrigel, suggesting that Hsp27 depletion does not affect the ability of B02 cells to degrade Matrigel.

In addition, we have tested the effect of Hsp27 downregulation on cellular proliferation state (Park *et al*, 2002; Lee *et al*, 2004). In previous studies using HeLa cells, we have shown that Hsp27 depletion decreased proliferation by interfering with G_2/M phase (Gibert *et al*, 2012). In contrast, analysis of the WST-1 proliferation assay of B02 cells revealed that Hsp27 depletion was devoid of significant effect (Figure 2D).

Hsp27 inhibition of expression reduces metastasis formation *in vivo*

Metastasis invasion is correlated with the transport of cancer cells by a circulatory system to distant sites. We next wanted to assess whether the Hsp27 depletion in B02 cells inhibited bone metastasis formation *in vivo*. Pools of B02-Sh27 or B02-Ms27 clones were therefore injected in anaesthetised nude mice by intravenous injection in the tail. B02 cells have been transfected to stably express a luciferase enzyme, which allows *in vivo* B02 cell detection by injection of luciferin. Luciferase activity as bioluminescence was then quantified. It correlated with metastasis expansion and dissemination, and was represented by an indicative colouration inlay in mice pictures (Figure 3A). Metastasis development was only detected in the bone marrow thigh of mice, confirming the specificity of B02 cells to form bone metastasis *in vivo*, in contrast to the MDA-MB-231 parental cells. Luciferase activity was statistically decreased by about 50% during the first 3 weeks ($P \leqslant 0.01$) in the group of B02-Sh27-inoculated mice as compared with the ones injected with B02-Ms27 cells. These results suggest that Hsp27 depletion delays metastasis formation by B02 cells (Figure 3B).

Bone metastasis progression is highly correlated with bone demineralisation and fractures due to cancer progression in this environment. Hence, in addition to the *in vivo* analysis of B02 cells' luciferase activity described above, radiographies of each mouse were performed to analyse metastasis expansion. Measurement of osteolytic lesions was achieved by quantification of bone destruction on hind limb radiograph (Figure 4A). As shown in Figure 4C, the extent of osteolytic lesions was dramatically decreased when animals were injected with B02 cells depleted in Hsp27 compared with control cells. There was a significant difference between the groups during all the experiments, including at day 28, when the luciferase activity was similar between mice injected with B02-Sh27 and those injected with B02-Ms27 cells.

Histomorphometric studies were performed at day 28 in order to quantify bone destruction and tumour burden in the animals. Similar to quantitative-PCR on tumour biopsies, Sh27 still invalidated Hsp27 expression after 4 weeks of xenografts (Supplementary Figure 1). As shown in Figure 4C, the results confirm that bone destruction is diminished in animals injected with Hsp27-depleted B02 cells compared with control B02-Ms27

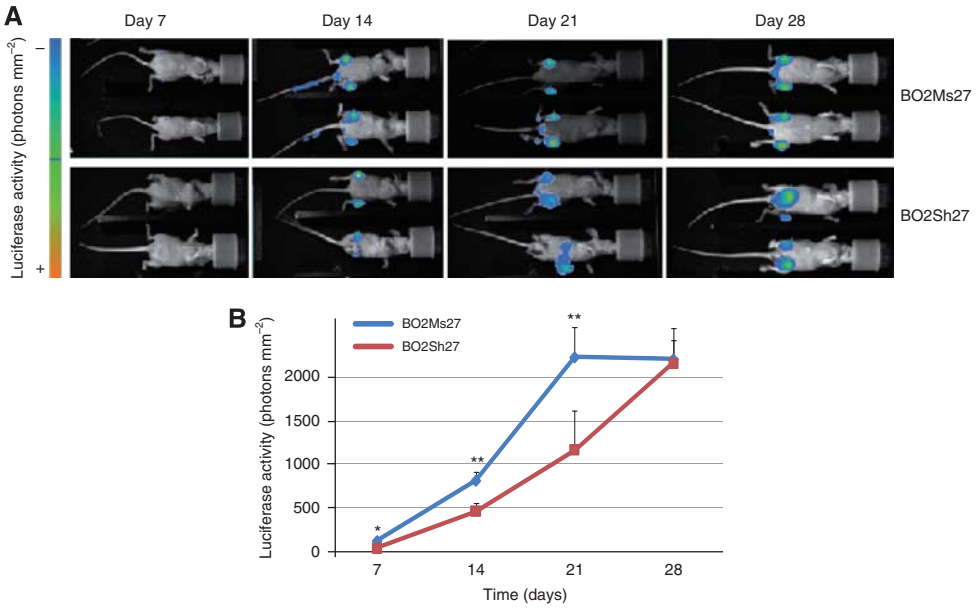
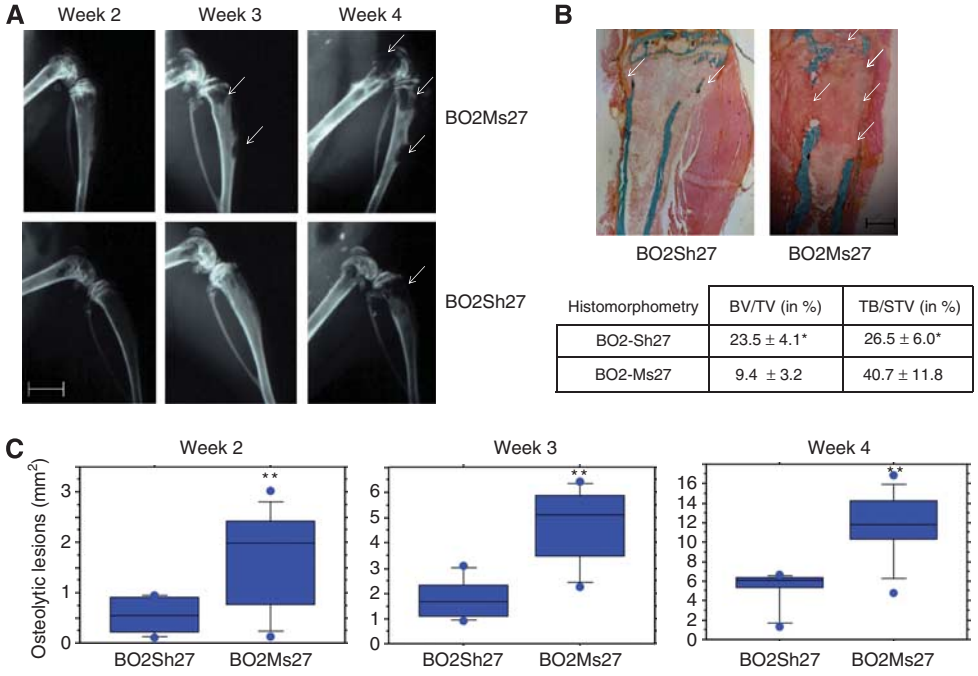


Figure 3 Effect of Hsp27 silencing on breast cancer bone metastasis formation in animals. B02Ms27 cells and cells silenced for Hsp27 expression (B02Sh27) were injected intravenously into nude mice. (**A**) Luciferase activity is quantified weekly using the night owl technology, as described in Materials and Methods, after a subcutaneous injection of luciferin. Luciferase activity is inlaid in the mice picture with the corresponding ladder. (**B**) Luciferase expression, weekly quantified in B02-SH27 clones and control cells, is reported in the graph. (**$P \leqslant 0.01$).

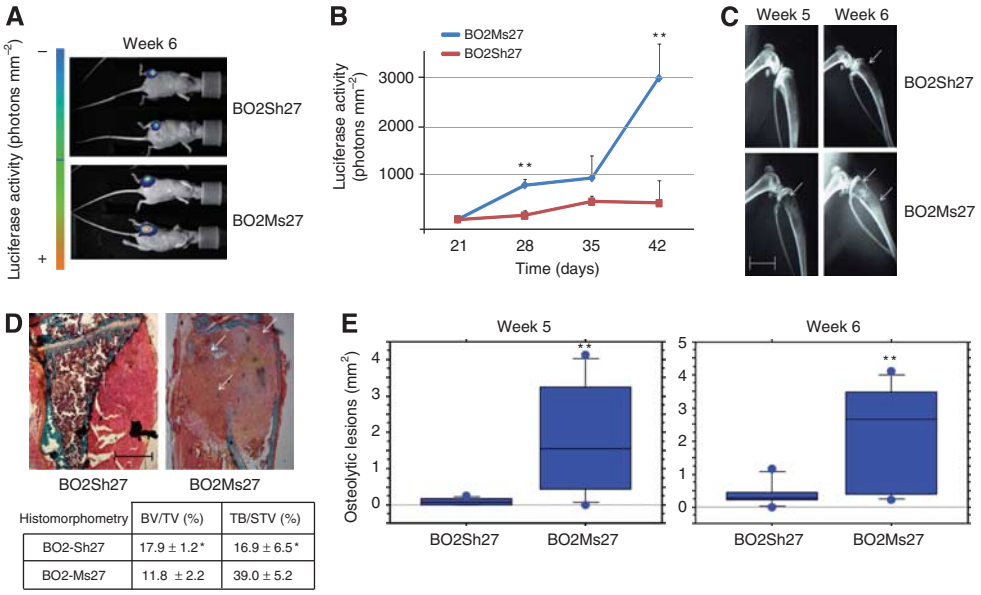


Histomorphometry	BV/TV (in %)	TB/STV (in %)
BO2-Sh27	23.5 ± 4.1*	26.5 ± 6.0*
BO2-Ms27	9.4 ± 3.2	40.7 ± 11.8

Figure 4 Effect of Hsp27 depletion on bone lesion formation. Radiographic, immunostaining and histomorphometric data were obtained on day 31 after tumour cell inoculation in nude mice. (**A**) Osteolytic lesions on representative radiographs are indicated by white arrows. Scale bar = 0.5 cm. (**B**) Representative bone histology of Goldner's trichrome-stained tibial metaphysis from metastatic animals. The bone is coloured in green; bone marrow and tumour cells are coloured in red. Scale bar = 0.5 mm. BV/TV: bone volume relative to tissue volume. TB/STV: skeletal tumour burden relative to soft tissue volume ratio (arrows: bone decalcification) (**$P \leqslant 0.01$). (**C**) Bone decalcification was quantified by software using Visiolab 2000, and the results are reported in the graph (**$P \leqslant 0.01$).

cells. It should be noted that trabecular bone was only partially destroyed in tibiae of mice injected with B02-Sh27 cells (BV/TV = 23.5%; TB/STV = 26.5%), whereas it had completely disappeared in the metaphysic tibiae of animals injected with B02-Ms27 cells (BV/TV = 9.4%; TB/STV = 40.0%).

This study also showed that at day 28, Sh27 tumour size is dramatically decreased in comparison with Ms27 tumours. This result is not in accord with the luciferase activity measured in these animals (see above; Figure 3), a discrepancy that could be the result of the fact that at day 28 the large size of the tumours



Histomorphometry	BV/TV (%)	TB/STV (%)
BO2-Sh27	17.9 ± 1.2*	16.9 ± 6.5*
BO2-Ms27	11.8 ± 2.2	39.0 ± 5.2

Figure 5 Hsp27 depletion decreases tumour formation in bone. B02-Ms27 cells and B02-Sh27 cells were injected in the left tibiae of female nude mice (**$P \leqslant 0.01$). (**A**) Luciferase activity was quantified using the night owl technology as described in Materials and Methods. Photographs presented are representative of the results for each animal group. (**B**) Luciferase activity was measured weekly during 2 months and are reported on graph. (**C**) Bone lesions were weekly quantified by radiography of the injected tibiae. Demineralisation is depicted with white arrows. Scale bar = 0.5 mm. (**D**) Histomorphometric data of tibiae were obtained 2 months after cell inoculation. The bone is coloured in green, whereas bone marrow and tumour cells (stars) are coloured in red. BV/TV: bone volume relative to tissue volume. TB/STV: skeletal tumour burden relative to soft tissue volume ratio. Scale bar = 0.5 mm (arrows: bone decalcification). (**E**) Quantification of osteolysis is performed on radiographs and is presented in a graph for each group of mice.

interfered with luciferase bioluminescence activity, which was then not precise enough to detect the differences observed in the histomorphometric study (El Hilali *et al*, 2002).

Hsp27 depletion inhibits bone tumour formation

In order to determine whether the decrease in bone metastasis formation observed in response to Hsp27 inhibition was only due to the inhibition of migration and invasion of the cells, we next injected the cells directly into the tibiae of nude mice. In this experiment, most of the mechanisms of migration could be ignored because the cells were already on the metastatic site. Xenograft development was evaluated weekly during 2 months by bioluminescence quantification and radiography. Luminescence activity was only detected in the injected tibiae, showing that the cells once injected in the tibiae did not disseminate in the organism (Figure 5A). Quantification of luciferase activity revealed a decrease in tumour burden in animals injected with B02 cells depleted of Hsp27 (Figure 5B). Therefore, this result was confirmed by histomorphometric analysis. Osteolytic lesions were also dramatically reduced in case of the inhibition of Hsp27, as also confirmed by histomorphometric analysis (Figure 5C and D). Moreover, quantification of bone destruction confirmed that osteolytic lesions were reduced after injection of the B02-Sh27 (BV/TV = 23.5%; TB/STV = 23.5%) cells compared with the B02-Ms27 cells (BV/TV = 9.4%; TB/STV = 40.7%).

Taken together, these experiments show that Hsp27 is not only important for increased migration and invasion properties of metastatic cancer cell, but it also has a major role in formation of the metastatic lesion *per se* at the secondary bone settings.

DISCUSSION

Hsp27 has been shown to be a major therapeutic target for cancer therapies against primary tumour formation by protecting cells against apoptosis. This stress protein is upregulated in many tumour types, including breast, ovarian, colon, head and neck cancer (Ciocca and Calderwood, 2005; Calderwood *et al*, 2006; Tsuruta *et al*, 2008; Huang *et al*, 2010), but was not well characterised during *in vivo* metastasis development. In our study, we show that Hsp27 is overexpressed in the aggressive forms of 53 human breast tumours. Thus, in tumours positive for lymph node invasion, the increase is 2.57-fold over the median expression, whereas it is 2.95-fold over the median in the metastatic ones (Figure 1A). This increased expression may have some correlation with the results presented in a recent study highlighting the role of Hsp27 in epithelial–mesenchymal transition (Wei *et al*, 2011).

To understand whether this overexpression is important in the development of metastasis in distant organs, we analysed the consequences of Hsp27 silencing in a mice xenograft model. We used the breast cancer cell line MDA-MB231/B02, characterised for its tropism for bone invasion (Pecheur *et al*, 2002). In breast cancer, bone metastases have been correlated with bone alteration-like demineralisation and fractures. The role of Hsp27 in breast cancer metastases, particularly in bone metastasis, is not clearly elucidated. We show here using two different techniques that Hsp27 silencing induces a decrease in metastasis formation (Figure 3 and 4). Luminescence detection was highly correlated with bone decalcification as revealed by radiographies, except in one case (at day 28, Figure 3A), probably because luciferase activity is altered in important metastases, leading to quantification defects as proven by other studies (El Hilali *et al*, 2002; Paroo *et al*, 2004).

Lemieux *et al* (1999) have shown that overexpression of Hsp27 leads to a decrease in cellular migration of MDA-MB231 cells, and that in the MDA-MB231 cells, overexpression of Hsp27 did not show a significant effect on bone dissemination and alteration *in vivo*. In our study, we silenced the expression of Hsp27 using a ShRNA approach and we characterised clones presenting a decrease of about 90% in Hsp27 expression. These depleted clones showed a decreased ability to migrate and invade *in vitro*.

Hence, it can be concluded that a decrease in bone metastasis formation can be observed following a depletion (our study) or overexpression (Lemieux *et al*, 1999) of Hsp27. These apparently conflicting results may suggest that the intracellular level of Hsp27 has a crucial role in this phenomenon. Consequently, a negative or positive change in Hsp27 balance level may alter the heterogenic structures formed by this protein (Paul *et al*, 2010) and destabilise its interactions with pro-metastatic client protein targets. In this regard, Hsp27 has been shown to stabilise F-actin filaments during cell migration (Pivovarova *et al*, 2007; Lee *et al*, 2008; Chen *et al*, 2009), confirming our results obtained with antisense strategy. Overexpression of Hsp27 could also have drastic effects on cytoskeleton architecture that may also lead to a decreased ability of cells to migrate. Future experiments are needed to test this hypothesis.

Our results also suggest that the decrease in cellular migration and invasion are not the only mechanisms responsible for the low level of bone metastasis formation. Indeed, we have observed that silencing of Hsp27 is associated with a decreased amount of tumour cells in the bone microenvironment (Figure 5). One possible hint for the effect is the fact that it is known that the bone microenvironment is a very stressful environment, owing mostly to intense mechanical stresses. We have previously shown that Hsp27 functional inhibition, by interfering peptides called aptamers, could modify the *in vivo* proliferation of tumours, whereas proliferation was not modified in cell culture conditions (Gibert *et al*, 2011). These results are in correlation with our presented data because proliferation is not modified in the *in vitro* context, whereas B02-Sh27 tumour growth was altered *in vivo*. It is thus tempting to propose that Hsp27 may have an important role in protecting bone cells against these kinds of stress (Muraoka *et al*, 2010).

Altogether, these results support the view that Hsp27 has a crucial role during metastasis and tumour implementation and expansion in the bone. Targeting Hsp27 may then prove to be a promising strategy to prevent bone metastasis, which is a terrible step towards patient death.

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Conflict of interest

The authors declare no conflict of interest.

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