Hsp-27 induction requires POU4F2/Brn-3b TF in doxorubicin-treated breast cancer cells, whereas phosphorylation alters its cellular localisation following drug treatment

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Abstract POU4F2/Brn-3b transcription factor (referred to as Brn-3b) is elevated in >60% of breast cancers and profoundly alters growth and behaviour of cancer cells by regulating distinct subsets of target genes. Previous studies showed that Brn-3b was required to maximally transactivate small heat shock protein, HSPB1/Hsp-27 (referred to as Hsp-27), and consequently, Brn-3b expression correlated well with Hsp27 levels in human breast biopsies. In these studies, we showed that Brn-3b is increased in MCF7 breast cancer cells that survive following treatment with chemotherapeutic drug doxorubicin (Dox) with concomitant increases in Hsp-27 expression. Targeting of Brn-3b using short interfering RNA reduced Hsp-27 in Dox-

treated cells, suggesting that Brn-3b regulates Hsp-27 expression under these conditions. Wound healing assays showed increased Brn-3b in Dox-treated migratory cells that also express Hsp-27. Interestingly, Hsp-27 phosphorylation and cellular localisation are also significantly altered at different times following Dox treatment. Thus, phospho-Hsp-27 (p-Hsp27) protein displayed widespread distribution after 24 hrs of Dox treatment but was restricted to the nucleus after 5 days. However, in drug-resistant cells (grown in Dox for >1 month), p-Hsp-27 was excluded from nuclei and most of the cytoplasm and appeared to be associated with the cell membrane. Studies to determine how this protein promotes survival and migration in breast cancer cells showed that the protective effects were conferred by unphosphorylated Hsp-27 protein. Thus, complex and dynamic mechanisms underlie effects of Hsp-27 protein in breast cancer cells following treatment with chemotherapeutic drugs such as Dox, and this may contribute to invasiveness and drug resistance following chemotherapy.

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Abbreviations

Brn-3b	POU transcription factor Brn-3b/POU4F2
HSP	Small heat-shock protein
AV	Adenovirus
WT	Wild type
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Introduction

Chemotherapeutic drugs such as doxorubicin (Dox) are commonly used for the treatment of a wide range of cancers, including breast, ovarian cancer and lung cancer. However, acquired resistance to such drugs represents a major health problem because surviving cancer cells become refractory to treatment and contribute to recurrence and metastatic spread. Moreover, these highly aggressive cancer cells can spread as metastasis to organs at distances from the original tumour, thereby contributing to the deadly nature of this disease (Harris and Hochhauser 1992; Jacks and Weinberg 2002). These properties of cancer cells arise as a consequence of changes in gene expression. However, the precise mechanisms through which malignant cells develop drug resistance and migratory potential are still not fully understood.

The Brn-3b/POU4F2 transcription factor (referred to as Brn-3b) is elevated in >60% of breast cancers compared with normal breast biopsies (Budhram-Mahadeo et al. 1999; Budhram-Mahadeo and Latchman 2006) and plays an important role in determining proliferation and growth characteristics of breast epithelial cells (Budhram-Mahadeo et al. 1999; Dennis et al. 2001). Two isoforms of Brn-3b have been identified (Theil et al. 1995), with the longer 43 kDa Brn-3b(l) protein containing an additional amino terminal domain that is absent in the shorter 30–35 kDa Brn-3b(s) isoform. Both Brn-3b isoforms have been detected in breast cancer cells (Budhram-Mahadeo et al. 1999). Elevated Brn-3b promotes cell proliferation and tumour growth (Dennis et al. 2001; Irshad et al. 2004), whereas reducing Brn-3b was sufficient to reverse these growth effects, suggesting that this regulator is important for controlling growth of such cancer cells (Dennis et al. 2001; Irshad et al. 2004). Cancer cells overexpressing Brn-3b continued to proliferate in the presence of growth-inhibiting retinoic acid, suggesting that this protein may also be associated with drug resistance. Cells with increased Brn-3b also showed enhanced migration in Matrigel assays, indicating increased migratory potential (Irshad et al. 2004).

As a transcription factor, Brn-3b gives rise to such diverse effects by its ability to regulate multiple target genes. For instance, Brn-3b may enhance proliferation by increasing transcription of cell cycle regulators, cyclinD1/CDK4 (Budhram-Mahadeo et al. 2008; Samady et al. 2004) whilst inhibiting the tumour suppressor gene BRCA1 (Budhram-Mahadeo et al. 1999), which induces cell cycle arrest in breast cancer cells. Brn-3b also regulates target genes that can alter cell motility and drug resistance, e.g. by repressing the adhesion molecule, γ -catenin (plakoglobin) (Samady et al. 2006) whilst transactivating the small heat-shock protein, Hsp-27. Thus, Brn-3b/POU4F2 can transactivate the Hsp-27 promoter either directly or indirectly by

interacting and cooperating with the oestrogen receptor (ER), a key modulator of Hsp-27 in breast cancer cells (Lee et al. 2005).

The importance of Brn-3b in regulating Hsp-27 was demonstrated by significant correlation (R , ~ 0.8) between Brn-3b and Hsp-27 in breast cancer biopsies (Lee et al. 2005). Elevated Hsp-27 is associated with aggressive, metastatic disease that is refractory to treatment, e.g. radiotherapy and chemotherapeutic drugs (Ciocca and Calderwood 2005), and high Hsp-27 often correlates with shorter disease-free survival/recurrence following treatment in patients with advanced breast cancer (Ciocca et al. 1993; Vargas-Roig et al. 1998) and is therefore a poor prognostic indicator. Hsp-27 is also increased in >70% of patients with lymph node metastasis even when primary tumours are negative for Hsp-27 (Storm et al. 1996). Hsp-27 enhances anchorage-independent growth (Rust et al. 1999) and increases resistance to drugs (Ciocca et al. 1992; Oesterreich et al. 1993; Yamamoto et al. 2001) and metastatic potential (Lemieux et al. 1997) by acting in different capacities in cells. For example, by acting as a molecular chaperone, Hsp-27 can maintain integrity of cellular proteins under stressful conditions (Hartl and Hayer-Hartl 2002; Nollen and Morimoto 2002; Tavaría et al. 1996) but can also enhance cell survival directly by modulating the apoptotic machinery (Concannon et al. 2003; Farooqui-Kabir et al. 2004; Latchman 2002). By associating with cytoskeletal proteins such as actin, Hsp-27 also regulates cell motility and integrity (Landry and Huot 1999). Therefore, increased transcription of HSP-27 gene by Brn-3b is likely to be important for the generation of aggressive, Dox-resistant recurrent tumours.

However, while increased expression of Hsp-27 in tumour cells is important for function, its effects are also highly regulated by its oligomerisation state, which alters the size of Hsp-27 complexes. This, in turn, depends on post-translational phosphorylation of Hsp-27 by kinases (MAPKAPK2/3 and p38) on specific serine residues (Landry et al. 1992) that alters its quaternary structure and, thereby, its oligomerisation state. Thus, unphosphorylated Hsp-27 proteins form large complexes (200–800 kDa) (Merck et al. 1993a,b) that can facilitate chaperone activity, enhance survival (e.g. by interaction with and inhibition of pro-caspases) or modulate cell motility by interaction with cytoskeletal protein (e.g. actin). In contrast, phosphorylated Hsp-27 proteins (p-Hsp-27) exist as smaller complexes (tetramers, dimers and monomers) with distinct functions. For example, p-Hsp-27 can inhibit actin polymerisation but also protect against actin fragmentation under conditions of oxidative stress (Huot et al. 1996). p-Hsp-27 also contributes to cell survival but appears to act through different mechanisms to the unphosphorylated form, e.g. it can interact with Daxx to inhibit apoptosis (Lambert et al. 1999).

Phosphorylation of Hsp-27 occurs as a transient, reversible and dynamic process whereby dephosphorylation by phosphatase, PP2a, can rapidly return Hsp-27 to its unphosphorylated state and thus change its function (Zantema et al. 1992). Some stresses cause Hsp-27 to re-localise to the nucleus (Arrigo et al. 1988), but whether this depends on phosphorylation is not clear (Kim et al. 1984). Here, we report that the Brn-3b transcription factor is induced in breast cancer cells that survived treatment with chemotherapeutic drug, doxorubicin (Dox), and this is required to increase Hsp-27 since Brn-3b siRNA blocked Hsp-27 expression in Dox-treated cells. Furthermore, Brn-3b and Hsp-27 are also highly expressed in migratory cells following treatment, and the localisation of Hsp-27 in cellular processes might suggest a role in migration and cell–cell contact. Dox also increases Hsp-27 phosphorylation, which alters localisation in cells depending on time after treatment. Use of phosphorylation mutants demonstrated distinct functions for phosphorylated and unphosphorylated Hsp-27 following Dox treatment in MCF7 breast cancer cells. These results and implications in drug resistance and migration of breast cancer cells are discussed.

Experimental procedures

Materials Tissue culture plastics and reagents were obtained from Life Technologies (UK) and general laboratory reagents from Merck (UK) and Sigma (UK). Primary antibodies (Ab) were as follows: α -Brn-3b rabbit pAb; (Abcam) or goat pAb (Santa Cruz Biotechnology Inc, USA); dilution, 1:1,500–2,000; anti-Hsp27 goat pAb (M20, Santa Cruz Biotechnology Inc, USA); dilution, 1:1,000; or anti-phospho HSP25 pAb (Cell Signalling); dilution, 1:1,000; anti-actin goat pAb (I-19), Santa Cruz: Insight Biotech Ltd; 1:1,500. horseradish peroxidase-conjugated secondary antibodies were obtained from Dako (UK) and used at 1:2,000–1:3,000. Doxorubicin (D-1515 from Sigma UK) was prepared in dimethyl sulphoxide (DMSO; stock–100 $\mu\text{g}/\mu\text{L}$). Brn-3b complementary DNAs cloned in pLTR expression vector was as described (Budhram-Mahadeo et al. 1998), and Brn-3b siRNAs [and non-silencing (scrambled) control] were obtained from Open Biosystems human pGIPZ Lentiviral short hairpin RNA (shRNA) library. Hsp-27 adenoviral particles were a kind of gift from Dr. D. Trott and Professor M. Rose (Imperial College London) and were originally made by Dr. J. Martin (Loyola University) (Martin et al. 1999)

Western blot analysis Total cellular proteins from cell lines (harvested directly in $2\times$ Laemmli buffer) were used for immunoblotting as described (Budhram-Mahadeo et al.

1999) using 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis. Blocking was for 1 h in phosphate-buffered saline containing 0.1% Tween 20 (PBST)/4% non-fat powdered milk. Primary and secondary antibodies were diluted (as specified) in 1% milk+PBST. Blots were developed using enhanced chemiluminescence reagent (Pearce, UK). Differences in total protein levels were adjusted using housekeeping genes, e.g. actin or GAPDH.

Cell culture and transient transfections MCF7 breast cancer cells (obtained from ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 1% non-essential amino acids and 1% penicillin/streptomycin (Budhram-Mahadeo et al. 1998). To analyse cells following Dox treatment, initial studies were set up using different doses of this drug (0.1–1 μmol), to determine concentration to be used for subsequent studies. Characterisation of cell death led to subsequent studies being undertaken using 0.5 μmol Dox for treatment. To generate Dox resistance, cells were treated with 0.5 μmol drug for 1 week to kill off sensitive cells, and surviving cells were then maintained in 0.1 μmol of Dox (3–4 days), alternating with growth in medium with no drug (3–4 days), which allowed very low levels of proliferation, required for maintenance over longer periods. Cells treated for >1 month were detached using trypsin, plated onto coverslips and treated with 0.5 μmol Dox before use for co-localisation (immunostaining) experiments.

For transient transfection experiments, cells were plated into six-well plates (5×10^4 cells/well). After 12 h, transfection was undertaken using GeneJuice® transfection reagent, according to manufacturer's protocol.

To manipulate the expression of wild-type Hsp-27 and analyse the effects of phosphorylation mutants in cells, adenovirus (AV) vectors were used, which expressed wild-type (WT), non-phosphorylatable mutant (3A), phosphorylation-mimic (3D) or antisense construct (to reduce Hsp-27). AV constructs were transduced into cells (in serum-free medium) by adding 200 MOI of viral particles onto cells plated at 5×10^5 cells/well (six-well plates). Full-growth medium was replaced after 3–4 h of treatment. After 24 or 48 h, cell viability was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or cells were harvested and total protein isolated for experiments.

Immunostaining for Brn-3b and Hsp-27 expression Immunostaining was carried out on MCF7 cells grown on coverslips and either treated with Dox or DMSO vehicle control for specified times. Cells were fixed in 4% paraformaldehyde (PFA) for 15 min, washed in $1\times$ Tris-

buffered saline (TBS) and pre-incubated in block solution [20% goat serum in TBS+0.1% triton-X100 (TBST)] for 30–60 min before incubation with primary antibody diluted in 1× TBST+1% serum (Brn-3b mAb, 1:500; total Hsp-27 mAb, 1:250; phospho-Hsp-27 pAb, 1:500) overnight at 4°C or 1 h at room temperature. For co-immunostaining, the next primary antibody was incubated sequentially after five washes in TBST. Following incubation with secondary antibody, final washes were performed and cells mounted in fluorescent mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Images were obtained either using Zeiss LSM 710 confocal laser scanning inverted microscope or with Zeiss Axioscop 2 fluorescent microscope and analysed using Image J or Axiovision software).

MTT viability assay Following drug treatment, cell viability was assessed by absorbance spectrometry using MTT assay, which measures conversion of MTT to formazan in living cells. Treated cells and untreated control were incubated with 250 µL of 0.5 mg/mL of MTT for 2 h. MTT was aspirated, and formazan crystals were dissolved in 250 µL DMSO and optical density measured at 560 nm.

Wound closure assay To analyse dynamic changes in migrating cells, 'wound-closure' assay was carried out as described (Doshi et al. 2008). Briefly, cells were plated out as a monolayer of confluent cells, and a pipette tip or cell scraper was used to make a 'wound' in this monolayer. Cells were then treated with Dox or vehicle control for different times before analyses were carried out. Changes in the morphology of cells migrating from the edge of the 'wound' were analysed using bright-field microscopy. Immunostaining, using fluorescent-tagged secondary antibody, was used to detect protein localisation in migrating cells, and this was analysed using epifluorescence microscopy (i.e. Zeiss inverted microscope and Axiovision software).

Results

Brn-3b expression is stimulated by Dox treatment of MCF7 cells and is required for Hsp-27 induction

Since Brn-3b TF regulates Hsp-27 expression in breast cancer cells and Hsp-27 is associated with drug resistance and migration, we tested whether this TF is increased in breast cancer cells treated with chemotherapeutic agents. Doxorubicin is a common chemotherapeutic drug used for treatment of many cancers, including breast cancer, so we tested the effects of this drug on Brn-3b and Hsp-27

expression in breast cancer cell line, MCF7. Initial studies were undertaken to determine the optimal concentration of Dox for these studies by analysing the effects of different concentrations of the drug on cell viability. Changes in cell survival were measured using MTT assays in MCF7 cells treated with 0.5 or 1 µmol Dox for 24 or 48 h. Both 0.5 µmol and 1 µmol Dox caused some cell death after 24 hours but after 48 hours of treatment, 1 µmol resulted in substantially more death and fewer cells surviving, compared with 0.5 µmol. FACS analysis showed that Dox treatment caused cell cycle arrest in G2 phase [Figure 1B (i)] and this was reflected by reduction in G1 cell cycle protein, cyclin D1, compared with untreated control [figure 1B (ii)].

Therefore, subsequent studies were undertaken using 0.5 µmol doxorubicin to facilitate analysis at later times (up to 5 days). Dox-resistant cells were generated as described (methods) after treatment with 0.5 µmol drug for 5 days, followed by alternating medium containing 0.1 µmol Dox with medium lacking this drug.

To analyse changes in Brn-3b and Hsp-27 expression in drug-treated cells, total cellular proteins were extracted from cells harvested at different times after addition of Dox and were used for immunoblotting. Figure 1c (i) shows a representative blot of Brn-3b protein with low basal levels in untreated cells that increased after 18 h of Dox treatment and was sustained for up to 72 h. This was particularly evident for Brn-3b(l) isoform, which is a better activator of the Hsp-27 promoter (Lee et al. 2005). Figure 1c (ii) shows concurrent induction of Hsp-27 protein in these cells. Furthermore, Hsp-27 was elevated in drug-resistant cells that survived treatment with Dox>1 month (iii), suggesting that this protein might contribute to longer term survival following drug treatment.

Cells grown in the presence of Dox did not proliferate but underwent cell cycle arrest (figure 1b) and FACS analysis confirmed that distinct morphological changes, evident in the flattened, enlarged cell body with multiple extensions [shown in Fig. 1d (i)]. To test for expression of Brn-3b and Hsp-27 protein following Dox treatment, co-immunostaining was undertaken in Dox-treated cells and compared with untreated controls. Figure 1d (ii) shows low basal levels of both Brn-3b and Hsp-27 in untreated confluent, non-dividing MCF7, which were increased in Dox-treated cells (iii). As expected for a transcription factor, Brn-3b was localised to the nuclei in surviving cells (top panel), whereas Hsp-27 displayed more generalised distribution throughout the cells (bottom panel). These results suggest that Brn-3b and its target gene, Hsp-27, are elevated in surviving cells following Dox treatment of MCF7.

To test if Brn-3b was necessary for Hsp-27 expression in cells treated with Dox, short interfering RNA was used to

Fig. 1 **a** MTT assay showing percentage of survival, following treatment of MCF7 cells with 0.5 μ M (black bar) or 1.0 μ M (grey bar) with doxorubicin for either 24 or 48 h. Loss of cell viability was expressed relative to the untreated control cells (set at 100%). **b** (i) Graphical representation of FACS analysis showing percentage of cells in different phases of the cell cycle following doxorubicin treatment. (ii) Expression of cyclin D1 in untreated cells (lane 1) or doxorubicin treated (Dox) cells (lane 2) after 48 h. **c** Representative Western blot analysis showing increased (i) Brn-3b protein or (ii) HSP-27 in MCF7 cells at different times following Dox treatment. Immunoblots to detect housekeeping gene, actin, was used to assess for variability in protein loading in different sample. (iii) Immunoblot to show changes in HSP-27 protein in Dox-resistant MCF7 cells (grown in doxorubicin for up to 2 months) with actin house keeping

gene controlling for protein loading. **d** (i) Photomicrograph showing morphological changes associated with Dox treatment of MCF7 cells with the surviving cells appearing flattened with enlarged cell body and multiple distinct processes. Immunostaining for Brn-3b (red) or HSP-27 (green) (ii) showing low protein levels in confluent, untreated cells (iii) with significant increases in Dox-treated cells. DAPI staining (blue), was used to identify cell nuclei. **e** Representative staining for HSP-27 protein in MCF7 transfected with (i) Brn-3b siRNA 3 (si3) that specifically silence Brn-3b. Cells expressing Brn-3b si3 that are identified by GFP (green and indicated with asterisk) show reduced HSP-27 protein expression (red). (ii) Control siRNA (NS) transfected cells stained for HSP-27 showed continued expression in GFP-positive cells to confirm specificity of si3 effects on reducing Hsp-27

Fig. 1 continued.

reduce Brn-3b protein. Cells were targeted by transfection of Brn-3b shRNA construct (si3) in pGIPZ vector, which also expresses green fluorescent protein (GFP) to mark transfected cells. Empty vector was used as a control. Immunostaining for Hsp-27 was carried out on transfected cells following treatment with Dox for 24 h. Figure 1e shows that Brn-3b siRNA (si3)-transfected (GFP positive) cells did not express Hsp-27, whereas cells transfected with non-silencing control (NS) continue to express this protein. These results confirm that loss of Brn-3b blocked Hsp-27 expression and suggests that it is required for Hsp-27 induction following Dox treatment.

Brn-3b and Hsp-27 expression in migratory cells is increased after Dox treatment

Cells that survived treatment with chemotherapeutic drugs such as doxorubicin can give rise to recurrence of aggressive cancer cells that migrate and grow as metastasis. Since Hsp-27 plays an important role in cell migration and is associated with metastatic cancers, we analysed whether increases in Brn-3b correlate with Hsp-27 expression in migratory breast cancer cells following Dox treatment.

Therefore ‘wound-closure’ assays, known to induce Hsp-27 expression (Doshi et al. 2008) were undertaken to study gene expression in cells migrating into a ‘wound’ made in a monolayer of confluent cells in the absence or presence of Dox. Figure 2a shows that Dox-treated cells appeared to be more migratory and displayed distinct morphology, with flattened cell bodies and extensive processes. Immunostaining, to look at protein expression, showed strong staining for Brn-3b and Hsp-27 in migrating Dox-treated cells (Fig. 2b). Brn-3b TF was localised to the nuclei, whereas

Fig. 2 **a** Photomicrograph showing ‘wound site’ in untreated MCF7 cells compared with Dox-treated cells demonstrates the distinct morphological changes following Dox treatment (1 μ mol) and increased numbers of migrating cells. **b** Confocal images showing localisation of Brn-3b (red) or HSP-27 (green) proteins at the wound site after immunostaining. Merged picture also shows DAPI staining on the nuclei. (i) Nuclear expression of Brn-3b is observed in cells that are positive for HSP-27 protein, which is localised primarily to the cytoplasm and in processes that make contact with other cells (indicated with *asterisk*). (ii) Cells without HSP-27 (marked with *asterisk*) do not have evident process formation compared with HSP-27-positive migrating cells. These cells are also positive Brn-3b. **c** Co-localisation of actin detected using phalloidin staining (red) in processes involved in cell–cell contact, which also express HSP-27 (green). Merged picture shows nuclear (DAPI) staining also

(A) Morphological changes at wound site after 72 hours

Hsp-27 protein was more widely distributed, with expression particularly evident in cellular processes of migrating cells (Fig. 2b). Interestingly, cells without Hsp-27 did not have migratory processes [Fig. 2b (ii) marked with an asterisk], suggesting that Hsp-27 may be required for the development of extensions involved in migration and cell–cell contact.

To determine whether Hsp-27 expression is associated with process formation in migrating cells that make cell–cell contact, we tested whether Hsp-27 co-localises with structural protein, actin, as previously shown (Huot et al. 1995). Therefore, co-immunostaining was undertaken for Hsp-27 and filamentous actin (phalloidin staining) in Dox-treated migrating MCF7 cells. Figure 2c shows localisation of Hsp-27 in cellular processes involved in cell–cell contact, which also stained for actin filaments, suggesting that Hsp-27 is associated with migrating MCF7 breast cancer cells following Dox treatment.

Dox treatment changes Hsp-27 phosphorylation and alters cellular localisation

Whilst stimulation of Hsp-27 promoter activity by TFs (e.g. Brn-3b) increases expression levels in cells, post-translational phosphorylation of the protein can also significantly alter function.

Since Dox treatment induces Hsp-27 in MCF7 cells, we also tested whether phosphorylation of the protein is also modified under these conditions. Therefore, phospho-specific Ab was used to detect phosphorylated Hsp-27 (p-Hsp-27) following immunoblotting and compared with total Hsp-27. Representative immunoblots, shown in Fig. 3a, demonstrates low levels of p-Hsp-27 protein in control, untreated MCF7 cells that is in contrast to the high basal expression detected using antibody to total Hsp-27 protein [Fig. 1b (ii)]. However, p-Hsp-27 proteins were increased after 6 h of Dox treatment and sustained for up to 72 h. We also analysed levels of p-Hsp-27 protein in different cellular compartments using isolated nuclear or cytoplasmic fractions extracted from cells treated with Dox for different times. Representative immunoblot, shown in Fig. 3b, suggest changes in localisation of p-Hsp-27 protein at different times following treatment. Thus, phospho-protein was detected in both cytoplasmic and nuclear fractions by 24 h after treatment but appears to be primarily nuclear after 5 days, suggesting a shift in localisation of p-Hsp-27 protein after prolonged drug treatment.

Immunostaining was next undertaken to analyse the localisation of p-Hsp-27 in cells treated with Dox for duration, compared with total Hsp-27. Figure 4a shows widespread distribution of p-Hsp-27 after 24 h of Dox treatment, which was similar to total Hsp-27. However, after 5 days of treatment, p-Hsp-27 was detected mainly in

Fig. 3 **a** Western blot analysis showing changes in p-HSP-27 protein in MCF7 cells treated with Dox for different times (*top panel*). Total HSP-27 in corresponding samples are also shown (*bottom panel*). Invariant actin protein is used to control for differences in protein loading between the different samples shown. **b** Immunoblot showing p-HSP-27 in cytoplasmic (c) and nuclear (n) fractions prepared from cells treated with Dox (+) or DMSO vehicle control (–). Cells were harvested at the times indicated after treatment for protein extraction

cell nuclei (Fig. 4b), whereas total Hsp-27 was found throughout the cells suggesting that cytoplasmic Hsp-27 protein must be unphosphorylated. Interestingly, Dox-resistant (DR) MCF7 cells (grown in medium containing Dox for >1 months) showed distinct and restricted localisation total and p-Hsp-27 for both protein, around the cell membrane but excluded from nuclei (Fig. 4c). There was a significant overlap of staining for total and phospho-specific Hsp-27, suggesting that the phospho-protein may be the predominant form expressed in these drug-resistant cells.

The changes in cellular distribution of p-Hsp-27 following Dox treatment of MCF7 cells indicate that function of this protein might depend on phosphorylation status under different growth conditions. Since previous studies in other cell types suggested that phosphorylated Hsp-27 can inhibit actin polymerisation (Guay et al. 1997; Lavoie et al. 1993, 1995), we analysed p-Hsp-27 localisation in migrating cells after Dox treatment and compared with total Hsp-27. Figure 4d shows representative photomicrographs for total Hsp-27, which demonstrate widespread protein distribution, including the cellular processes that make contact with other cells (see also Fig. 2). However, p-Hsp-27 expression in these cells was confined to the nuclei suggesting that following drug treatment, unphosphorylated proteins localised in cellular processes will have different functions in the cells from nuclear p-HSP-27.

Survival effects of Hsp-27 in Dox-treated MCF7 cells require unphosphorylated protein

Both phosphorylated and unphosphorylated Hsp-27 can give rise to anti-apoptotic effects but via different mechanisms of action. For example, p-Hsp-27 is associated with

Fig. 4 Changes in localisation of phospho-HSP-27 (red) in MCF7 cells treated with Dox for different times indicated compared with expression of the total HSP-27 (green). Representative immunostaining shows **a** generalised expression of total and phospho-HSP-27 after 24 h of Dox treatment. **b** Continued widespread distribution of total

HSP-27 after 5 days Dox treatment, but phospho-HSP-27 becomes primarily nuclear after 5 days. **c** Restricted expression of both total and phospho-HSP-27 protein in Dox-resistant cells. **d** Migratory cells treated with Dox show widespread distribution of total HSP-27 but nuclear localisation of p-HSP-27

inhibition of Daxx-mediated apoptosis (Charette et al. 2000; Charette and Landry 2000), whereas unphosphorylated Hsp-27 can inhibit activation of pro-caspases or modify cytochrome C release to block apoptosis (Guay et al. 1997; Lavoie et al. 1993, 1995; Rogalla et al. 1999). Since surviving Dox-treated cells express both unphosphorylated and phosphorylated Hsp-27, we analysed which form of this protein is likely to contribute to cell viability following Dox treatment. These studies were undertaken using recombinant adenovirus (rAV) expressing either wild-type (WT) or mutant Hsp-27 in which three serine residues were altered to glutamic acid (3D) to mimic constitutive p-Hsp-27 or to alanine (3A) to act as non-phosphorylatable protein (Martin et al. 1999). rAV construct expressing antisense to Hsp-27 was also included to test the effects of reducing expression.

Immunoblots shown in Fig. 5a demonstrate increased Hsp-27 protein following transduction of rAV-expressing

WT or mutant Hsp-27 (3A or 3D) constructs but reduction with antisense, as expected. Subsequent viability studies were undertaken using MTT assay to analyse the effects of either mutating, increasing or decreasing Hsp-27 on cell survival by transducing cells with different Hsp-27 rAV or empty vector (control) for 48 h, followed by Dox treatment for 24 h. Results of MTT assays to measure cell viability in the absence and presence of Dox are shown in Fig. 5b. Although there was no change in viability in the absence of drug, the wild-type and mutant Hsp-27 proteins had distinct effects on survival following Dox treatment. Thus, cells expressing WT Hsp-27 showed enhanced survival, whereas the antisense construct, to reduce Hsp-27 (Fig. 5a), blocked survival and increased cell death. Interestingly, the non-phosphorylatable mutant, Hsp-27-3A, was protective in cells treated with Dox since viability was similar to that observed with WT Hsp-27, whereas constitutively phosphorylated mimic, Hsp-27-3D, did not protect cells against Dox induced

Fig. 5 **a** Western blot analysis showing increased expression of HSP-27 following transduction of cells with AV expressing either WT, 3A or 3D mutants or reduction with antisense (AS). **b** Results of MTT assay to analyse changes in viability of MCF7 cells following transduction with different Hsp-27 constructs. Black bars show cell viability in untreated cells, and grey bars show changes in viability following Dox treatment in cells are expressed as mean (SD) of three independent experiments. Increased viability in cells with wild-type (WT) HSP-27 and non-phosphorylatable mutants are statistically significant ($p < 0.05$) compared with SR control. Reduction in viability in cells expressing α -sense and non-phosphorylatable mutant (27-3D) are compared to survival in absence of Dox

death (Fig. 4b). These results indicate that survival of Dox-treated MCF7 cells requires unphosphorylated Hsp-27.

Discussion

The acquisition of drug resistance by cancer cells following treatment presents a significant problem since this leads to recurrence of highly aggressive tumours that are refractory to treatment and consequently have poor prognosis and increased mortality. It is evident that changes in gene expression contribute to acquisition of drug resistance and subsequent metastatic spread of tumour cells, but the mechanisms controlling such events are still to be fully elucidated. In this study, we show that the Brn-3b transcription factor is significantly up-regulated in breast cancer cells treated with the chemotherapeutic drug, Dox, and its expression is sustained in surviving cells that are maintained in presence of this drug for >1 month. Treatment of cells with Dox resulted in cell cycle arrest, which is evident from the distinct morphological changes and fluorescence-activated cell sorting (FACS) analysis show-

ing G2 arrest. Although Brn-3b is elevated in Dox-treated cells, its proliferative target gene, cyclin D1, is expressed at low levels, under these conditions, suggesting that the effects of Brn-3b are mediated via other target genes, such as Hsp-27. This was further supported by studies showing that RNA interference to silence Brn-3b successfully blocked Hsp-27 expression following Dox treatment. Therefore, increased Brn-3b TF in breast cancer cells can control distinct aspects of cell growth and behaviour, depending on target genes that it regulates under different cellular conditions.

Previous studies had shown that Hsp-27 controls the survival and behaviour of breast cancer cells, and increased Hsp-27 is associated with enhanced anchorage-dependent and independent growth, tumour invasion and resistance to chemotherapeutic drugs (Hansen et al. 1999; Oesterreich et al. 1993). The increased expression of Hsp-27 in Brn-3b-positive Dox-treated cells is not surprising since we previously showed that this transcription regulator is necessary for maximal stimulation of Hsp-27 in breast cancer cells (Lee et al. 2005). Thus, Brn-3b binds to DNA sequence elements in the Hsp-27 promoter and can either directly transactivate the promoter or cooperate with the ER to maximally stimulate promoter activity in MCF7 cells. Two Brn-3b isoforms [longer Brn-3b(l) and short Brn-3b(s)], which differ in the N-terminal region, are expressed in breast cancer cells and although both isoforms transactivate Hsp-27, Brn-3b(l) is a more potent transcriptional activator of this promoter (Lee et al. 2005). Interestingly, Dox treatment resulted in significant increases in the longer Brn-3b(l) protein and, to a lesser extent, Brn-3b(s) isoform. Since Hsp-27 is important for drug resistance, then the observation that Brn-3b regulates Hsp-27 expression following Dox treatment suggests that Brn-3b expression is important for responses of these cells after treatment with such chemotherapeutic drugs and may be likely to be important for development of drug resistance in breast cancer cells. In this regard, it is interesting that cells that survive long-term treatment with Dox, do indeed express high levels of Brn-3b. To generate such Dox-resistant cells (>1 month), we alternated medium containing Dox with drug-free medium, which facilitated low levels of growth, suggesting that Dox-resistant cells can re-enter the cell cycle and grow, albeit slowly once the drug is removed from the medium. Elevated expression of Brn-3b and Hsp-27 in cells that survive and grow under these conditions might suggest that increases of such factors in drug resistance cells will be associated with recurrent cancers.

Dox-treated cells expressing elevated Brn-3b and Hsp-27 also demonstrated enhanced migration in wound healing assays. Furthermore, in these migrating cells, Hsp-27 expression overlapped with f-actin (shown by phalloidin staining) in processes of cells making contact with other

cells. This is interesting because previous studies showed that Hsp-27 can associate with and stabilise actin, thereby facilitating polymerisation and migration. These results support a role for Hsp-27 (following induction by Brn-3b) in contributing to migratory potential of breast cancer cells, following drug treatment.

Although increases in gene transcription and subsequent expression of Hsp-27 are critical for its effects in cells, post-translation modifications such as phosphorylation, can also significantly alter its organisation and hence cellular function. Thus, unphosphorylated Hsp-27 exist as large oligomeric complexes (200–800 kDa) that are required for specific functions such as its action as a molecular chaperone (Garrido 2002; Rogalla et al. 1999). However, phosphorylation at specific serine residues (ser-15, 78 and 82) causes these large complexes to dissociate and form smaller oligomers (tetramer, dimer or monomer), which act in a different manner to larger unphosphorylated complexes. In this regard, p-Hsp-27 was shown to stabilise actin filaments but inhibit actin polymerisation by acting as a barbed-end actin capping protein. Both phospho- and unphosphorylated Hsp-27 can also inhibit apoptosis, but their effects are achieved through distinct mechanisms, e.g. phosphorylated Hsp-27 is required for inhibition of Fas receptor (fas R) mediated apoptosis via Daxx (death domain-associated protein) since the smaller oligomers can bind to and inhibit Daxx (Charette et al. 2000; Charette and Landry 2000). p-Hsp-27 may also modulate the pro-survival AKT pathway (Mearow et al. 2002; Zheng et al. 2006). However, Hsp-27 also inhibits mitochondrial mediated apoptosis by binding and sequestering cytochrome C. This anti-apoptotic effect appears to require unphosphorylated Hsp-27 (Guay et al. 1997; Lavoie et al. 1993, 1995; Rogalla et al. 1999), which disrupt apoptosome formation with pro-caspases thus inhibiting activation of effector caspases such as caspase-3. Therefore, changes in Hsp-27 phosphorylation are likely to alter its effects in cells. In this study, we demonstrated that in addition to induction of Hsp-27 mRNA and protein, Dox treatment also increased phosphorylation. Of particular interest were the striking changes in cellular localisation of p-Hsp-27 compared with the unphosphorylated protein, at different times following Dox treatment. Thus, after 24 h of treatment, total Hsp-27 (representing unphosphorylated and phosphorylated proteins) was detected throughout the cell, and p-Hsp-27 showed similar cytoplasmic and nuclear distribution. However, after Dox treatment for 5 days, p-Hsp-27 was primarily localised to the cell nuclei even though total Hsp-27 was detected in cytoplasm also, suggesting distinct function for these different forms. Interestingly, in Dox-resistant cells, Hsp-27 protein is only detected at the periphery of cells possibly associated with cell membrane and is no longer evident in the nucleus. In these cells, p-

Hsp-27 and total Hsp-27 showed considerable overlap in localization, suggesting that phosphorylated protein may be the predominant form in drug-resistant cells. The distinct localisations of Hsp-27 in Dox-resistant cells compared with cytoplasmic and/or nuclear expression in cells treated for short periods would suggest that this protein might have different and unique functions in dox-resistant cells compared with its effects in cells immediately after they are subjected to this stress. However, this is still to be elucidated.

These observations raise some interesting questions about the function of the phosphorylated and unphosphorylated forms of Hsp-27 following Dox treatment of MCF7 cells because of the distinct mechanisms of action (Bruey et al. 2000; Garrido 2002; Parcellier et al. 2003, 2005). In our study, phosphorylated Hsp-27 did not appear to be directly involved in migration following Dox treatment because p-Hsp-27 was localised to the nuclei of migrating cells, whereas total Hsp-27 was markedly increased in processes of migratory cells that were involved in cell–cell contact. This is interesting since previous studies suggested that p-Hsp-27 inhibits actin polymerisation by acting as a barbed end actin capping protein so may inhibit migration (Benndorf et al. 1994; Miron et al. 1991). As such, restricted expression of p-Hsp-27 to the nuclei of these cells is likely to prevent this inhibition whilst allowing unphosphorylated Hsp-27 to enhance remodelling and migration. Our results also demonstrated that unphosphorylated Hsp-27 confers protection against Dox-mediated death in MCF7 cells since non-phosphorylatable Hsp-27-3A mutant protected cells against Dox-induced death similar to wild-type Hsp-27, but this was not seen with constitutively phosphorylated mimic, Hsp-27-3D mutant. This was in keeping with results shown by Martin et al. (1997, 1999) who demonstrated that the protective effects of Hsp-27 following ischaemic insult in cardiac myocytes was dependent on unphosphorylated Hsp-27, whereas p-Hsp-27 did not affect survival. Therefore, survival and migration of MCF7 cells treated with Dox appears to require the unphosphorylated Hsp-27. However, because Dox treatment increases protein phosphorylation, this would suggest an important role for such modification in drug-treated cells. Although the mechanism through which Hsp-27 act under these conditions is still unclear, the nuclear localisation of p-Hsp-27 after drug treatment is intriguing since results from others have identified distinct functions for nuclear HSP-27 from its effects in the cytoplasm. Thus, heat-shock proteins, including Hsp-27, have been implicated in regulation of gene transcription, possibly through chaperone effects that can modulate TF conformation, thereby affecting stability of the transcription initiation complex (Freeman and Yamamoto 2002; Zoubeidi et al. 2007). Moreover, recent studies have

shown that Hsp-27 in cell nuclei can interact with TF such as SP1 and directly regulate transcription of target genes, e.g. NGFR and TrkA (Friedman et al. 2009). It is therefore possible that nuclear p-Hsp-27 in Dox-treated cells could act in a similar manner to control gene expression, thereby contributing to the aggressive nature of Hsp-27 over-expressing tumours following drug treatment, but this is still to be elucidated.

Thus, increased expression of the transcription factor, Brn-3b, in response to Dox treatment of breast cancer cells may control survival and subsequent cell migration by controlling the expression of key target genes such as Hsp-27. Our results also suggest that control of heat shock protein function is quite dynamic since Hsp-27 is actively regulated at the level of transcription, by TFs such as Brn-3b, as well as post-transcriptionally by phosphorylation. Such modifications, which profoundly alter protein localisation, are also likely to affect its function in these cells, thereby contributing to acquisition of drug resistance and metastasis in highly refractory tumours that over-express Hsp-27.

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