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Hsp27-Induced MMP-9 Expression Is Influenced by the Src Tyrosine Protein Kinase Yes

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The small heat shock protein hsp27 is associated with aggressive tumor behavior in certain subsets of breast cancer patients. Previously we demonstrated that hsp27 overexpression in breast cancer cells increased *in vitro* and *in vivo* invasiveness, suggesting that hsp27 influences the metastatic process. To investigate this role for hsp27, we have utilized MDA-MB-231 breast cancer cells that overexpress hsp27 and cDNA expression array technology. We demonstrate that hsp27 overexpression up-regulates MMP-9 expression and activity and down-regulates Yes expression. Furthermore, our results suggest that Yes may be involved in regulating MMP-9 expression, as well as *in vitro* invasion. Reconstitution of Yes expression by transfection into hsp27-overexpressing cells decreased MMP-9 expression, and increased *in vitro* invasiveness, abrogating the phenotype conferred by hsp27 overexpression. Therefore, our results provide a new potential mechanism by which hsp27 affects the metastatic cascade—through regulation of MMP-9 and Yes expression.

Key Words: **hsp27; MMP-9; Yes; MDA-MB-231; cDNA expression array; breast cancer; heat shock protein; invasion; matrix metalloproteinase; src kinase.**

Hsp27 belongs to the family of small heat shock proteins, is expressed constitutively in a number of tissues, and is expressed at high levels after various types of stress (1). In stressed cells, hsp27 modulates cell survival (2), apoptosis (3–7), and microfilament organization (8). These functions are regulated by phosphorylation of the hsp27 protein, which is induced not only by chemical or physical stresses, but also by mitogens, cytokines, and other agents that activate signal transduction pathways (1). Hsp27 phosphorylation is controlled by MAPKAP kinases 2 and 3, which are directly downstream of p38 in the MAP kinase signaling cascade (9).

In addition to its involvement in stress resistance, hsp27 may play a regulatory role in other cellular processes. For example, it is well established that hsp27 regulates proliferation and differentiation (10, 11), but hsp27 may also modulate cell motility. Evidence for hsp27's role in motility comes from studies involving endothelial cells, which normally express high levels of hsp27 (11–13). In these cells, hsp27 mediates vascular endothelial growth factor-induced microfilament reorganization and cell motility by affecting the generation of lamellipodia microfilaments. Besides proliferation, differentiation, and cell motility, hsp27 may also play a role in signal transduction. Recent studies have demonstrated a specific and direct physical interaction between hsp27 and protein kinase B/Akt serine/threonine kinase (PKB/AKT) (14, 15). While the precise relationship between PKB/AKT activation and hsp27 is not clear, these studies suggest that hsp27 might be necessary for the activation (or inactivation) of certain kinases, and thus may play a role in signal transduction. Finally, hsp27 may have some as yet other unknown functions in the cell. Preliminary evidence suggests that novel hsp27-binding proteins may exist, and that some of these may exhibit cell-specific expression (16).

Understanding the many functions of hsp27 in the cell will contribute to our understanding of its role in malignancy, since hsp27 is overexpressed in a number of human tumors. In breast cancer, high hsp27 levels are associated with a shorter disease-free survival and a higher proliferation rate in a subset of patients (17). *In vitro* evidence indicates that hsp27 overexpression confers resistance to certain chemotherapeutic agents frequently used in the treatment of breast cancer (5, 18). Furthermore, in osteosarcoma and ovarian tumors, hsp27 is a marker of poor prognosis, and in

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ovarian tumors, hsp27 expression increases with advanced stage (19, 20).

It is not surprising that hsp27 is associated with poor prognosis and resistance to therapy, since hsp27 regulates proliferation and plays a protective role in cells under stress. However, hsp27 may also play a role in the metastatic process, which is associated with poor prognosis as well. Studies in our laboratory indicate that hsp27 overexpression increases the metastatic potential of human breast cancer cells grown in nude mice (21). These studies suggest that hsp27 may participate in metastasis by regulating several steps in the metastatic process. We have shown that overexpression of hsp27 increases *in vitro* invasiveness and cell adhesion, processes important in metastasis. Furthermore, we hypothesized that hsp27 may affect metastasis by regulating signal transduction pathways controlling actin microfilament dynamics, and may also alter the expression of other proteins that participate in metastasis. In the present study, we examined the relationship between hsp27 expression and breast cancer metastasis using hsp27-overexpressing MDA-MB-231 breast cancer cells and cDNA array technology. This technology provided the opportunity to profile the expression of a large number of gene sequences, and allowed us to identify differentially expressed genes that might be involved in hsp27's effect on metastasis. We show that hsp27 overexpression alters the expression of specific genes, in particular MMP-9 and the Src kinase Yes, known to be involved in cell adhesion, motility, and invasion. Furthermore, we demonstrate that reconstitution of Yes expression can reverse the invasive phenotype of our hsp27-overexpressing cells.

MATERIALS AND METHODS

Materials. Anti-hsp27 monoclonal antibody was purchased from Neomarkers (Freemont, CA), anti-TIMP-1 and anti-MMP-2 antibodies were obtained from Oncogene Research Products (Cambridge, MA), anti-TIMP-2 was acquired from Chemicon (Temecula, CA), anti-MMP-9 was purchased from R & D Systems (Minneapolis, MN), and anti-Yes antibody was obtained from Transduction Laboratories (Lexington, KY). All other chemicals were from Sigma.

DNA plasmids. The human MMP-9 promoter/SEAP reporter plasmid (MMP-9) used in this study contains the SEAP gene (pSEAP2 basic; Clontech, Palo Alto, CA) under the transcriptional control of the 670 bp human MMP-9 promoter (22). A 1.8 kb Yes cDNA was obtained from Dr. Tadashi Yamamoto (University of Tokyo, Japan), and subcloned into the *Eco*RV site of the pZeoSV2 expression vector (YesZeo) (Invitrogen, Carlsbad, CA) (23).

Cells and cell culture. The MDA-MB-231 human breast cancer cells used in this study, the pCDNA1 vector control transfectants C1 and C2, and the stable hsp27-overexpressing clones 19 and 12(2), were generated as previously described (21), and maintained in MEM supplemented with 10% fetal bovine serum, 6 ng/ml insulin, and 25 μg/ml gentamicin sulfate. The pZeoSV2 vector control transfectants C3 and C4, and three stable Yes-overexpressing clones (Clones 2, 22, and 25) were generated by stable transfection of the hsp27 overexpressing clones similarly maintained in MEM additionally supplemented with 250 μg/ml Zeocin (Invitrogen, Carlsbad, CA). All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and periodically tested for Mycoplasma contamination (Bonique Laboratories, Saranac Lake, NY).

Microarray analysis. Cells were plated at a density of 2.5×10^6 cells per T-175 flask. After 72 h of growth, cells were harvested and total RNA prepared with RNeasy kits (Qiagen Inc., Valencia, CA) as per the manufacturer's instructions. Messenger RNA was isolated from the total RNA on Dynabeads (Dynal, Oslo, Norway), and used to synthesize ^{32}P-radiolabeled cDNAs for hybridization to the Atlas Human Cancer cDNA expression array (CLONTECH, Palo Alto, CA), according to the manufacturer's instructions. The Atlas cDNA expression arrays are positively charged nylon membranes (8×12 cm) that are spotted in duplicate with 200- to 600-base-pair cDNA fragments representing 588 genes of like function (i.e., oncogenes, assorted receptors, etc.) grouped together geographically. The hybridization data were collected with a Molecular Dynamics PhosphoImager (Molecular Dynamics, Sunnyvale, CA), and statistical analysis using principle components analysis (PCA) of the data was performed as previously described (24).

Western blot analysis. For immunoblot analysis of hsp27 and Yes levels in various transfectants, total cellular extracts were prepared by solubilization in 5% SDS. Conditioned medium was prepared as described in Materials and Methods for zymogram analysis and also used for the immunoblot analysis of MMPs and TIMPs. In all cases, equal amounts of protein were subjected to electrophoresis in 10% polyacrylamide gels, and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Equal protein loading was ascertained by Ponceau S (Sigma, St. Louis, MO) staining of blotted membranes, and immunoblots were developed using the Enhanced Chemiluminescence (ECL) system (DuPont, Boston, MA). Fold protein expression differences were determined by densitometric scanning of immunoblots from two independent experiments.

Gelatin zymography. Cells were plated at a density of 1.25×10^6 cells per T-75 flask. After 48 h of growth, cells were washed twice with phosphate buffered saline and treated with serum-free Improved MEM Zinc Option (Gibco BRL, Gaithersburg, MD) supplemented with 1 g/L glucose, 1× trace elements (Biofluids Inc., Rockville, MD), 1 μg/ml fibronectin, 10 mM Hepes and 1 μg/ml transferrin (Gibco BRL, Gaithersburg, MD). After 48 h, conditioned medium was collected and concentrated using Amicon Centricon 10 columns (Millipore Corporation, Bedford, MA) as per the manufacturer's instructions. Zymogram gels and reagents were obtained from Novex (San Diego, CA) and used according to the manufacturer's instructions. Samples were mixed with an equal volume of 2× Tris-Glycine SDS sample buffer, and incubated at room temperature for 10 min before being loaded onto a 10% Tris-Glycine gel containing 0.1% gelatin. After running, gels were renatured for 30 min in 1× renaturing buffer, equilibrated for 30 min with 1× developing buffer at room temperature, then incubated at 37°C in fresh 1× developing buffer for 48 h. The gels were stained with Coomassie Brilliant Blue R-250, and zones of enzymatic activity visualized as clear areas were shown by negative staining. Fold expression differences for zymography were determined by densitometric scanning of gels from a minimum of two independent experiments.

Transient and stable transfections. Transfections were performed using FuGENE 6 transfection reagent (Boehringer Mannheim, Indianapolis, IN) following the manufacturer's instructions. For transient transfections, cells were plated at a density of 1×10^5 cells per well in 6-well cluster plates (Falcon, Franklin Lakes, NJ). After 24 h, cells were washed twice with phosphate buffered saline, and treated with serum-free Improved MEM Zinc Option (Gibco BRL, Gaithersburg, MD) supplemented with 1 g/L glucose, 1× trace elements (Biofluids Inc., Rockville, MD), 1 μg/ml fibronectin, 10 mM Hepes, and 1 μg/ml transferrin (Gibco BRL, Gaithersburg, MD). Transfections of individual wells were performed using 2 μg of the SEAP reporter plasmid, pSEAP2 basic (vector control; Clontech, Palo Alto, CA) or MMP-9, and 200 ng of the pCMV-β-galactosidase plas-

mid (Promega, Madison, WI) to normalize for transfection efficiency. After 48 h, assays for SEAP and β-galactosidase activity were performed.

Conditioned medium was withdrawn and used to assay for MMP-9 SEAP promoter activity, as per the manufacturer's instructions (CLONTECH Chemiluminescent Assay, CLONTECH, Palo Alto, CA). In addition, cells were washed twice with phosphate buffered saline, and harvested using 1× cell culture lysis reagent (Promega, Madison, WI), and the lysate was used for the β-galactosidase assays. β-galactosidase activity was determined using the method of Rouet *et al.* (1992) (25) with a Bio-Rad microplate reader (Bio-Rad Laboratories, Hercules, CA). Data shown are representative of two independent experiments, each performed in triplicate wells.

For stable transfections, cells were plated at a density of 1×10^6 cells per 10 cm dish (Falcon, Franklin Lakes, NJ). After 24 h, cells were transfected with 20 μg of YesZeo or pZeoSV2 plasmids, and after 48 h, they were split into four 15 cm dishes (Falcon, Franklin Lakes, NJ). Colonies were picked with cloning cylinders approximately two weeks after selection in MEM supplemented with 10% fetal bovine serum, 6 ng/ml insulin, 25 μg/ml gentamicin sulfate, and 350 μg/ml Zeocin (Invitrogen, Carlsbad, CA), then expanded and maintained on Zeocin for two weeks before further experimentation.

Invasion assays. Invasion experiments were conducted using Blind Well Chambers (Neuro Probe Inc., Cabin John, MD) and Nucleopore 13 mm 8 μm PVP-free filters (Neuro Probe Inc., Cabin John, MD) coated with Matrigel (Collaborative Biomedical Research, Bedford, MA). Each well insert was layered with 50 μl of dilute Matrigel (50 μg). After drying, the filters were placed in a Blind Well Chamber previously loaded with 220 μl 3T3-conditioned medium. Meanwhile, cells were washed with Dulbecco's phosphate buffered saline without calcium chloride or magnesium chloride (Gibco BRL, Gaithersburg, MD), harvested using Versene (Gibco BRL, Gaithersburg, MD), and resuspended at a concentration of 1×10^6 cells per ml in Dulbecco's modified Eagle medium (Gibco BRL, Gaithersburg, MD) containing 0.1% BSA. The cell suspension was immediately added to the upper compartment of the chamber, and the wells were incubated at 37°C for 24 h. The filters were then removed from the chamber, and stained with Diff-Quick (Baxter Scientific, McGaw Park, IL). Invasion was assessed by counting the cells that traversed the filter and were attached to the bottom side. Results shown are representative of three independent experiments.

Statistical analyses. As previously described (24), Principal Components Analysis (PCA) was used to analyze log-transformed expression data from the array experiments. A robust 95% confidence interval on the second principal component was used to identify genes that exhibited over- or underexpression in an hsp27 overexpressing clone relative to a control transfected clone. Fold differences in expression were estimated by back-transforming the second principal component. P-values indicate the likelihood of such a value occurring by chance, using a normal distribution and a robust estimator of variance.

Analysis of variance of log-transformed invasion cell-counts was used to compare the invasiveness of Yes transfected clones expressing low levels of MMP-9 to control clones, and in a separate analysis, to compare the invasiveness of a high MMP-9 expressing Yes transfected clone to control clones. Geometric means and 95% confidence intervals were computed by back transforming means and 95% confidence intervals of log-transformed data.

RESULTS

Regulation of MMP-9 expression by hsp27. We have previously shown that hsp27 overexpression increases *in vitro* invasiveness and *in vivo* metastatic behavior of breast cancer cells (21), using MDA-MB-231 cells stably transfected with a human hsp27 cDNA. In an attempt to identify genes involved in invasion or metastasis whose expression is altered with hsp27 overexpression, we utilized cDNA expression array technology. ^{32}P-labeled cDNAs were synthesized by reverse transcription of mRNA isolated from control vector-alone transfected (Clone C2), or hsp27-overexpressing cells (Clone 19) (21), and hybridized to Atlas cDNA arrays. The expression of specific genes was determined by densitometric scanning and Principal Components Analysis (PCA) of hybridized signals (24). Of the 588 genes contained on the Atlas Human Cancer cDNA Expression Array, at least 121 are associated with the cellular processes of cell adhesion, motility, and invasion. Using a 5% cut-off, PCA identified 20 candidate genes from this group whose expression was affected by hsp27 overexpression, many of which were either MMPs or TIMPs (data not shown). These genes included MMP-9 and the tissue inhibitor of metalloproteinases-1, TIMP-1, which have been associated with breast tumor progression and metastasis (26). Array analysis showed that there was a higher expression of MMP-9 RNA (1.75-fold, $P < 0.01$) and a lower expression of TIMP-1 RNA (0.61-fold, $P < 0.03$) in the hsp27-transfected cells compared to control cells. There were no significant differences in expression of two other breast cancer metastasis-associated genes, MMP-2 ($P < 0.08$) and tissue inhibitor of metalloproteinases-2, TIMP-2 ($P < 0.09$), in our cDNA array analysis.

We chose first to confirm the cDNA expression array results for MMP-9 and TIMP-1, as well as MMP-2 and TIMP-2, at the protein level using Western blot analysis. We confirmed that MMP-9 was differentially expressed at the protein level (Fig. 1A, upper panel) between control-transfected (Clones C1 and C2) and hsp27-transfected (Clones 19 and 12(2)) MDA-MB-231 cells, with approximately a twofold higher expression in hsp27-overexpressing cells. We also confirmed the RNA data, that neither MMP-2 nor TIMP-2 were differentially expressed at the protein level as a result of hsp27 overexpression, though there was a slight but not significant change in protein expression of TIMP-2 (0.5-fold) in one of the hsp27-transfected clones (Clone 12(2)). In contrast to these validations, however, the lower TIMP-1 levels predicted by our RNA array data in hsp27-transfected cells were not confirmed using Western blot analysis (Fig. 1A).

To evaluate the functional activity of MMP-9 in our control and hsp27-transfected cells, we next utilized gelatin zymography (Fig. 1B). Hsp27-overexpressing cells (Clones 19 and 12(2)) secreted a 2- to 3-fold higher level of enzymatically active MMP-9 than control cells (Clones C1 and C2). In agreement with our protein expression results, we found no difference in MMP-2 activity (Fig. 1B), or in TIMP-1 or TIMP-2 activity (data not shown), concomitant with hsp27 overexpression. Therefore, we conclude that hsp27 overexpression specifically increases expression and activity of

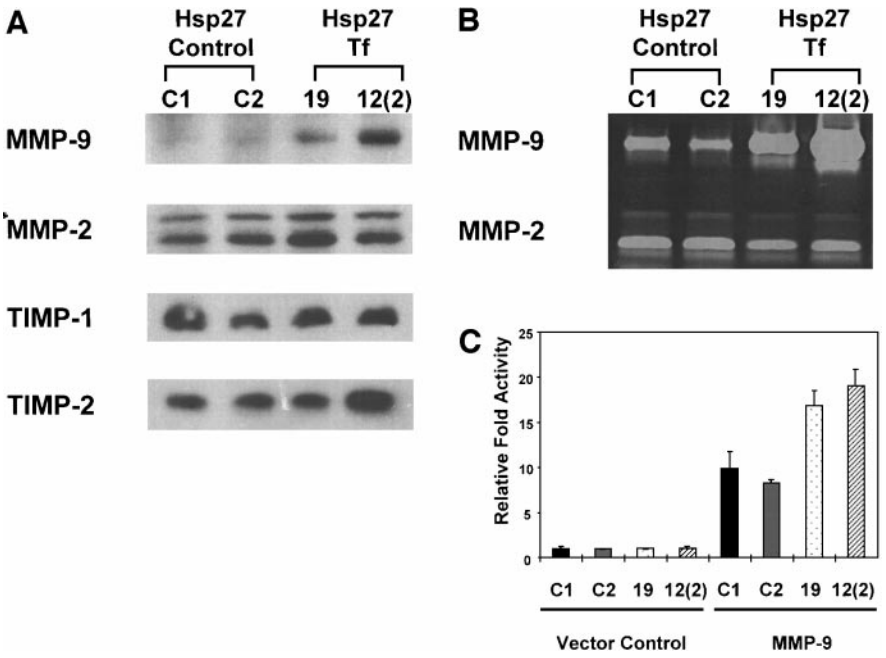

FIG. 1. Increased MMP-9 expression and activity with hsp27 overexpression. (A and B) Control (C1, C2) and hsp27-transfected (19, 12(2)) cells were plated at a density of 1.25×10^6 cells per T-75 flask. After 48 h of growth, the media was replaced with serum-free media. After an additional 48 h, the conditioned media was harvested and concentrated as described under Materials and Methods, and used for Western blot and zymographic analysis. (A) Western blot analysis of MMP-9, MMP-2, TIMP-1, and TIMP-2 levels in control (C1, C2) and hsp27-transfected (19, 12(2)) cells was performed in 10% polyacrylamide gels. (B) Zymographic analysis of MMP-9 and MMP-2 activity in control (C1, C2) and hsp27-transfected (19, 12(2)) cells was performed in 10% polyacrylamide gels containing 0.1% gelatin. Data shown in A and B are representative of three independent experiments. (C) Analysis of MMP-9 promoter activity in control (C1, C2) and hsp27-transfected (19, 12(2)) cells. Cells (triplicate wells) were cotransfected with 2 μg of the empty SEAP reporter vector (vector control) or 2 μg of the MMP-9 SEAP reporter (MMP-9) and 200 ng β-galactosidase reporter plasmid using the FuGENE 6 transfection reagent as described under Materials and Methods. After 48 h the conditioned media was withdrawn and used to assay SEAP activity; the cells were harvested and protein extracts assayed for β-galactosidase activity. Data shown in C are representative of two independent experiments, and presented as fold expression over the empty reporter vector alone $\pm$SE.

MMP-9, but not MMP-2 in MDA-MB-231 breast cancer cells, and that this increase is probably not due to decreased inhibitor expression, since levels of TIMP-1 and TIMP-2 were unaltered.

Since hsp27 influences both MMP-9 RNA and protein expression, we next examined MMP-9 promoter transcriptional activity by transiently transfecting control and hsp27 stable transfectants with a 670 bp fragment of the MMP-9 promoter. This region of the proximal promoter, located directly 5′ of the transcriptional start site, regulates transcription of MMP-9 (22, 27–29). In these studies, control and hsp27-overexpressing MDA-MB-231 cells were cotransfected with a SEAP reporter driven by the 670 bp MMP-9 promoter fragment (MMP-9), or the empty SEAP reporter (vector control), and a β-galactosidase reporter. MMP-9 promoter activity was increased 9.9- and 8.3-fold in the control clones C1 and C2, respectively, and 16.8- and 18.9-fold higher in the two hsp27-overexpressing clones 19 and 12(2) (Fig. 1C). Therefore, overexpression of hsp27 in these cells yielded an approximate two-fold increase in transactivation of the MMP-9 promoter. This suggests that the increased MMP-9 RNA and protein synthesis demonstrated in our array and Western blot analysis may be due to increased transcriptional activity directly resulting from hsp27 overexpression.

Hsp27 overexpression decreases Yes expression. To further explore the mechanism of hsp27 overexpression on MMP-9 activity, we examined other differentially expressed genes that were identified in our cDNA array analysis. We were especially interested in genes participating in signaling pathways involved in cell adhesion, motility, and invasion. One potential candidate we identified in the array analysis was Yes, a member of the Src family of tyrosine protein kinases. There was an approximate 2.6-fold decrease in Yes RNA expression between control and hsp27-transfected cells in an array analysis (results not shown). These results were confirmed at the protein level using Western blot analysis (Fig. 2A); Yes protein expression in control transfectants (HspC, Clones C1 and C2) was approximately 3- to 5-fold higher than in hsp27-overexpressing cells (HspTf, Clones 19 and 12(2)).

To define the role of Yes on the phenotype conferred by hsp27 overexpression, we next reconstituted Yes expression by stably transfecting a plasmid coding for

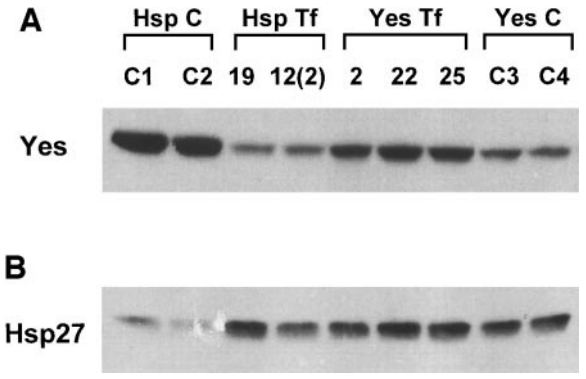

FIG. 2. Decreased Yes expression with hsp27 overexpression. (A and B) Hsp27 control (C1, C2), hsp27-transfected (19, 12(2)), Yes control (C3, C4), and Yes-transfected (2, 22, 25) cells were plated at a density of 1.25×10^6 cells per T-75 flask. After 72 h of growth, cells were harvested and total cellular extracts prepared in 5% SDS. (A) Yes, and (B) hsp27 Western blot analysis was performed in 10% polyacrylamide gels as described under Materials and Methods. Data shown are representative of two independent experiments.

human Yes cDNA in the sense orientation into both of our original hsp27-overexpressing clones, Clones 19 and 12(2). The resulting double-transfectant clones were screened by Western blot analysis for Yes and hsp27 protein expression (Figs. 2A and 2B, respectively). The range of Yes expression was increased 2- to 3-fold (Clones 2, 22, and 25), as evaluated by densitometric scanning of the immunoblots as compared to control-transfected cells (Clones C3 and C4). The three Yes-transfected clones were generated by transfection of hsp27-overexpressing clone 12(2). The control cells (Clones C3 and C4) were generated by transfection of the empty plasmid vector (pZeoSV2) into hsp27 transfectant 12(2). Both the control and Yes transfectant clones maintained high levels of hsp27 expression (Fig. 2B). The Yes reconstituted clones 2, 22, and 25 expressed similar levels of Yes protein (Fig. 2A, Yes Tf) compared to the noticeably high levels of Yes in the hsp27 control C1 and C2 clones (Fig. 2A, Hsp C).

Reconstitution of Yes abrogates hsp27's effect on MMP-9 activity. We next examined whether Yes reexpression could alter MMP activity in our cells, as assessed by gelatin zymography, since MMP activity was altered by hsp27 overexpression. As shown in Fig. 3, the level of MMP-9 activity was 2- to 3-fold lower in the Yes-expressing transfectants (Yes Tf, Clones 2, 22, 25) compared to control cells (Yes C, Clones C3, C4), whereas the levels of MMP-2 activity were relatively similar between these two transfectant groups. Furthermore, MMP-9 expression in the Yes transfectants (Yes Tf) and controls (Yes C) mimics that seen in the hsp27 controls (Hsp C) and hsp27 transfectants (Hsp Tf), respectively. These results suggest that increased MMP-9 expression and activity concomitant with hsp27 overexpression are mediated through the Yes signaling pathway.

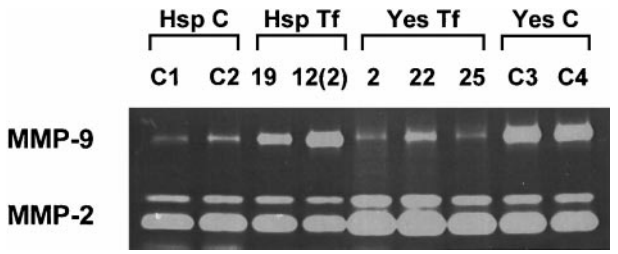

FIG. 3. Decreased MMP-9 enzymatic activity with Yes overexpression. Hsp27 control (C1, C2), hsp27-transfected (19, 12(2)), Yes control (C3, C4), and Yes-transfected (2, 22, 25) cells were plated at a density of 1.25×10^6 cells per T-75 flask. After 48 h of growth, the media was replaced with serum-free media. After an additional 48 h, the conditioned media was harvested and concentrated as described under Materials and Methods, and used for zymographic analysis in 10% polyacrylamide gels containing 0.1% gelatin. Data shown are representative of two independent experiments.

Yes expression reverses hsp27's effect on cell invasion. To address the biological role of Yes reexpression further in breast cancer cells, we next examined the ability of Yes to influence properties associated with tumor metastasis using Matrigel invasion assays. Two of the Yes transfected clones (Clones 2 and 25) also had the lowest MMP-9 activity, as assessed by zymography (Fig. 3). These two clones with the lowest MMP-9 activity (Clones 2 and 25) exhibited decreased invasiveness as compared to the control transfected clones (Clones C3 and C4; $P = 0.002$), while the third clone (Clone 22; $P = 0.77$) did not (Table 1). This suggests that hsp27's effect on MMP-9 activity might be modulated by Yes expression in human breast cancer cells.

DISCUSSION

Hsp27 is overexpressed in a number of malignancies, including breast cancer, although its exact role in metastasis is unknown. In the present study, we show for the first time that hsp27 overexpression up-regulates MMP-9 expression and activity in human breast cancer cells. These results extend our previous observa-

TABLE 1

Yes Expression Correlates with Invasion of Breast Cancer Cells

Transfection	Clone	Mean (95% CI)*
Control transfected	C3	301 (180–503)
	C4	791 (430–1452)
Yes transfected	2	109 (49–247)
	25	168 (62–452)
	22	430 (180–1025)

* Geometric mean number of invading cells determined by Boyden chamber assay, and corresponding 95% confidence interval for each clone.

tions that hsp27-overexpressing cells exhibit altered morphology, increased *in vitro* invasiveness and adhesion, and increased *in vivo* metastatic behavior (21). Furthermore, we demonstrate that hsp27 overexpression decreases Yes expression, and reconstitution of Yes into hsp27-overexpressing cells reverses the invasive phenotype of breast cancer cells. Cells overexpressing both hsp27 and Yes exhibited decreased MMP-9 activity, and decreased *in vitro* invasiveness. Therefore, our results provide a new potential mechanism for hsp27 effects on the metastatic cascade through Yes signal transduction processes in breast cancer cells.

MMPs are a family of zinc-dependent endopeptidases that have been associated with tumor cell invasion and metastasis due to their ability to hydrolyze a variety of extracellular matrix proteins (26). These enzymes have been correlated with metastasis in a large variety of systems, and in general, can be produced by either the tumor cells themselves or the stromal cells surrounding the lesion. In breast tumors, two members of the MMP family, MMP-2 and MMP-9, are highly expressed in both the stroma and cancer cells (22, 30, 31). MMP-2 and MMP-9 are thought to degrade a major structural protein in basement membrane called type IV collagen, and thus contribute to the invasive ability of breast cancer cells (32), although the mechanisms regulating MMP expression in breast cancer cells are largely unknown.

Our results demonstrate that hsp27 regulates MMP-9, but not MMP-2 RNA and protein expression in the human breast cancer cell line MDA-MB-231. Similar to our study, another specific hsp, Chlamydial hsp60, was correlated with up-regulation of MMP-9 expression in macrophages, while it had no effect on MMP-2 (33). Additional evidence for a role for hsps in MMP regulation comes from a study examining the effect of heat shock treatment on collagenase (MMP-1) gene expression (34). Heat shock caused a rapid increase in hsp expression, followed by a delayed increase in MMP-1 mRNA levels in both normal human synovial and dermal fibroblasts. Transcriptional activation of MMP-1 due to heat shock was mediated through an AP-1 binding site, in agreement with Carter (1997) (35) that hsps may play a modulatory role in the regulation of AP-1 responsive genes. The MMP-9 promoter, like that of MMP-1, contains an AP-1 binding site which is important for its expression (22), suggesting that hsp27 might up-regulate MMP-9 through increased AP-1 binding. Our unpublished observations, however, indicate that hsp27 overexpression may not affect AP-1 activity, and may thus regulate MMP-9 expression in breast cancer cells through other mechanisms.

Our results suggest that hsp27's effect on MMP-9 expression is mediated through the Src-family tyrosine protein kinase Yes. The Src family members are non-receptor tyrosine kinases that associate with the plasma membrane. These kinases have been implicated as essential signaling components in a diverse array of cellular functions, including extracellular matrix-promoted adhesion and spreading, focal adhesion formation and disassembly, formation of lamellipodia or membrane ruffles, and migration (36), although the precise role of various family members in these cellular events and in cancer is largely unknown. In human tumors, increased Src kinase activity can occur, and it has been hypothesized that Src may play an important role in the malignant progression of various types of cancer, including colon (37), bladder (38), breast (39), and skin (40). In breast cancer, Src activity is increased in both human primary breast tumor cells and breast tumor cell lines, and has been correlated with the tumorigenicity of human breast tumors (39, 41, 42).

We would like to suggest that Yes can modulate MMP-9 activity and the invasiveness of breast cancer cells, implying a role for Yes in breast cancer progression. This is also supported by other studies that have associated Src family member expression with MMP activity. For instance, transformation of normal chicken embryo fibroblasts with Src or Yes stimulates MMP activity and increases the invasiveness of these cells (43). One mechanism for Src- or Yes-regulated MMP expression may involve altered protein binding at MMP promoters, since Src stimulation of the MMP-9 promoter in human fibrosarcoma and hepatoma cells is mediated through an AP-1 site and a GT box (44). These elements are distinct from those responsible for induction by TNFα and TPA (22). Our results suggest that in breast cancer cells, Yes expression inhibits MMP activity, and that decreased Yes expression in our hsp27-transfected MDA-MB-231 cells may facilitate altered transcription factor binding on the MMP-9 promoter, resulting in an increase in MMP-9 transcription.

This paper demonstrates an inverse relationship between Yes and hsp27 expression and function in breast cancer cells. There are a number of functional ways Yes and hsp27 might be interrelated and affect signal transduction. First, hsp27 may modulate other kinase activities (e.g., PKB/AKT) through its chaperone function, and thus might influence Yes expression. Second, increased hsp27 expression may affect the dynamics of actin polymerization/depolymerization and microfilament organization, and regulate the activities of the diverse array of kinases found at the cellular membrane such as Yes. Third, Yes may regulate hsp27 phosphorylation/dephosphorylation, since hsp27 is phosphorylated by p38-regulated MAPKAP kinases 2 and 3 (9), and is dephosphorylated principally by phosphatase PP2A (45). In fact, Yes is found complexed with a 38-kDa phosphoprotein (46), as well as PP2A (47).

In summary, our results suggest that the signaling pathway(s) involving hsp27, Yes, MMP-9, and invasion in breast cancer cells are interregulated and possibly coregulated, and thus may operate as a dynamic signaling network. Identification of other proteins that participate in this pathway, as well as those directly regulating Yes and MMP-9 expression remains to be done, but may provide further therapeutic targets for clinical intervention in the metastatic cascade of breast cancer cells.

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