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HSP27 is required for invasion and metastasis triggered by hepatocyte growth factor

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The hepatocyte growth factor (HGF) also known as scatter factor activates cancer cell invasion and metastasis. We show that in ovarian cancer cells HGF induced the phosphorylation of the small heat shock protein of 27 kDa (HSP27) by activating the p38MAPK. HSP27 is increased in many cancers at advanced stage including ovarian cancer and associated with cancer resistance to therapy and poor patients' survival. The phosphorylation of HSP27 regulates both its chaperone activity and its control of cytoskeletal stability. We show that HSP27 was necessary for the remodeling of actin filaments induced by HGF and that motility *in vitro* depended on the p38MAPK-MK2 axis. *In vivo*, HSP27 silencing impaired the ability of the highly metastatic, HGF-secreting ovarian cancer cells to give rise to spontaneous metastases. This was due to defective motility across the vessel wall and reduced growth. Indeed, HSP27 silencing impaired the ability of circulating ovarian cancer cells to home to the lungs and to form experimental hematogenous metastases and the capability of cancer cells to grow as subcutaneous xenografts. Moreover, HSP27 suppression resulted in the sensitization of xenografts to low doses of the chemotherapeutic paclitaxel, likely because HSP27 protected microtubules from bundling caused by the drug. Altogether, these data show that the HSP27 is required for the proinvasive and prometastatic activity of HGF and suggest that HSP27 might be not only a marker of progression of ovarian cancer, but also a suitable target for therapy.

Genetic, experimental and clinicopathological data show that the hepatocyte growth factor (HGF) and its tyrosine kinase receptor MET play a fundamental role in human cancer, by promoting growth, cell survival, angiogenesis and metastasis.[1–3] In particular, HGF is also known as scatter factor as it causes tumor cells to lose contacts with neighbors and become highly motile, initiating tumor invasion of adjacent tissue and, ultimately, the growth of distant tumors. The HGF/MET couple elicits these biological activities by initiating several downstream signaling pathways, such as those involving the mitogen-activated protein kinases (MAPK), the phosphatidylinositol 3-kinase/AKT and the AKT substrate mammalian target of rapamycin (mTOR).[4]

The HGF receptor, encoded by the MET oncogene is expressed in most epithelial cancers. In ovarian cancer, it is expressed in ~70% and overexpressed in 30% of cases.[5,6] It has been shown that HGF promotes the spread and metastasis of ovarian cancer cells in preclinical models *in vitro* and *in vivo*.[7–9] High level of HGF was measured in fluids of both benign and malignant ovarian cysts, as well as in ovarian cancer ascites.[10] Increased levels of HGF were found in sera of patients with advanced ovarian cancers and associated to poor prognosis.[11]

The small heat shock protein of 27 kDa (HSP27) encoded by the *HSPB1* gene, belongs to the family of small heat shock proteins (HSPs), which are involved in a wide range of cellular processes and are specially known for their ability to help cells to survive under stress conditions, for example, after heat shock. HSP27 is a molecular chaperone with antiaggregation property, as it participates in sequestering damaged proteins[12] and is involved in the proteasomal degradation of certain proteins under stress conditions.[13] Besides its role in stressed cells, HSP27 plays crucial roles within the cell under unstressed conditions as it provides cytoskeletal structural stability[14,15] and exerts an antiapoptotic function by binding

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What's new?

Hepatocyte growth factor, HGF, induces cancers to spread. Recently, researchers learned that it activates a small heat shock protein, HSP27, which is increased in advanced stage cancers, and associated with resistance to chemotherapy. In this paper, the authors showed that silencing HSP27 stopped ovarian cancer cells from metastasizing. Suppressing HSP27 also sensitized the tumors to Paclitaxel. The results show that HSP27 is important for metastasis and invasion, and could make a promising target for therapy.

crucial apoptotic proteins.[16,17] The role of HSP27 in the stabilization of the cytoskeleton might help promoting cell motility and invasiveness and thereby metastasis. Together with the frequent report of increased expression of HSP27 in human cancers at advanced stage,[18] these concepts suggested that HSP27 might be a marker of cancer progression and bad prognosis, and that targeting HSP27 could be a therapeutic strategy.

HSP27 is regulated at transcriptional and post-transcriptional levels. The synthesis of HSP27 can be induced not only by heat shock and other stress conditions, but also by physiological stimuli such as those regulating differentiation.[19] HSP27 can oligomerize[12] and oligomerization is regulated by phosphorylation.[20,21] Specific changes in oligomerization and phosphorylation allow HSP27 to interact with definite polypeptides and to modulate their folding, activity and half-life. This links HSP27 to multiple and specific cellular functions.[20] For example the small phosphorylated HSP27 oligomers appear to be the active form having F-actin capping activity and negatively modulating the growth of F-actin fibers,[15] while larger but unphosphorylated oligomers of different size bind and stabilize polypeptides such as STAT-3, procaspase-3 and histone deacetylase 6 preventing their degradation.[22]

HSP27 phosphorylation is catalyzed by a large number of kinases, among which the MAPKAPK2 mitogen-activated protein kinase-activated protein kinase 2 (MK2).[21] MK2 is a substrate of the 38 kDa mitogen-activated protein kinase (p38MAPK) that is activated by the HGF and is necessary for HGF triggered motility.[23] Altogether these data prompted us to investigate the role played by HSP27 in the HGF triggered metastasis.

Material and Methods

Cell lines and reagents

TOV-21G and SK-OV-3 cell lines were from the American Type Culture Collection (Manassas, VA), acquired from LGC Standards (Teddington, London, UK). OVCAR-3 cells were from the NCI-60 collection, acquired from Charles River (Wilmington, MA). All cell lines were purchased in 2011 and grown as indicated by the provider. HGF was purchased from RayBiotech (Norcross, GA). Paclitaxel (PTX) was obtained from Teva Pharmaceutical Industries (Petah Tikva, Israel).

Lentiviral infection

Stable silencing of HSP27 was achieved using the shRNAs transduced with lentiviral vectors. MISSION® shRNAs for HSP27 and the control one were purchased from Sigma-Aldrich (Saint Louis, MO). SK-OV-3 cells were engineered to produce and secrete HGF as previously described.[24] pGIPZ shRNAs for HSP27 and the control one were purchased from Thermo Fisher Scientific (Waltham, MA) and used to silence HSP27 for the study of spontaneous metastases *in vivo* (see below). SK-OV-3 cells were transduced with the dominant negative p38MAPK mutant as previously described.[25] To study hematogenous metastases, the stable expression of luciferase and GFP was obtained using the pRRL.sin.PPT.CMV.Luciferase.iresEMCVwt.eGFP.pre lentiviral vector kindly provided by Dr. Elisa Vigna. The LV vectors were produced and used as reported previously.[25]

Western blotting and antibodies

Western blotting was carried out as described previously.[25] The HSP27, antitubulin phospho-HSP27 (Ser15, 78 and 82), phospho-p38MAPK, phospho-MK2 and phospho-c-Met antibodies were from Cell Signaling Technology (Beverly, MA); the c-Met antibody was from Invitrogen (Paisley, Scotland, UK); the actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); the vinculin antibody was from Sigma-Aldrich. Primary antibodies were detected using the appropriate peroxidase-conjugated secondary antibody (Jackson Immunoresearch, Newmarket, Suffolk, UK), and revealed by Pierce® ECL Western Blotting Substrate (Rockford, IL).

Quantitative RT-PCR

Quantitative RT-PCR was performed as described.[26] Primer sets and PCR cycling conditions are available from the authors.

Immunofluorescence

SK-OV-3 cells grown on glass coverslips, left untreated or treated for 6 hr with HGF or PTX, were fixed with paraformaldehyde and permeabilized. After blockade, cells were incubated for 1 hr at room temperature with the primary antibody, for 30 with the secondary antibodies (donkey anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 555, Invitrogen) and then stained with DAPI. For F-actin staining, the Alexa Fluor 555-phalloidin was used (Invitrogen). Coverslips were then mounted using the fluorescence mounting medium (Dako, Glostrup, DK) and analyzed using a confocal laser scanning microscope (TCS SPE II; Leica, Wetzlar, D) equipped with 63×/1.40 oil-immersion objective. For F-actin

quantification, three independent experiments were performed and four fields per coverslip were scanned for each experiment. To quantify the fluorescent signal associated to 555-phalloidin the ImageJ software was used (Wayne Rasband, National Institute of Health, Bethesda, MD). The fluorescence values were then normalized to the number of nuclei for each field.

Motility and migration assays

For the scatter assay, SK-OV-3 cells were seeded in triplicate at 1,500 cells/well in 24-well plates in complete medium. Once cells formed compact colonies, they were treated with 50 ng/ml HGF for 24 hr. For the motility assay, 6×10^4 SK-OV-3 were seeded in duplicate in the upper compartment of the Transwell chamber (BD Falcon™, Franklin Lakes, NJ) and medium containing 50 ng/ml HGF was added in the lower compartment for 5 hr. In both assays, after stimulation cells were fixed with 11% glutaraldehyde and stained with 0.1% crystal violet in 20% methanol solution (Sigma Aldrich); photographs were taken by optical microscope. For both assays, three independent experiments were performed. For the motility assay four fields/insert/experiment were photographed and analyzed with ImageJ software.

In vivo experiments

For the measure of spontaneous metastasis, SK-OV-3 cells were engineered to secrete HGF and, when indicated, to express either a control or a HSP27 specific shRNA using lentiviral vectors carrying the GFP transgene under a different promoter. Cells expressing GFP uniformly were sorted using MoFloTM XDP (Beckman Coulter, High Wycombe, UK). A total of 5×10^5 sorted cells were injected subcutaneously (s.c.) in the right posterior flank of 6-week-old NOD/SCID mice (Charles River Laboratories). Animals (seven mice per group) were sacrificed and the lungs examined when tumors reached the volume of 300–500 mm^3 (*i.e.*, ~6 weeks after the injection). The lungs were removed and fixed. Fluorescent acquisition images were collected with IVIS® Lumina II (Caliper Life Sciences, Hopkinton, MA), using excitation wavelength of 465 nm and detection of 520 nm for GFP, a field of view of 5 cm, a time exposure of 0.5 min, with medium sensitivity binning. Images were processed using Life Science's Living Image software and the total number of lung surface metastasis foci was counted.

For the measure of hematogenous metastases, control and HSP27-silenced SK-OV-3 were transduced with lentiviral vectors containing the luciferase and GFP expression vector. Cells were sorted according to the GFP expression using MoFloTM XDP (Beckman Coulter, High Wycombe, UK). Subsequently, 3×10^5 sorted control and HSP27-silenced SK-OV-3 were injected in the tail vein of female CD1 nude mice (six mice per group, Charles River Laboratories). To monitor the formation lung colonies through time, bioluminescent acquisitions images were collected by means of the IVIS® Lumina II imaging system, after intraperitoneal injection of luciferine (150 mg/kg body weight). Detection of bioluminescent signal was acquired using a field of view of 12.5 cm, a time exposure of 1 min, with high sensitivity binning. After 2 months from cells injection, mice were sacrificed immediately after luciferine injection and lung explants were visualized by IVIS® Lumina II. Images were processed using Life Science's Living Image software and the total number of metastatic foci at lung surface was determined.

Short-term lung colonization assay was carried to evaluate cell homing to the lungs by labeling 5×10^5 SK-OV-3 control cells with cellTrace™ CFSE (Molecular probes, USA) and 5×10^5 HSP27 silenced SK-OV-3 cells with cellTrace™ VIOLET (Molecular probes). Cells were mixed in 200 μl PBS and three mice (NOD/SCID) for each time point were injected intravenously. Lungs were explanted 2 and 24 hr after injection and fixed. Microscopy imaging was performed using a Leica TCS SP5-AOBS 5-channel confocal and multiphoton system (Leica Microsystems) equipped with a pulsed femtosecond Ti:Sapphire (Ti-Sa) Chameleon Vision II laser (Coherent), tunable for excitation from 680 to 1,080 nm. Violet, CFSE were captured by two detection channels. The two-photon laser was tuned to a wavelength of 700 and 985 nm for the first and second channel respectively. Emission wavelengths of 400–476 nm (blue, violet) and 490–539 nm (green, CFSE) were collected. Images of the tissues were taken using a HCX PL APO CS 20×/0.7 NA oil immersion objective. Series of *x-y-z* images (typically $0.75 \times 0.75 \times 2.5$ μm^3 voxel size) were collected along the *z*-axis at 2.5 μm intervals throughout the tissues (30–50 μm) depth, images were then analyzed with ImageJ software.

To study the sensitization of HSP27-silenced tumors to PTX, 3×10^6 control and HSP27-silenced SK-OV-3 were injected s.c. in the right posterior flank of 6-week-old female CD1 nude mice (Charles River Laboratories). When tumors were palpable, mice were divided in experimental groups of six animals and treated with 20 mg/kg PTX by i.v. injection once a week for 4 weeks. Control animals were treated with vehicle. Tumor dimensions were evaluated using a caliper and were calculated using the formula $(D \times d^2)/2$, where D is the major tumor axis and d is the minor tumor axis. All animal procedures were approved by the Veterinary Ethical Commission of the I.R.C.C. and by the Italian Ministry of Health.

Results

HGF triggers HSP27 phosphorylation in ovarian cancer cell lines

We studied three ovarian cancer cell lines, representative of the genetic heterogeneity of human ovarian cancer: SK-OV-3, TOV-21G and OVCAR-3. Their genetic profiles are reported at http://www.sanger.ac.uk/perl/genetics/CGP/core_line_viewer?action=cell_lines and Ref. [27]. For example, SK-OV-3 cells show deletions of the *CDKN2a* and *TP53* genes and a *PIK3CA* mutation; TOV-21G cells have wild-type *TP53* and

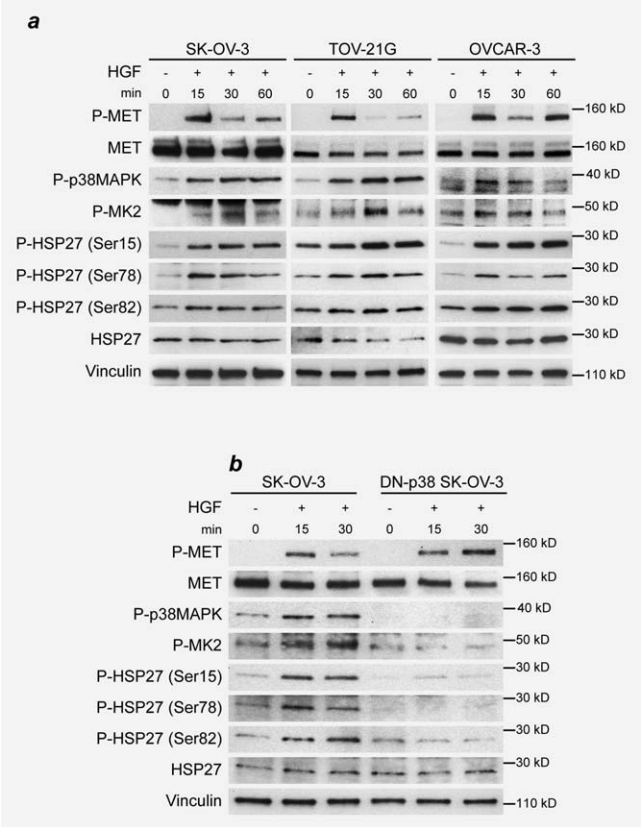

Figure 1. HGF triggers HSP27 phosphorylation in ovarian cancer cell lines. Expression and phosphorylation of proteins in the SK-OV-3, TOV-21G and OVCAR-3 ovarian cancer cell lines were evaluated with Western-blot analysis of total cell extracts. (*a*) HGF-induced HSP27 phosphorylation (P-HSP27) was evaluated with Western-blot analysis using antibodies specific for each phosphorylated residue (serines 15, 78 and 82). Activation of MET (P-MET), p38MAPK (P-p38MAPK) and MK2 (P-MK2) were evaluated by labeling the phosphorylated forms with phospho-specific antibodies. All ovarian cancer cell lines were stimulated with 50 ng/ml HGF at different time points are indicated. When possible, the same blots were probed sequentially. All blots were re-probed for vinculin to confirm equal loading. (*b*) The dependence of the phosphorylation of HSP27 on p38MAPK was assessed by transducing SK-OV-3 cells with the dominant negative p38MAPK mutant (DN-p38) and stimulating cells with 50 ng/ml HGF at different time points as indicated. Western-blot analysis and antibodies used were as in panel (*a*).

FANCF inactivated by methylation and OVCAR-3 cells carry a *TP53* mutation and show *PIK3CA* amplification.

In all three ovarian cancer cell lines, both the MET receptor for HGF and HSP27 are consistently expressed (Fig. 1*a*). HGF stimulation induced the phosphorylation of HSP27. Basally, HSP27 phosphorylation was barely detectable, while after stimulation with HGF phosphorylation was observed at the three main phospho-acceptor serine residues (Fig. 1*a*), which are Ser15, Ser78 and Ser82 in humans.[21] HSP27 phosphorylation was associated to the phosphorylation of the p38MAPK and MK2, which are the best characterized kinases leading to HSP27 phosphorylation. We confirmed the involvement of the p38MAPK-MK2 cascade by transducing cells with the dominant negative p38MAPK mutant.[25] In the absence of a functional p38MAPK, neither MK2 nor HSP27 were phosphorylated upon HGF stimulation (Fig. 1*b*), indicating that the HGF-triggered phosphorylation of HSP27 at the Ser15, Ser78 and Ser82 depended on the activation of p38MAPK.

HGF-triggered ovarian cancer cell motility is impaired by HSP27 silencing and by inhibition of the p38MAPK/MK2 axis

HGF, also known as scatter factor, is able to induce epithelial-to-mesenchymal transition and cell motility.[1,4] As HSP27 controls actin filament polymerization,[15] we evaluated if HSP27 is involved in the motility induced by HGF in ovarian cancer cells. HSP27 was stably silenced with specific short-hairpin RNAs carried by lentiviral vectors (Fig. 2*a*) in the SK-OV-3 ovarian cancer cells and cell motility was evaluated in the scatter and in the migration assays (Figs. 2*b* and 2*e*).

Sparse SK-OV-3 cells grew on plastic forming packed islands with cell-to-cell contacts mimicking the structure of an epithelial sheet. Addition of HGF to cell culture medium causes loss of cell junctions and cell dispersal throughout the culture dish (scatter). Figure 2*b* shows that HSP27 silencing impaired the capability of SK-OV-3 cells to scatter upon HGF stimulation.

As it is known that HSP27 affects the polymerization of actin filaments, confocal analysis and phalloidin staining were used to measure the amount of F-actin in HSP27-silenced and control cells left untreated or treated with HGF (Fig. 2*c*). As shown by the phalloidin staining and the relevant quantification (Figs. 2*c* and 2*d*, respectively), HSP27 silencing resulted in the decrease of F-actin.

The migratory capability of HSP27-silenced and control cells was measured with the modified Boyden chamber assay that uses the Transwell® permeable support. Figure 2*e* shows that HSP27 silencing impaired the ability of HGF to induce cell migration through permeable filters. Moreover, to confirm the role of the p38MAPK/MK2 axis in linking MET activation by HGF to HSP27 function, we inhibited either p38MAPK or MK2, by expressing a dominant negative p38MAPK form (see above) or by treating cells with the MK2 biochemical inhibitor III, which inhibits not only MK2 but also the MK5 and MK3 substrates of p38MAPK. As shown in Figure 2*f*, the inhibition of either components of the axis resulted in abrogation of HGF stimulated cell migration. Altogether these data suggested that the p38MAPK/MK2/HSP27 pathway plays a key role in the control of HGF-dependent ovarian cancer cells motility.

HSP27 silencing impairs the prometastatic activity of HGF in ovarian cancer cells

Cell motility is a prerequisite for cancer cell to disseminate and to home in a distant site in the multistep process of

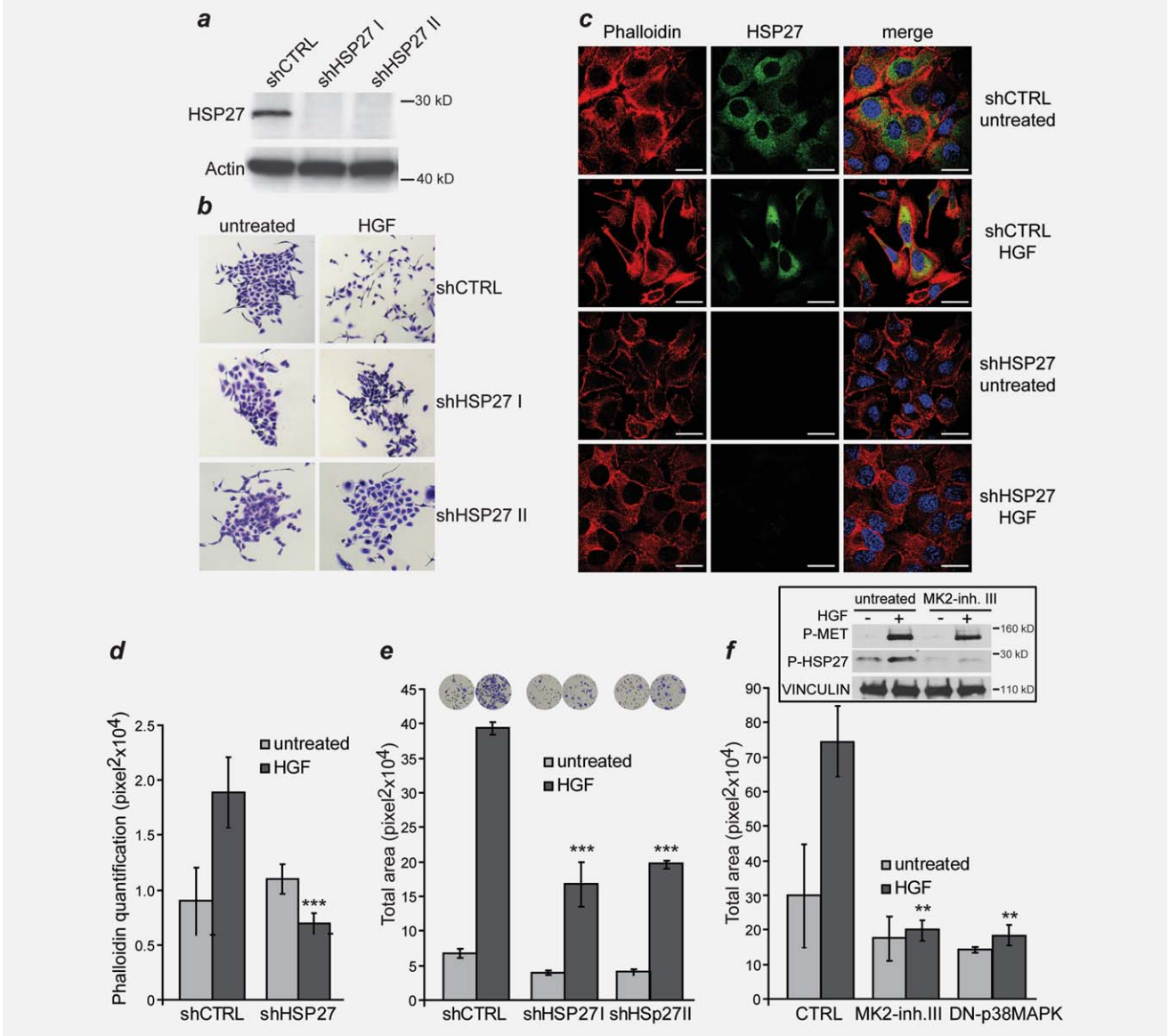

Figure 2. HSP27 silencing and inhibition of the p38MAPK-MK2 pathway impair HGF-triggered ovarian cancer cell motility. (*a*) HSP27 was silenced stably in SK-OV-3 cells with two different short-hairpin RNAs (shHSP27 I and II). As a control, cells were transduced to express a scramble short-hairpin RNA sequence (shCTRL). Expression of HSP27 was assayed in control and HSP27-silenced cells with Western-blot analysis. The blot was re-probed for actin to confirm equal loading. (*b*) Scatter assay was performed to evaluate the HGF-induced motility of SK-OV-3 cells. Colonies formed by control and HSP27-silenced were left untreated or treated with 50 ng/ml HGF for 24 hr. Photographs were taken with an optical microscope. The results of a representative experiment are shown. HGF triggered the dispersal of cells (scattering) was impaired in HSP27 silenced cells. (*c*) HGF-triggered actin polymerization was evaluated by confocal microscopy. Control and HSP27-silenced SK-OV-3 cells were left untreated or treated with 50 ng/ml HGF for 6 hr. The antibody for human HSP27 was used together with phalloidin that binds F-actin. Nuclei were stained with DAPI. Scale bar: 15 μm. (*d*) Phalloidin fluorescence signal was quantified by ImageJ software to evaluate the amount of F-actin. Data are the mean ± SEM. The *p* value was calculated *vs*. control cells (***$p < 0.001$) by ANOVA test. (*e*) Transwell® permeable supports were used to evaluate the migratory capability of SK-OV-3 toward a chemotactic stimulus such as HGF. Control and HSP27-silenced SK-OV-3 cells were plated in the upper chamber and left untreated or treated with 50 ng/ml HGF for 5 hr. Photographs of the lower side of the filters (insets in the graph) were taken with an optical microscope and ImageJ software was used to quantify the area covered by cells. Five independent experiments were performed and data are the mean ± SEM. The *p* value was calculated *vs*. control cells (***$p < 0.001$) by ANOVA test. (*f*) Transwell® permeable supports were used as in panel (*e*) to evaluate the effect of inhibiting either p38MAPK or MK2 onto the migratory capability of SK-OV-3 toward HGF. Control SK-OV-3 cells or SK-OV-3 cells expressing DN-p38MAPK were plated in the upper chamber and left untreated or treated with 50 ng/ml HGF for 5 hr. The suppression of HSP27 phosphorylation by DN-p38MAPK is shown in Figure 1*b*. Alternatively, SK-OV-3 cells in the upper chamber were treated with the MK2 inhibitor III (20 μM) for 16 hr and, when indicated, with HGF. Photographs of the lower side of the filters (insets in the graph) were taken with an optical microscope and ImageJ software was used to quantify the area covered by cells. Three independent experiments were performed and data are the mean ± SEM. The *p* value was calculated *vs*. control cells (**$p < 0.01$) by ANOVA test. The inhibition of HSP27 phosphorylation by the MK2 inhibitor III is shown in the inset.

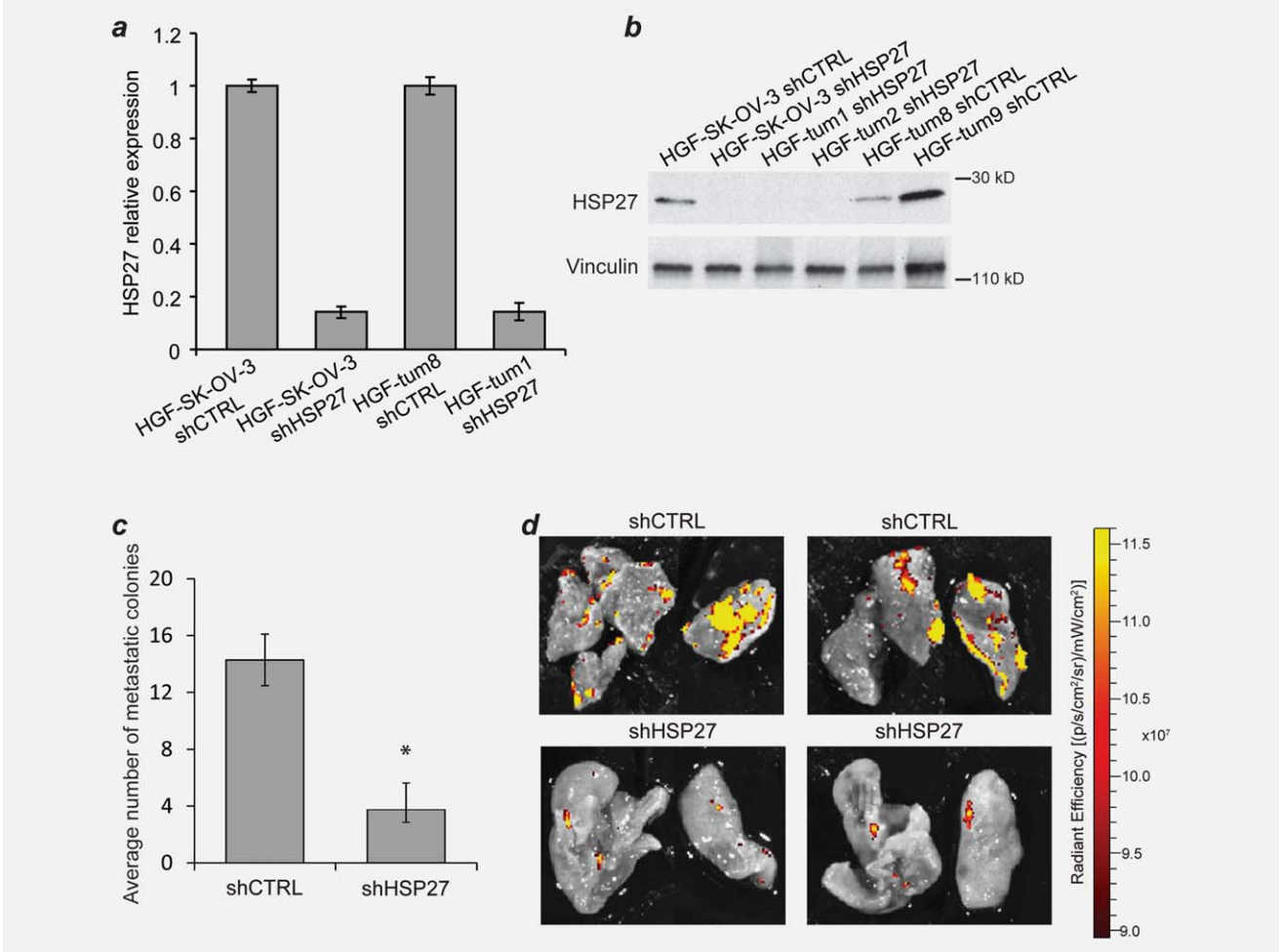

Figure 3. HSP27 silencing impairs the ability of ovarian cancer cells to give rise to spontaneous metastases. SK-OV-3 cells were engineered to secrete HGF and to express either HSP27 specific (shHSP27) or control shRNA (shCTRL) driven by lentiviral vectors carrying also the GFP transgene. Cells were injected subcutaneously in immune-compromised mice and spontaneous metastases formed by SK-OV-3 xenografts were evaluated, by examining lungs when primary tumors have reached similar volumes. (*a*) Silencing of HSP27 in the HGF-secreting, GFP expressing cells, measured with quantitative RT-PCR. (*b*) Silencing of HSP27 in the HGF secreting injected cells and in tumors grown subcutaneously, evaluated using Western-blot analysis of HSP27. (*c*) Quantification of lung metastatic foci visualized as fluorescent dots by means of the IVIS® Lumina II imaging system. The average number of metastases in seven mice in a representative experiment is shown. Student's *t*-test $*p < 0.05$; all average values of data were expressed as mean ± SEM. (*d*) Representative *ex vivo* fluorescent images of the lungs (left and right lobes are separated) of mice with metastatic foci. Fluorescent intensity is expressed as radiance efficiency (p/s/cm^2/sr/μW^{-1}/cm^2).

metastasis. Thus, we evaluated whether HSP27 was involved in the prometastatic activity of HGF in ovarian cancer cells *in vivo*.

SK-OV-3 cells were engineered to produce and secrete HGF as described.[24] HSP27 silencing was obtained (Figs. 3*a* and 3*b*) using a specific shRNA driven by a lentiviral vector carrying also the GFP transgene to allow cell sorting and to facilitate the detection of metastases. Cells were injected subcutaneously in immune-compromised mice. HGF-secreting cells formed tumors that showed a high propensity to give spontaneous metastases (Figs. 3*c* and 3*d*). After HSP27 silencing, the HGF-secreting tumors showed a slightly delayed growth (data not shown) and formed a smaller number of spontaneous metastases (Figs. 3*c* and 3*d*). Notably, no correlation was detectable between the volume of each primary tumor and the number of metastases formed (data not shown).

These data suggest that HSP27 plays an important role in the HGF-triggered metastatic process and possibly in the process of metastasis in general. HSP27 silencing might have impaired some of the biological properties required for the accomplishment of the process, such as local invasion, intravasation, survival in the bloodstream, extravasation and growth at distant sites.

HSP27 silencing impairs the ability of ovarian cancer cells to form experimental metastases

To evaluate the role of HSP27 in the cell capability to survive in the bloodstream, to migrate across the vessel wall and to

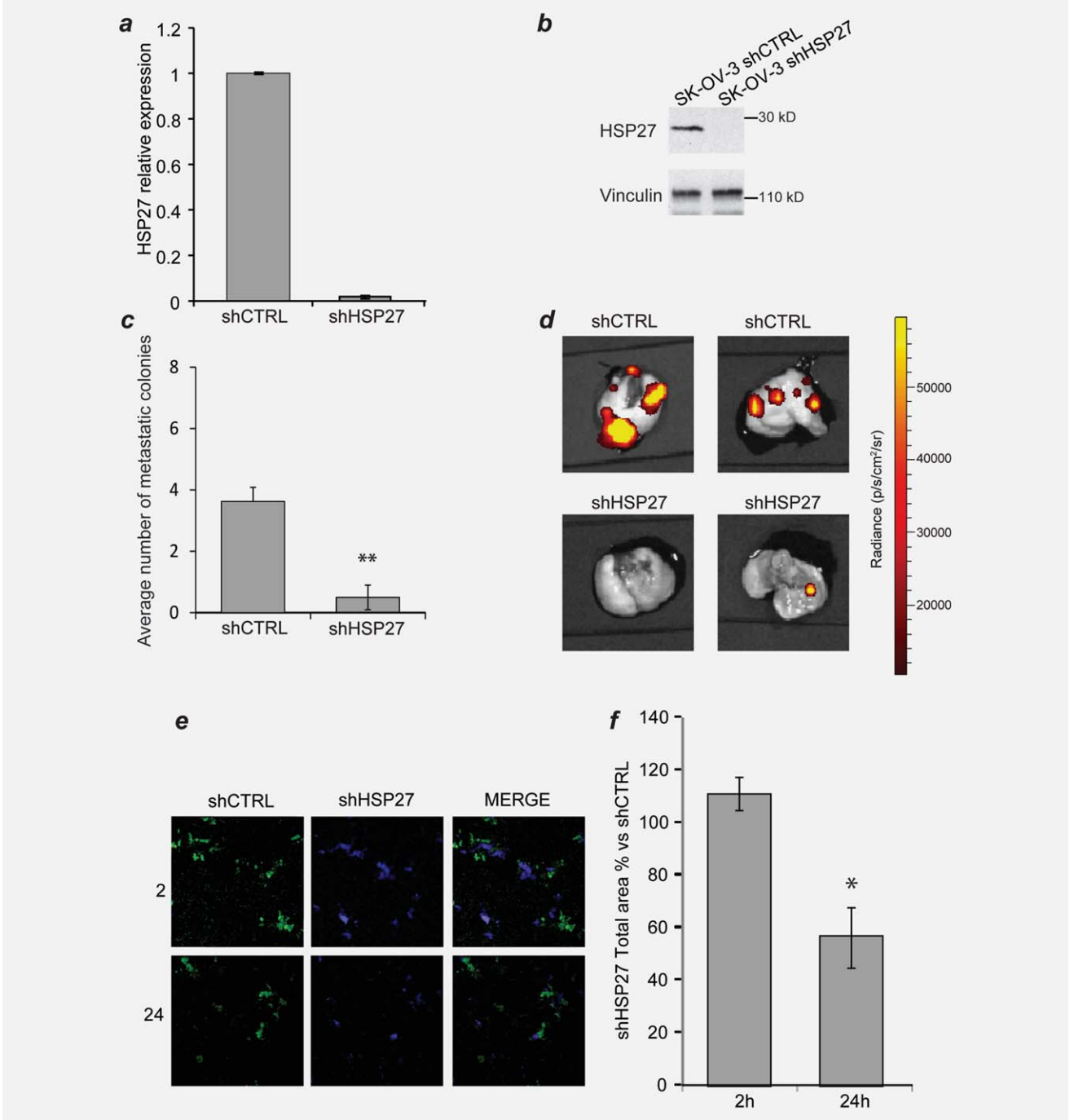

Figure 4. HSP27 silencing diminishes cell ability to form experimental hematogenous metastases and impairs cell homing to the lungs. (*a*) Silencing of HSP27 in the injected SK-OV-3 cells expressing luciferase and GFP, measured with quantitative RT-PCR. (*b*) Silencing of HSP27 in the injected cells, evaluated using Western-blot analysis of HSP27. (*c*) Quantification of lung metastatic foci visualized as bioluminescent dots by means of the IVIS® Lumina II imaging system. The average number of metastases in five mice in a representative experiment is shown. Student's *t*-test $**p < 0.01$; all average values of data were expressed as mean ± SEM. (*d*) Representative *ex vivo* bioluminescent images of lungs of two mice, visualized 2 months after the inoculum of luciferase-positive SK-OV-3 cells into the tail vein; bioluminescence intensity is expressed as radiance (p/s/cm^2/sr). (*e*) Detection in the lung of control and HSP27 silenced SK-OV-3 cells (silencing is shown in Fig. 2) labeled with cellTrace™ CFSE (green) and VIOLET (violet), respectively; cells were mixed and injected intravenously; after 2 and 24 hr (h) lungs were explanted and fixed; cells were visualized with confocal microscopy and representative fields are shown. (*f*) Ratio between green and violet fluorescent cells in the lungs. Lungs of three mice for each time point were examined and at least four fields per lung were acquired. For each field a Z-stack acquisition of several slices was made. For image analysis with ImageJ software, a maximum intensity projection was made for all fields before quantifying the area covered by cells for each single channel. Data are the mean ± SEM The *p* value was calculated using the Student's *t*-test ($*p < 0.05$).

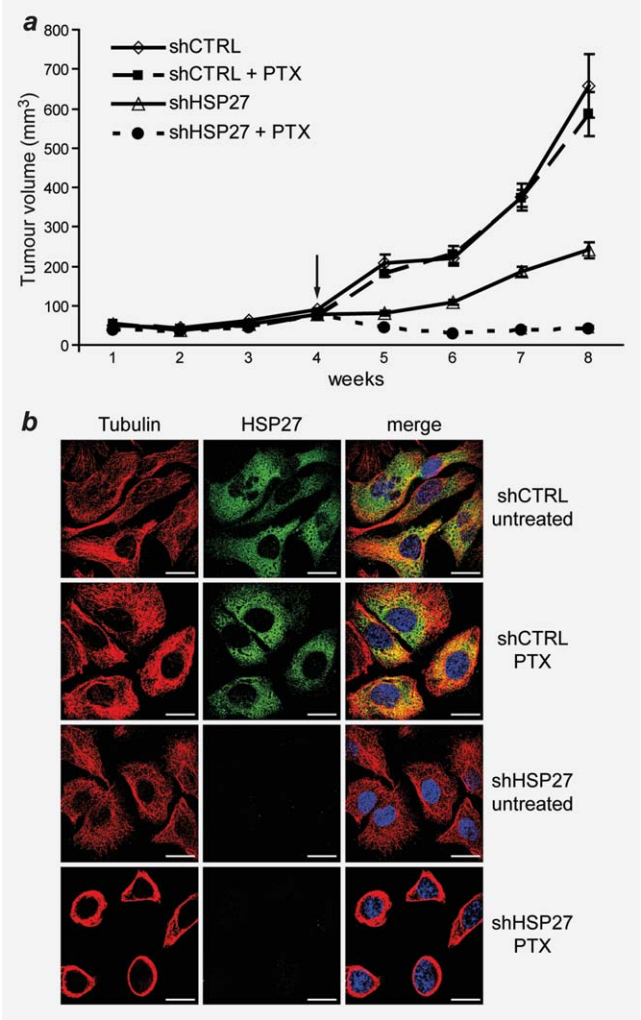

Figure 5. HSP27-silencing impairs ovarian cancer cell growth and sensitizes them to PTX treatment *in vivo*, by affecting microtubule architecture. (*a*) The involvement of HSP27 in the growth of SK-OV-3 and in their response to PTX was evaluated *in vivo*. Control and HSP27-silenced SK-OV-3 cells were injected in the flank of CD1 nude mice. When tumors were overtly palpable, mice were left untreated or treated with 20 mg/kg i.v. PTX once a week for 4 weeks. The black arrow indicates the beginning of treatment. Data are the mean ± SEM. (*b*) Microtubule architecture upon PTX treatment was studied by confocal microscopy. Control and HSP27-silenced SK-OV-3 cells were left untreated or treated with 100 nM PTX for 6 hr. The antibodies for human HSP27 and tubulin were used together with DAPI for nuclear staining. Scale bars: 15 μm.

form lung colonies, cell ability to form experimental hematogenous metastases was measured. HSP27-silenced and control SK-OV-3 cells (Figs. 4*a* and 4*b*) were stably transduced with lentiviral vectors carrying a luciferase-IRES-GFP transgene, so that GFP-expressing cells were isolated by cell sorting. Sorted cells were injected into the tail vein of immunecompromised mice and the formation of lung colonies was evaluated after 21 days. Luciferine injection allowed the monitoring of animals in time and the measure of lung colonization. Figures 4*c* and 4*d* show that control SK-OV-3 cells formed experimental metastases, which were almost abrogated by HSP27 silencing.

To assess the role played by the homing of cells to the lung *versus* cell ability to proliferate in the formation of experimental metastases, short-term colonization was evaluated by injecting in the tail vein simultaneously the HSP27 silenced and control cells differentially labeled with two compounds that, once inside the cells, are cleaved to generate two different fluorescent dyes, violet and green, respectively. Two hours after injection, confocal microscopy allowed the detection of the same number of cells in the lungs, while after 24 hr green cells, *i.e.*, control cells, were more numerous than the violet-labeled HSP27 silenced cells (Figs. 4*e* and 4*f*).

HSP27 is necessary for the growth of ovarian cancer cells *in vivo* and protects cells from the chemotherapeutic PTX

The inability of HSP27 silenced cells to form lung colonies might be due also to their inability to proliferate. *In vitro*, HSP27 silencing did not affect the proliferation rate of control and HGF-secreting ovarian cancer cells (data not shown). Conversely, *in vivo* HSP27 silencing reduced significantly the subcutaneous growth of control ovarian cancer cells (Fig. 5*a*). As mentioned before, also the growth *in vivo* of the HGF-secreting cells was slightly delayed.

Nonetheless, even *in vivo* the growth of control ovarian cancer cells was not fully abrogated by HSP27 suppression. Thus, we wondered whether suppression of HSP27 might sensitize cells to PTX *in vivo*, as it sensitizes bladder[28] and prostate[29] cancer cells *in vivo* and an ovarian cancer cell line *in vitro*.[30] We found that also SK-OV-3 cells are sensitized to PTX *in vitro* (not shown). We thus studied the effect of PTX on the *in vivo* growth of HSP27-silenced ovarian cancer cells as subcutaneous tumors. PTX was administered at low doses, to which control cells were completely unresponsive (Fig. 5*a*). HSP27-silencing blocked the *in vivo* growth in response to PTX (Fig. 5*a*). As HSP27 binds microtubules and likely stabilizes them,[31] we investigated whether HSP27 loss and PTX affected microtubules of ovarian cancer cells, using tubulin staining and confocal microscopy (Fig. 5*b*). HSP27 silencing alone did not affect microtubules. PTX at the used doses did not change the architecture of microtubules. Conversely, in response to PTX, HSP27-silenced cells showed a round shape indicative of cell shrinkage likely due to the bundling of microtubules (Fig. 5*b*). These results underscored the importance of HSP27 in the response of ovarian cancer cells to PTX.

Discussion

This article shows that HGF elicits phosphorylation of HSP27, through the p38MAPK-MK2 pathway, and that this phosphorylation is necessary for the prometastatic capability of HGF. This is in line with the notion that stimuli other than heat shock can induce phosphorylation of HSP27.[21] Phosphorylation induced by HGF occurred at all three best characterized serines, which are the phospho-acceptor sites *in*

vivo and are all involved in regulating HSP27 functions.[21] Several studies have addressed the contribution of single phosphorylation of Hsp27 at Ser-15, Ser-78 or Ser-82 in biological processes but did not allow ascribing restricted functions to each residue. Depending on cell context and stimulus, HSP27 phosphorylation is regulated by several protein kinases involved in cell survival, proliferation and motility, such as MK2, MK3, MK5, AKT, RSK, PKA, PKC and PKD.[21] Here, we show that, in ovarian cancer cells, HSP27 phosphorylation induced by HGF at all three serines was suppressed by the expression of a dominant negative form of the p38MAPK. This was not surprising as it was already reported that p38MAPK plays a key role in the motility triggered by HGF.[23]

It is known that the phosphorylation of HSP27 results in change of conformation of the protein and thus impairment of aggregation and release of the inhibition of actin polymerization, *i.e.*, increased actin dynamics.[15] In agreement, in ovarian cancer cells HSP27 silencing suppressed the HGF dependent remodeling of actin filaments and the scattering and motility triggered by HGF through permeable filters. The role of HSP27 in the dynamics of other cytoskeletal components such as microtubules and intermediate filaments might be also important.[14] The gain-of-function mutations of the HSP27-encoding *HSPB1* gene, which cause the axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy,[32] result in an increased stabilization of microtubules that is incompatible with motility and mitosis.[31] Moreover, HSP27 interacts also with intermediate filaments and regulates their aggregation.[33]

HSP27 suppression inhibited the formation of spontaneous metastases triggered by HGF. Data show that HSP27 is important in both the regulation of extravasation *in vivo*, as homing and experimental hematogenous metastasis are impaired by HSP27 silencing. Moreover, HSP27 regulates *in vivo* growth of ovarian cancer cells. These data suggest that HSP27 might be involved in the control of ovarian cancer metastasis. A role of HSP27 in metastasis has been suggested also by preclinical models of head and neck squamous cell carcinoma[34] and breast carcinoma.[35,36] Moreover, clinical data correlated tumor progression and expression of HSP27 in several tumor types. HSP27 is highly expressed in many cancers, such as breast, prostate, colorectal and pancreatic carcinomas and in many instances has been associated with aggressive behavior, metastasis and poor prognosis.[18] However, HSP27 expression correlates with good prognosis in some tumors, like endometrial and esophageal carcinomas.[37,38] In line with the possible Janus-like properties of HSP27,[39] a genetic variant of the promoter of the HSP27-encoding *HSPB1* gene has been associated to both an increased lung cancer risk and a favorable survival in patients with advanced lung cancer, as this variant results in a reduced level of the HSP27 protein.[40]

In ovarian cancer too, the role played by HSP27 is controversial. A higher HSP27 content was found in tumors from patients with advanced stage cancers and was associated with reduced survival and tumor progression after chemotherapy.[41–44] Nevertheless, a correlation between high level of HSP27 and better prognosis of ovarian cancer patients was also described.[45] It is important to assess the role played by HSP27 in the progression of ovarian cancer, because this is the most lethal gynecological malignancy, which is often diagnosed at a late stage, after tumor cells are widely metastasized within the peritoneal cavity. Despite aggressive treatment, which includes surgical cytoreduction followed by combination chemotherapy with taxanes, such as PTX and platinum drugs, such as carboplatin, more than two-thirds of all patients succumb to the disease.[46] Preclinical data shown here suggest that in ovarian cancer HSP7 is more likely a marker of progression and resistance to chemotherapy. Indeed, we show also that HSP27 suppression sensitized ovarian cancer cells to treatment with PTX *in vivo*, as well as it sensitizes prostate and bladder carcinoma cells to PTX *in vivo*[28,47] and breast cancer cells to doxorubicin *in vitro*.[48] Sensitization is likely due to the bundling of microtubules, which is thought to be the correlate of the lethal effect of PTX.[49] Interestingly, PTX caused bundling of microtubules only in HSP27-silenced ovarian cancer cells. The lack of effectiveness of PTX *in vivo* and on microtubules *in vitro* in HSP27-expressing cells was likely due to their partial resistance to the used doses of PTX. This is important in the case of ovarian cancer, as first-line therapy includes taxanes and these drugs are also frequently used in the subsequent lines of treatment. Thus, HSP27 targeting might be envisaged to circumvent resistance to this drug commonly used in ovarian cancer treatment.

HSP27 silencing was alone able to delay the growth of ovarian cancer cells *in vivo*, while HSP27 silenced cells survived and proliferated *in vitro*, suggesting that the microenvironment causes cellular stress *in vivo* that requires HSP27. This finding was unexpected, as prostate cancer cells growing *in vivo* display less expression of HSP27 and other HSPs than *in vitro*.[50] It is also possible that the role of HSP27 in cytoskeletal dynamics is more crucial in cells growing *in vivo* than *in vitro*. Alternatively, it might be hypothesized that HSP27 is necessary to circumvent physiological proapoptotic signals *in vivo* as HSP27 phosphorylation and/or expression are upregulated by several cytokines and by a number of transcription factors other than HSF1, respectively.

As HSP27 functions as a regulatory "hub" in multiple signaling and transcriptional pathways, it is an attractive therapeutic target. HSP27 inhibition may simultaneously suppress many pathways implicated in cancer progression and resistance to hormone and chemotherapy. Suppression of HSP27 reduces the growth *in vivo* of androgen resistant prostate cancer cells[29] and urinary bladder cancer cells,[28] reduces progression of breast cancer cells[51] and sensitizes pancreatic cancer cells to chemotherapy.[52] In preclinical models, suppression was obtained with small interfering RNAs and peptide aptamers.[53] The newly developed modified HSP27

antisense has proved its effectiveness in preclinical models[28,29,47,52] and is undergoing clinical trials. In the Phase I trials, reduction in tumor markers was observed in patients with prostate and ovarian cancer.[18]

In conclusion, data presented here suggest that HSP27 is not only required for the HGF triggered invasion and metastasis, but is also a necessary part of the machinery required for the progression of ovarian cancer and for sensitization to chemotherapeutics. Therefore, HSP27 is likely a biomarker of advanced stage ovarian cancer and of cancer resistance to therapy. More importantly, the preclinical data shown here proved that the growth and dissemination of ovarian cancer cells require HSP27 and, thus, that HSP27 might be targeted to improve the treatment of ovarian cancer patients.

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References

1. Birchmeier C, Birchmeier W, Gherardi E, et al. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* 2003;4:915–25.
2. Gherardi E, Birchmeier W, Birchmeier C, et al. Targeting MET in cancer: rationale and progress. *Nat Rev Cancer* 2012;12:89–103.
3. Trusolino L, Bertotti A, Comoglio PM. MET signalling: principles and functions in development, organ regeneration and cancer. *Nat Rev Mol Cell Biol* 2010;11:834–48.
4. Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. *Nature Rev Cancer* 2002;2:289–300.
5. Di Renzo MF, Olivero M, Katsaros D, et al. Overexpression of the Met/HGF receptor in ovarian cancer. *Int J Cancer* 1994;58:658–62.
6. Sawada K, Radjabi AR, Shinomiya N, et al. c-Met overexpression is a prognostic factor in ovarian cancer and an effective target for inhibition of peritoneal dissemination and invasion. *Cancer Res* 2007;67:1670–9.
7. Huntsman D, Resau JH, Klineberg E, et al. Comparison of c-met expression in ovarian epithelial tumors and normal epithelia of the female reproductive tract by quantitative laser scan microscopy. *Am J Pathol* 1999;155:343–8.
8. Maggiora P, Lorenzato A, Fracchioli S, et al. The RON and MET oncogenes are co-expressed in human ovarian carcinomas and cooperate in activating invasiveness. *Exp Cell Res* 2003;288:382–9.
9. Cai J, Tang H, Xu L, et al. Fibroblasts in omentum activated by tumor cells promote ovarian cancer growth, adhesion and invasiveness. *Carcinogenesis* 2012;33:20–9.
10. Sowter HM, Corps AN, Smith SK. Hepatocyte growth factor (HGF) in ovarian epithelial tumour fluids stimulates the migration of ovarian carcinoma cells. *Int J Cancer* 1999;83:476–80.
11. Aune G, Lian AM, Tingulstad S, et al. Increased circulating hepatocyte growth factor (HGF): a marker of epithelial ovarian cancer and an indicator of poor prognosis. *Gynecol Oncol* 2011;121:402–6.
12. Garrido C, Paul C, Seigneuric R, et al. The small heat shock proteins family: the long forgotten chaperones. *Int J Biochem Cell Biol*, 2012;44:1588-92.
13. Lanneau D, Wettstein G, Bonniaud P, et al. Heat shock proteins:cell protection through protein triage. *Sci World J* 2010;10:1543–52.
14. Wettstein G, Bellaye PS, Micheau O, et al. Small heat shock proteins and the cytoskeleton: an essential interplay for cell integrity? *Int J Biochem Cell Biol*, 2012;44:1680-6.
15. Doshi BM, Hightower LE, Lee J. The role of Hsp27 and actin in the regulation of movement in human cancer cells responding to heat shock. *Cell Stress Chaperones* 2009;14:445–57.
16. Acunzo J, Katsogiannou M, Rocchi P. Small heat shock proteins HSP27 (HspB1), αB-crystallin (HspB5) and HSP22 (HspB8) as regulators of cell death. *Int J Biochem Cell Biol*, 2012;44:1622-31.
17. Garrido C, Schmitt E, Candé C, et al. HSP27 and HSP70:potentially oncogenic apoptosis inhibitors. *Cell Cycle* 2003;2:579–84.
18. Zoubeidi A, Gleave M. Small heat shock proteins in cancer therapy and prognosis. *Int J Biochem Cell Biol*, 2012;44:1646-56.
19. Mehlen P, Mehlen A, Godet J, et al. Hsp27 as a switch between differentiation and apoptosis in murine embryonic stem cells. *J Biol Chem* 1997;272:31657–65.
20. Arrigo AP, Gibert B. HspB1 dynamic phosphooligomeric structure dependent interactome as cancer therapeutic target. *Curr Mol Med*, 2012;12:1151-63.
21. Kostenko S, Moens U. Heat shock protein 27 phosphorylation: kinases, phosphatases, functions and pathology. *Cell Mol Life Sci* 2009;66:3289–307.
22. Gibert B, Eckel B, Fasquelle L, et al. Knock down of heat shock protein 27 (HspB1) induces degradation of several putative client proteins. *PLoS One* 2012;7:e29719.
23. Rousseau S, Dolado I, Beardmore V, et al. CXCL12 and C5a trigger cell migration via a PAK1/2-p38alpha MAPK-MAPKAP-K2-HSP27 pathway. *Cell Signal* 2006;18:1897–905.
24. Bardella C, Dettori D, Olivero M, et al. The therapeutic potential of hepatocyte growth factor to sensitize ovarian cancer cells to cisplatin and paclitaxel in vivo. *Clin Cancer Res* 2007;13:2191–8.
25. Coltella N, Rasola A, Nano E, et al. p38 MAPK turns hepatocyte growth factor to a death signal that commits ovarian cancer cells to chemotherapy-induced apoptosis. *Int J Cancer* 2006;118:2981–90.
26. Bardella C, Olivero M, Lorenzato A, et al. Cells lacking the fumarase tumor suppressor are protected from apoptosis through a hypoxia-inducible factor-independent, AMPK-dependent mechanism. *Mol Cell Biol* 2012;32:3081–94.
27. Samouelian V, Maugard CM, Jolicoeur M, et al. Chemosensitivity and radiosensitivity profiles of four new human epithelial ovarian cancer cell lines exhibiting genetic alterations in BRCA2, TGFbeta-RII, KRAS2, TP53 and/or CDNK2A. *Cancer Chemother Pharmacol* 2004;54:497–504.
28. Kamada M, So A, Muramaki M, et al. Hsp27 knockdown using nucleotide-based therapies inhibit tumor growth and enhance chemotherapy in human bladder cancer cells. *Mol Cancer Ther* 2007;6:299–308.
29. Rocchi P, So A, Kojima S, et al. Heat shock protein 27 increases after androgen ablation and plays a cytoprotective role in hormone-refractory prostate cancer. *Cancer Res* 2004;64:6595–602.
30. Song TF, Zhang ZF, Liu L, et al. Small interfering RNA-mediated silencing of heat shock protein 27 (HSP27) Increases chemosensitivity to paclitaxel by increasing production of reactive oxygen species in human ovarian cancer cells (HO8910). *J Int Med Res* 2009;37:1375–88.
31. Almeida-Souza L, Asselbergh B, d'Ydewalle C, et al. Small heat-shock protein HSPB1 mutants stabilize microtubules in Charcot-Marie-Tooth neuropathy. *J Neurosci* 2011;31:15320–8.
32. Evgrafov OV, Mersiyanova I, Irobi J, et al. Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. *Nat Genet* 2004;36:602–6.
33. Perng MD, Muchowski PJ, van Den Ijssel P, et al. The cardiomyopathy and lens cataract mutation in alphaB-crystallin alters its protein structure, chaperone activity, and interaction with intermediate filaments in vitro. *J Biol Chem* 1999;274:33235–43.
34. Kang S, Elf S, Lythgoe K, et al. p90 ribosomal S6 kinase 2 promotes invasion and metastasis of human head and neck squamous cell carcinoma cells. *J Clin Invest* 2010;120:1165–77.
35. Gibert B, Eckel B, Gonin V, et al. Targeting heat shock protein 27 (HspB1) interferes with bone metastasis and tumour formation in vivo. *Br J Cancer* 2012;107:63–70.
36. Bausero MA, Bharti A, Page DT, et al. Silencing the hsp25 gene eliminates migration capability of the highly metastatic murine 4T1 breast adenocarcinoma cell. *Tumour Biol* 2006;27:17–26.
37. Langer R, Ott K, Specht K, et al. Protein expression profiling in esophageal adenocarcinoma patients indicates association of heat-shock pro-

tein 27 expression and chemotherapy response. *Clin Cancer Res* 2008;14:8279–87.

38. Geisler JP, Geisler HE, Tammela J, et al. A study of heat shock protein 27 in endometrial carcinoma. *Gynecol Oncol* 1999;72:347–50.
39. Calderwood SK, Ciocca DR. Heat shock proteins:stress proteins with Janus-like properties in cancer. *Int J Hyperthermia* 2008;24:31–9.
40. Guo H, Bai Y, Xu P, et al. Functional promoter-1271G>C variant of HSPB1 predicts lung cancer risk and survival. *J Clin Oncol* 2010;28: 1928–35.
41. Langdon SP, Rabiasz GJ, Hirst GL, et al. Expression of the heat shock protein HSP27 in human ovarian cancer. *Clin Cancer Res* 1995;1: 1603–9.
42. Arts HJ, Hollema H, Lemstra W, et al. Heat-shock-protein-27 (hsp27) expression in ovarian carcinoma: relation in response to chemotherapy and prognosis. *Int J Cancer* 1999;84:234–8.
43. Elpek GO, Karaveli S, Simşek T, et al. Expression of heat-shock proteins hsp27, hsp70 and hsp90 in malignant epithelial tumour of the ovaries. *APMIS* 2003;111:523–30.
44. Zhao M, Shen F, Yin YX, et al. Increased expression of heat shock protein 27 correlates with peritoneal metastasis in epithelial ovarian cancer. *Reprod Sci* 2012;19:748–53.
45. Geisler JP, Tammela JE, Manahan KJ, et al. HSP27 in patients with ovarian carcinoma: still an independent prognostic indicator at 60 months follow-up. *Eur J Gynaecol Oncol* 2004;25: 165–8.
46. Cannistra SA. Cancer of the ovary. *N Engl J Med* 2004;351:2519–29.
47. Andrieu C, Taieb D, Baylot V, et al. Heat shock protein 27 confers resistance to androgen ablation and chemotherapy in prostate cancer cells through eIF4E. *Oncogene* 2010;29:1883–96.
48. Zhang D, Putti TC. Over-expression of ERp29 attenuates doxorubicin-induced cell apoptosis through up-regulation of Hsp27 in breast cancer cells. *Exp Cell Res* 2010;316:3522–31.
49. Rowinsky EK, Donehower RC. Paclitaxel (taxol). *N Engl J Med* 1995;332:1004–14.
50. Tang D, Khaleque MA, Jones EL, et al. Expression of heat shock proteins and heat shock protein messenger ribonucleic acid in human prostate carcinoma in vitro and in tumors in vivo. *Cell Stress Chaperones* 2005;10:46–58.
51. Straume O, Shimamura T, Lampa MJ, et al. Suppression of heat shock protein 27 induces long-term dormancy in human breast cancer. *Proc Natl Acad Sci USA* 2012;109:8699–704.
52. Baylot V, Andrieu C, Katsogiannou M, et al. OGX-427 inhibits tumor progression and enhances gemcitabine chemotherapy in pancreatic cancer. *Cell Death Dis* 2011;2:e221.
53. Gibert B, Hadchity E, Czekalla A, et al. Inhibition of heat shock protein 27 (HspB1) tumorigenic functions by peptide aptamers. *Oncogene* 2011;30: 3672–81.