Resistance to Paclitaxel Increases the Sensitivity to Other Microenvironmental Stresses in Prostate Cancer Cells

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ABSTRACT

The microenvironment is central to many aspects of cancer pathobiology and has been proposed to play a role in the development of cancer cell resistance to therapy. To examine the response to microenvironmental conditions, two paclitaxel resistant prostate cancer (PCa) cell lines (stable and reversible) and one reversible heat resistant cell line were studied. In comparison to their parental cell lines, both paclitaxel resistant cell lines (stable and reversible) were more sensitive to microenvironmental heat, potentially yielding a synergistic therapeutic opportunity. In the two phenotypic cells repopulated after acute heat or paclitaxel treatments, there was an inverse correlation between paclitaxel and heat resistance: resistance to paclitaxel imparted sensitivity to heat; resistance to heat imparted sensitivity to paclitaxel. These studies indicate that as cancer cells evolve resistance to single microenvironmental stress they may be more sensitive to others, perhaps allowing us to design new approaches for PCa therapy. J. Cell. Biochem. 112: 2125–2137, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: PROSTATE CANCER; MICROENVIRONMENTAL STRESS; DRUG-RESISTANCE; HEAT; MICROENVIRONMENT

Prostate cancer (PCa) is the most common malignancy and the second leading cause of cancer-related deaths in men in the United States [Jemal et al., 2010]. In men with advanced disease, hormonal therapy (i.e., androgen deprivation) initially induces anti-tumor response in more than 90% of treatment-naïve patients. However, hormonal therapy eventually fails and the PCa progresses to a castrate-resistant stage that is essentially incurable [Gopalkrishnan et al., 2001]. Chemotherapy plays an increasingly important role in the management of castrate-resistant metastatic PCa. Recently, taxanes (paclitaxel or docetaxel—DTX) in combination with other agents, such as estramustine phosphate (EMP), or dexamethasone, have been shown to result in significant anti-tumor responses in this population of men [Petrylak et al., 2004; Tannock et al., 2004; Oudard et al., 2005]. Although castrate-resistant PCa often initially responds to paclitaxel-based chemotherapy, the disease eventually becomes resistant. Evolution of resistance is perhaps the largest problem in cancer therapy.

In our previous studies we have established stable paclitaxel-resistant DU145-TxR and PC-3-TxR cells from DU145 and PC-3 cell lines and investigated the mechanisms of drug resistance in DU145-TxR and PC-3-TxR cells. The multiple drug resistance gene (MDR-1)-encoded P-glycoprotein was demonstrated to be overexpressed in the DU145-TxR cells, and enhanced F-actin polymerization via down regulation of CTEN expression was identified as a paclitaxel resistant mechanism in the PC-3-TxR cells [Li et al., 2010; Takeda et al., 2007]. In addition to these molecular changes the microenvironment has been hypothesized to be a major component in the development of resistance. Microenvironmental factors, including: heat, pH, oxygen, and glucose, are central factors influencing both normal and cancer cells. Many studies have documented epigenetic changes in tumor cells, and there are limited data to support that epigenetic changes in the "normal" cells may be related to the tumor microenvironment [Hu et al., 2005; Fiegl et al., 2006]. The hypoxic conditions due to a high metabolic rate and

abnormal blood vessels in tumors accumulate lactic acid and promote accelerated oxidation of CO_2, providing an acidic extracellular tumor microenvironment (pH 5.8–7.1) [Vaupel et al., 1981; Swietach et al., 2007; Mashima et al., 2009]. In addition, glucose distribution in tumors is thought to follow similar patterns to that of oxygen. Acute and chronic hypoglycemia most likely exist in much the same way as acute and chronic hypoxia caused by transient alterations in blood supply, and diffusion-limitations, respectively [Horsman, 1995]. While oxygen and glucose concentration may be determined by diffusion into tumor tissue, pH may partly be determined by diffusion of ions out of tissue into blood vessels. Thus within the viable regions of tumors, there is a great cellular heterogeneity in oxygen concentrations, glucose content, and pH values. Furthermore the tumor microenvironment may modulate apoptosis indirectly via other processes that affect apoptosis such as proliferation, repair, energy metabolism, and signal transduction [Amellem and Pettersen, 1991; Yuan et al., 2000; Belka et al., 2004]. The microenvironment may also modify the radiation-induced apoptotic response of tumors [Hunter et al., 2006]. Elevation in environmental temperature has been shown to increase the cancer cell's susceptibility to chemo- and radiation therapy [Zeng et al., 2009], and we therefore hypothesized that as the cells become resistant to one type of therapy, such as chemotherapy (taxol), they might develop sensitivity to other types of microenvironmental stresses suggesting that combination of available and new therapies might be an effective treatment approach.

In the present work, we have investigated the ability of the previously established paclitaxel-resistant cell lines (PC-3-TxR and DU145-TxR) to respond to microenvironmental stresses. In addition, the responses of cell lines representing the spectrum of "normal" prostate to metastatic disease to these microenvironmental conditions were also studied. Overall these studies demonstrated that the development of chemotherapeutic resistance results in cells being less able to respond to stresses and may be the Achilles' heel of aggressive lethal cancers.

MATERIALS AND METHODS

CELL LINES, CULTURE, AND ISOLATION

The human prostate cell lines prostate epithelial cells (PrEC), LNCaP, PC-3, and DU145 were purchased from the American Type Culture Collection (Manassas, VA). The PrEC cells were cultured in PrEBM medium with PrEGM supplements (Lonza, Walkersville, MD). The OPCT cells were purchased from Asterand (Detroit, MI) and cultured in phenol-red free RPMI medium with PrEGM supplements. The LN96 cell line was established previously [Pflug et al., 1999], and cultured routinely in phenol-red free RPMI supplemented with 10% charcoal-stripped (CS) FBS (Hyclone, Logan, UT). The paclitaxel-resistant PC-3-TxR and DU145-TxR cells were generated and maintained as described previously [Li et al., 2010]. The PC-3-TxR cells were cultured in 10 nM paclitaxel (Sigma–Aldrich, St. Louis, MO) to maintain their drug-resistant phenotype. Prior to each experiment, these cells were grown for a minimum of 1 week in normal medium. The PC-3 and PC-3-TxR cells were maintained in RPMI1640 (Sigma) supplemented with 10% fetal bovine serum (FBS)

and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). The DU145 and DU145-TxR cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% FBS. The PC-3 and DU145 cells were treated with 100 nM paclitaxel for 48 h and after 30 days of recovery from the treatment, the cells were harvested and designated as PCAF48 and DUAF48, respectively. PC-3 and DU145 cells were treated with 43°C heat for 24 h and then harvested after 30 days of recovery and designated as PCAF24 and DUAF24, respectively.

PROLIFERATION ASSAY

Cell growth or growth inhibition assays were performed by plating 2×10^5 cells in six-well plates. After culturing for 24 h, the cells were treated with the indicated concentrations of paclitaxel. The cells that survived this treatment were cultured for an additional 48 h. At the end of the culture period, the cells were trypsinized and counted using a hemocytometer. The relative cell numbers compared with untreated controls were plotted as cell viability.

HEATING, ULTRAVIOLET RADIATION C (UVC), DIFFERENT pH VALUE, AND LOW GLUCOSE EXPERIMENTS

A typical tissue culture incubator was set to 43°C under normal humidity conditions for the heat experiments, and the UVC light (TUV 30W T8) in the cell culture hood was used for UVC exposure studies. NaOH and HCl (Sigma–Aldrich) were used to conduct the pH stress experiments, by modifying the pH values of the culture medium. The 1 mg/ml glucose concentration DMEM and no Glucose DMEM were utilized in the glucose stress studies.

GENERATION OF REVERSIBLE DRUG RESISTANT CELLS

Drug-sensitive cells PC-3 and DU 145 were treated with paclitaxel, at concentrations of 100 nM for 48 h. The viable cells remained attached on the dish at the end of 30 days recovery without paclitaxel.

GENERATION OF REVERSIBLE HEAT RESISTANT CELLS

PC-3 and DU 145 cells were treated at 43°C for 24 h, and the viable cells that remained attached on the dish at the end of a 30 day recovery at 37°C.

CELL SURVIVAL ASSAYS

Cells were plated in each well of a 24-well cluster dish. Forty-eight hours after plating, media were removed and replaced with media containing 10 nM paclitaxel. Forty-eight hours later, the cells were treated at 37 or 43°C for 2 h, with fresh media being replaced every 2 days until untreated cells reached confluence. The media were then removed, and cells were washed with phosphate-buffered saline (PBS) and then stained with 0.5% typan blue in PBS.

IMMUNOBLOTTING

Twenty-four hours after plating the cells, total protein was extracted using the M-PER Mammalian Protein Extraction Reagent (Thermo scientific, Rockford, IL). Fifteen micrograms of total protein was separated using 10–20% SDS–PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA), which were blocked with 5% nonfat dry milk, and immunoblotted with primary antibodies. The

following primary antibodies were used: rabbit polyclonal anti-PARP, anti-caspase-3, anti-NA$^+$ K$^+$-ATPase, anti-calcium binding protein CALB1, and anti-actin (Cell Signaling Technology, Inc., Danvers, MA). These primary antibodies were recognized by a goat anti-rabbit secondary antibody (Bio-Rad) and visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

FLOW–CYTOMETRY ASSAY

Expression of the CD133 on cell surface was assessed in the PC-3, PCAF48, PCAF24, DU145, DUAF48, and DUAF24 cells. The cells were washed with PBS before they were removed from the culture dish with trypsin and EDTA, counted, resuspended in 5 ml of fresh medium, incubated at 37°C for 30 min, and then transferred to Eppendorf tubes (1×10^6 for PC-3 and DU145 cells, 1×10^4 cells per tube). After washing with PBS containing 0.5% bovine serum albumin, the cells were centrifuged at 500g, and the supernatant was removed. The appropriate fluorochrome-conjugated antibody was added at 1:10 dilution (5–50 μl) in PBS and 0.5% bovine serum albumin and incubated on ice in the dark for 30 min. Briefly, 50 μl of the positive control beads and 50 μl of the negative control beads (BD Biosciences) were added, followed by 5 μl of the specific fluorochrome-conjugated antibody, and the cells were processed as described above.

DATA ANALYSIS

The statistical significance of differences in proliferation was determined by two-way ANOVA with post-hoc test. A Dunnett's test was also performed to determine the significance of intensity differences on immunoblotting analysis; differences noted $^*P < 0.05$ were considered to be statistically significant. The data represent mean values of three replicates with standard deviation for standard errors indicated.

RESULTS

CHANGING PHENOTYPES OF CELLS UNDER SELECTIVE PRESSURE OF MICROENVIRONMENTAL STRESS FACTORS

The key objective of this study centers on how the cell lines resistant to paclitaxel respond to the various microenvironmental stresses. To examine sensitivity of the cell lines to different stress environments, we exposed the PC-3-TxR and DU145-TxR cells along with their respective parental cells to various microenvironmental conditions. The evaluation of the resistance of the PC-3-TxR and DU145-TxR cells to paclitaxel was performed by estimating cell survival rates at different concentrations. In comparison to their parental cells, the IC$_{50}$ of the PC-3-TxR cells was increased from 5.16 to 56.39 nM, and the IC$_{50}$ of the DU145-TxR cells increased from 5.15 to more than 100 nM (Fig. 1A; Table I). Perhaps the microenvironmental factor that has been demonstrated to have the largest influence on cells is heat. In order to assess the effects of heat exposure, the cell lines (resistant and sensitive) were exposed to 43°C for 1, 2, or 4 h. After 48 h of recovery, the survival rates of the paclitaxel resistant PC-3-TxR and DU145-TxR were compared with their parental cells; PC-3 and DU145, respectively. As a single modality the paclitaxel resistant PC-3-TxR and DU145-TxR cells showed a greater

sensitivity to heat treatment. Compared to their parental cells, the IC$_{50}$ of the resistant cell lines were decreased from 2.39 to 0.61 and 2.87 to 0.61 h (Fig. 1B; Table I). In addition to heat, pH, UV, and glucose concentration play very important roles in the tumor microenvironment. The response of the resistant cell lines to these microenvironment stresses was also examined. In the pH stress experiments, PC-3-TxR and DU145-TxR cells exhibited a differential response to being exposed to higher pH values (1 value higher) for 48 h, but not to lower pH values (1 value lower) (Fig. 1C). The PC-3-TxR and DU145-TxR cell survival rates were not influenced significantly in comparison with their parent cells in response to UVC exposure. IC$_{50}$ were from 16.39 to 11.74 and 16.34 to 14.36 s (Fig. 1D; Table I). When cultured at low glucose concentration the paxitaxel-resistant cells had a lower survival rate in comparison to their parent cells. The IC$_{50}$ increased from 0.19 to 0.86 and 0.15 to 0.96 mg/ml (Fig. 1E; Table I). The proliferation rates were examined in the PC-3 with PC-3-TxR and DU145 with DU145-TxR cells, but the data did not reveal significant differences (Fig. 3A,B). The results suggest that as cancer cells achieve resistance to chemotherapy they develop sensitivities to other types of microenvironmental stresses.

DIFFERENTIAL SURVIVAL RATES AFTER EXPOSURE TO VARIOUS STRESSES IN NORMAL AND PROSTATE CANCER CELL LINES

In order to study the evolution of PCa resistance at each stage, primary cancer, metastasis, hormone-sensitive, hormone-refractory, chemotherapy-sensitive, and chemotherapy resistant, different cell models were utilized. As anticipated, the normal PrEC had significantly higher survival rates when treated with paclitaxel as compared to the primary PCa cells (OPCT). The IC$_{50}$ in PrEC was over 100 nM, but in the OPCT cells was 1.01 nM (Fig. 2A, left; Table I). The PrEC cells were also more viable in response to heat exposure longer than 2 h (Fig. 2B, left), more stable at a higher pH value (Fig. 2C left) and more resistant to UV treatment (The IC$_{50}$ was 15.43 s in PrEC, and 6.98 s in OPCT) (Fig. 2D, left; Table I). The OPCT cells were less viable at low glucose concentrations (IC$_{50}$ in PrEC was 1.50 mg/ml, but in OPCT was 1.01 mg/ml) (Fig. 2E, left; Table I). In order to confirm that the differences in the response to glucose (Fig. 2E, left) are not confounded by the use of DMEM medium for PrEC cells, we compared cell proliferation in PrEBM and DMEM media. There were no obvious differences (Fig. 3E). Furthermore, the hormone-therapy responsive cell, LNCaP, was compared to the refractory line LN96. In contrast to LNCaP, the LN96 cells were relatively more resistant to paclitaxel. The IC$_{50}$ was 1.54 nM in the LNCaP cells, but in LN96 was 4.41 nM (Fig. 2A, right; Table I) and was significantly more viable in low glucose conditions. (IC$_{50}$ was 0.48 and 0.12 mg/ml, respectively) (Fig. 2E, right; Table I). However, the LN96 cells were more significantly sensitive to heat. The IC$_{50}$ of LNCaP was over 4 h, but 3.44 h in the LN96 (Fig. 2B, right; Table I), high pH media (Fig. 2C, right), and UVC treatment (Fig. 2D, right; Table I). To ensure that differences in the proliferation capability did not influence these experiments, we compared the proliferation rates of these cells without treatment. The results suggest that there are no significant differences between the cell lines with the exception of slower proliferation of the PrECs compared to OPCT cells (Fig. 3C,D). To compare the observed phenotypes of all cells we estimated the IC$_{50}$ values for paclitaxel and heat. The result summarized in Table I
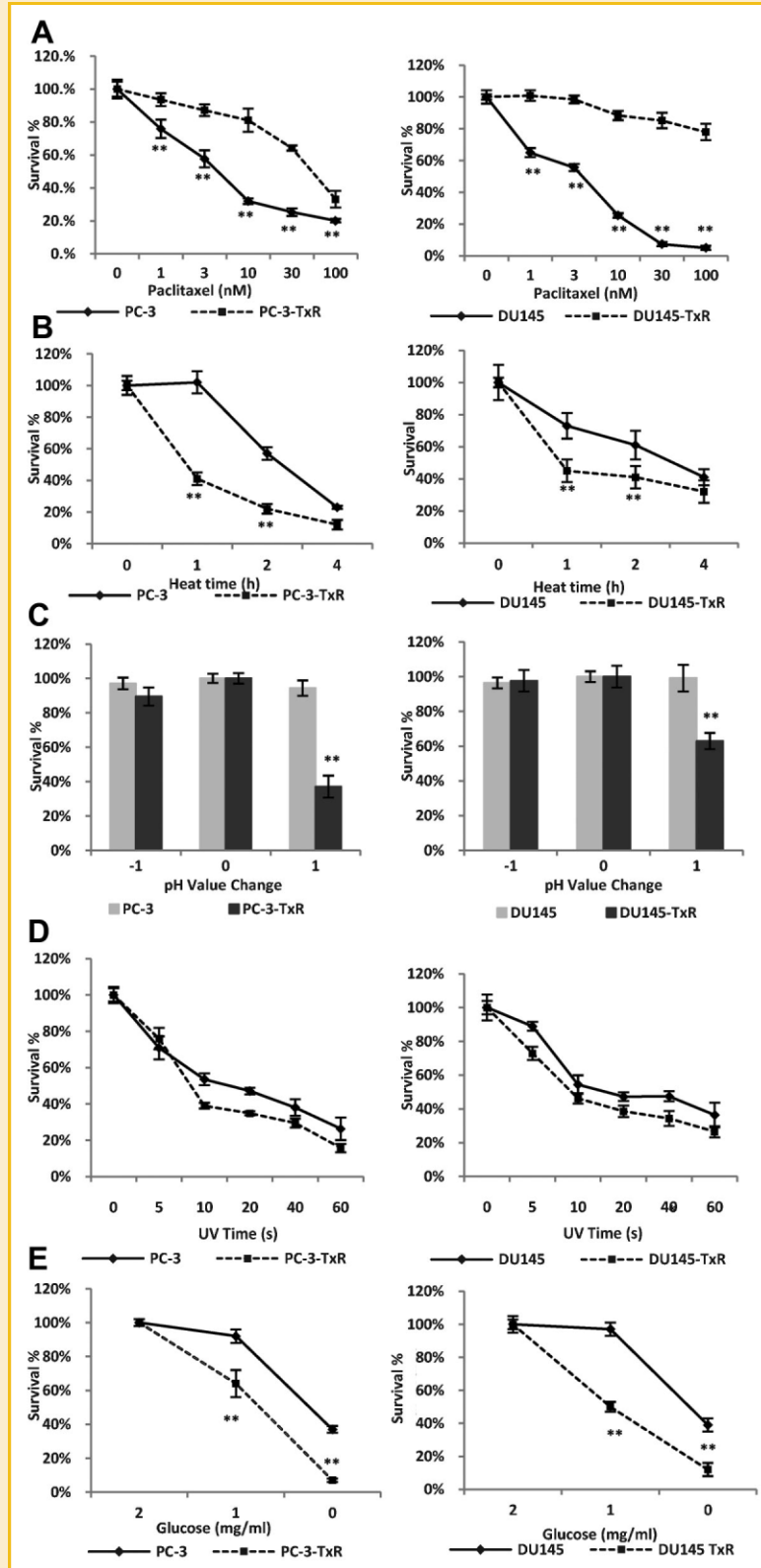
Fig. 1. Resistance to chemotherapy imparts a greater sensitivity to other microenviromental stresses. Evaluation of PC-3/TxR and DU145/TxR cell lines' survival rates at different concentrations of paclitaxel (A); exposure to 43°C heat for 0, 1, 2, and 4 h (B); at different ±1 pH values (C); exposure to UVC for 0, 15, 30, and 60 s (D); at different glucose concentration, 2, 1, and 0 mg/ml (E). *$P < 0.05$, **$P < 0.01$.

TABLE I. IC$_{50}$ Values of Prostate Cancer Cell Lines

Treatment	PrEC	OPCT-1	LNCap	LN96	PC-3	PC-3-TxR	DU145	DU-145TxR
Paclitaxel (nM)	>100	1.01	1.54	4.41	5.16	56.39	5.15	>100
Heat (h)	>4	>4	>4	3.44	2.39	0.61	2.87	0.61
UVC (s)	15.43	6.98	16.41	8.87	16.39	11.74	16.34	14.36
Glucose (mg/ml)	1.50	1.01	0.48	0.12	0.19	0.86	0.15	0.96

suggest that as PCa progresses, from the primary cell line to paclitaxel-resistant cell lines, together with the development of resistance to paclitaxel, an increased sensitivity to heat develops (Table I). The ability of cancer cells to acquire resistance to therapies such as hormone deprivation, or chemotherapy appears to make them vulnerable to other types of stresses to which they were resistance before. Normal cells are much less sensitive to these stresses.

DIFFERENT APOPTOSIS PATHWAY (CASPASE-3–DEPENDENT AND INDEPENDENT) ARE UTILIZED IN CELLS EXPOSED TO DISTINCT TYPES OF STRESSES

Cell apoptotic pathways are basic mechanisms in tissue homeostasis, cancer, and the induction of tolerance. As described above, the cell lines demonstrated differential viabilities in the context of different stress environments. Therefore, we next investigated the potential underlying apoptotic mechanisms of exposure to these distinct microenvironments. The effects of the stresses on individual apoptotic pathways in paclitaxel sensitive and paclitaxel resistance cell lines were examined. In the paclitaxel sensitive PC-3 and DU145 cells, after drug, or UV treatment, the apoptosis pathway in which caspase-3 is involved appears to be activated, as opposed to the heat, basic pH, and low glucose treatments (Fig. 4A,B, left). In the paclitaxel-resistant cell lines, PC-3-TxR and DU145-TxR, no evidence of apoptosis was observed after paclitaxel and UV treatment, whereas in PC-3 and DU145 (parental cells) evidence of caspase-3-induced apoptosis was present. However, after exposure to heat, or culturing at basic pH or in low glucose medium, we observed changes in PARP expression but not caspase-3 (Fig. 4A,B, right). These results suggest that in contrast to paclitaxel and UVC treatments, exposure to heat, high pH value, and low glucose may activate an alternative, caspase-3-independent apoptosis pathway. Based on the apoptosis pathway results, the relationship between caspase-3-induced apoptosis and noncaspase-3 apoptotic pathways where different microenvironmental stresses were examined.

CELLS THAT SURVIVE AND REPOPULATE AFTER EXPOSURE TO HEAT AND PACLITAXEL STRESS DEVELOP RESISTANCE TO THE SAME STRESSES BUT REMAIN SENSITIVE TO OTHERS

To further investigate the phenotypic changes observed in cells as they develop resistance, the PC3 and DU145 (parental cell lines) were exposed to 100 nM of paclitaxel for 48 h and then cultured at 43°C for 24 h. After 30 days of recovery, the surviving cells generated single colonies (Fig. 5A). The visualization of these cells by light microscopy showed distinct morphologic characteristics in comparison to their parental cells, and these cell lines were designated as PCAF48, DUAF48, PCAF24, and DUAF24 (Fig. 5B). The viability of

the cell lines undergoing "acute" stress exposure (PCAF48 and DU145AF48) in comparison to those in which resistance was developed by "chronic" stress exposure (PC3-TxR and DU145-TxR), was compared along with the parental (PC3 and DU145) lines, by exposure to different concentrations of paclitaxel. The results indicate that the resistant cells developed by chronic exposure had the highest survival rates, the parental cells had the lowest and the cells that acutely developed resistance had intermediate survival rates, with the IC50 values increasing from 5.16 to 10.01 nM in the PC-3 cells, and from 5.15 to 16.5 nM in the DU145 cells (Fig. 6A). As anticipated, the PCAF48 and DU145AF48 cells had lower survival rates when exposed to heat as compared to their parental cell lines (Fig. 6B). However, after 2 weeks of culture and four passages the "PCAF48–2" and "DU145AF48 -2" demonstrated differential survival rates after exposure to paclitaxel or heat showing a trend toward restoration of the phenotype they had before exposure to the acute stresses (Fig. 6C,D). Similar effects were observed for PCAF24 and DUAF24, which appeared to be more resistant to heating and more sensitive to paclitaxel compared to their parental cell lines (Fig. 6E,F). And again, when retested after 2 weeks and 4 passages they tended toward restoration of their previous phenotype (Fig. 6G,H).

The above results suggest that the cancer cell population contains cells capable of surviving after acute extreme stress exposure by adaptive changes in their phenotype. At the same time the cells lose the ability to persist in the presence of other factors. However, after the acute stress factor has been withdrawn, the cells tend to restore the phenotype suited for better fitness and more efficient proliferation. These cells have more potent ability to survive and repopulate after acute stress exposure, could be the stem-like cells of the tumor. As CD133 is considered to be a tentative marker of the PCa stem cell, we investigated expression of this surface molecule on the parental cells and the cells after repopulation following exposure to acute stress. The flow cytometry results that however only a small fraction from the repopulated cells have higher CD133 expression (Fig. 5C).

OVEREXPRESSION OF Na$^+$ K$^+$–ATPase AND CALCIUM–BINDING PROTEIN IN PC-3 AND DU145 PACLITAXEL SENSITIVE CELLS AFTER HEAT TREATMENT

Standard cancer therapies eradicate tumor cells, at least in part, by indirectly activating the apoptotic machinery. To investigate apoptotic pathways in both acute and chronic stress-induced resistant cell lines, cell extracts were obtained after 2 and 4 h of exposure to heat or 10 nM concentrations of paclitaxel and apoptotic pathway component expression was revealed by immunoblot analysis. Similar to the previous experiments (Fig. 4), we observed caspase-3 independent apoptosis in the PC-3-TxR and DU145-TxR cells after 2 h of heat that was more
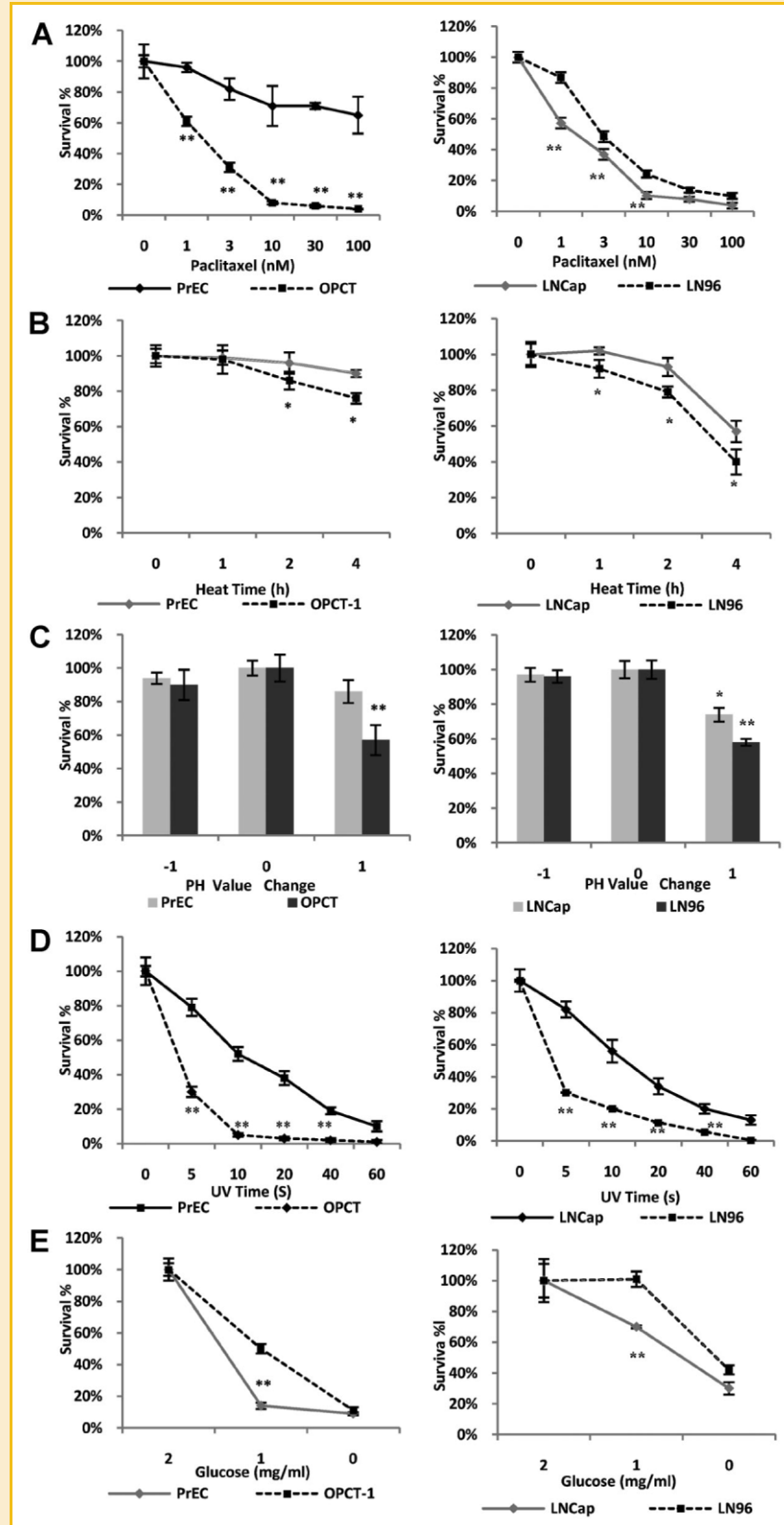
Fig. 2. Evaluation of PrEC, OPCT, LNCap, and LN96 cell lines' survival rates at different concentrations of the paclitaxel (A); exposure to 43°C heat for 0, 1, 2, and 4 h (B); at different ±1 pH values (C); exposure to UVC for 0, 15, 30, and 60 s (D); at different glucose concentration, 2, 1, and 0 mg/ml (E). $^*P < 0.05$, $^{**}P < 0.01$.
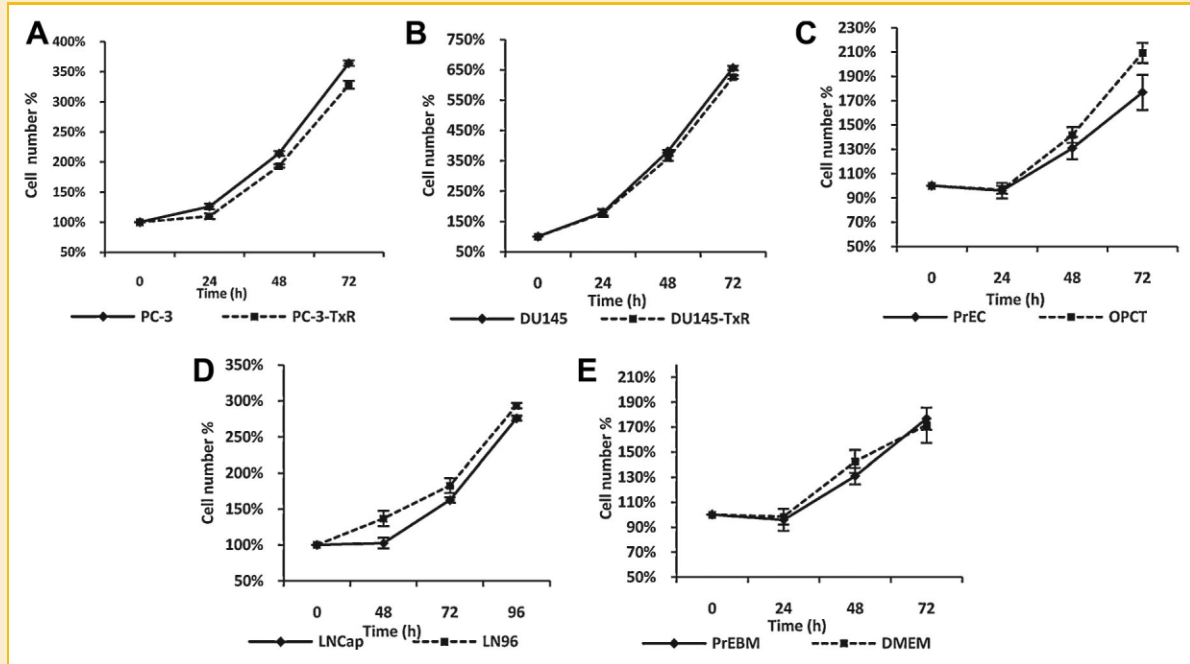
Fig. 3. Time point proliferation assay in prostate cancer cell lines. A–D: PrEC, OPCT, LNCap, LN96, PC-3, PC-3-TxR, DU145, and DU145-TxR were cultured as described in the "Materials and Methods" Section. The cells were seeded and counted in a hemocytometer. E: PrEC cells (2.5×10^5) were cultured in DMEM and PrEBM medium with PrEGM supplements.
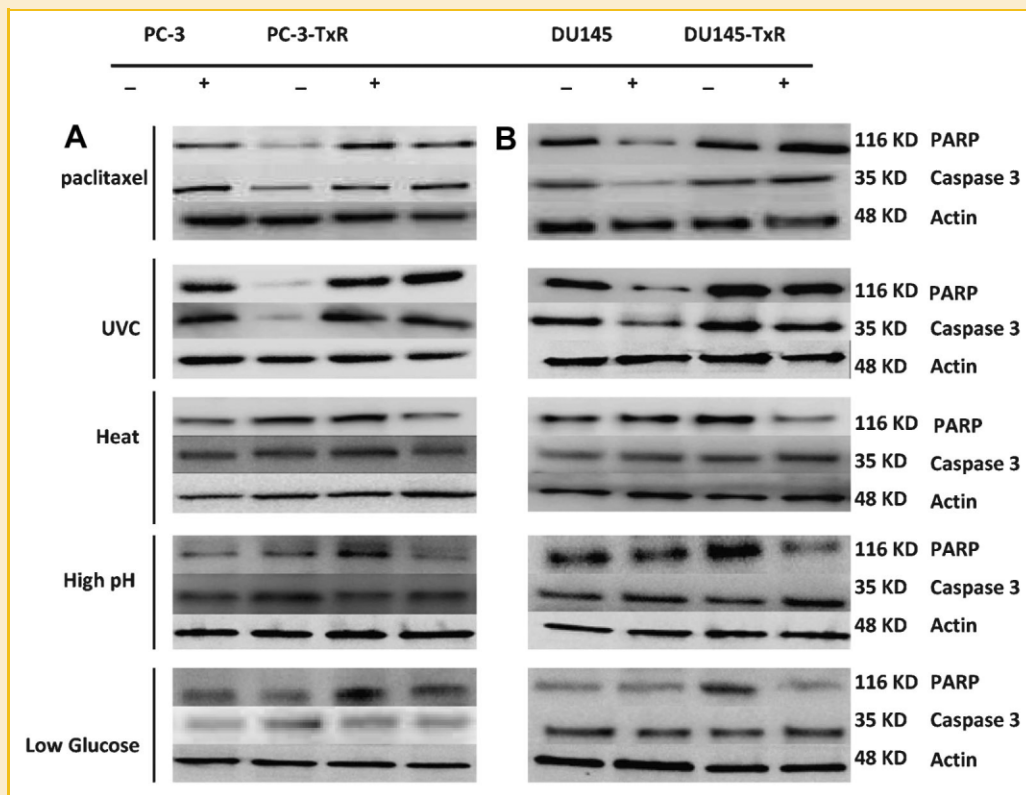

Fig. 4. Apoptotic pathways markers analyzed in total protein from PC-3, PC-3-TxR, DU145, and DU145-TxR cell lines with ($+$) and without exposure ($-$) to different treatment (10 nM paclitaxel, 30 s UVC, 2 h 43°C Heat, $+1$ pH, and 1 mg/ml glucose).
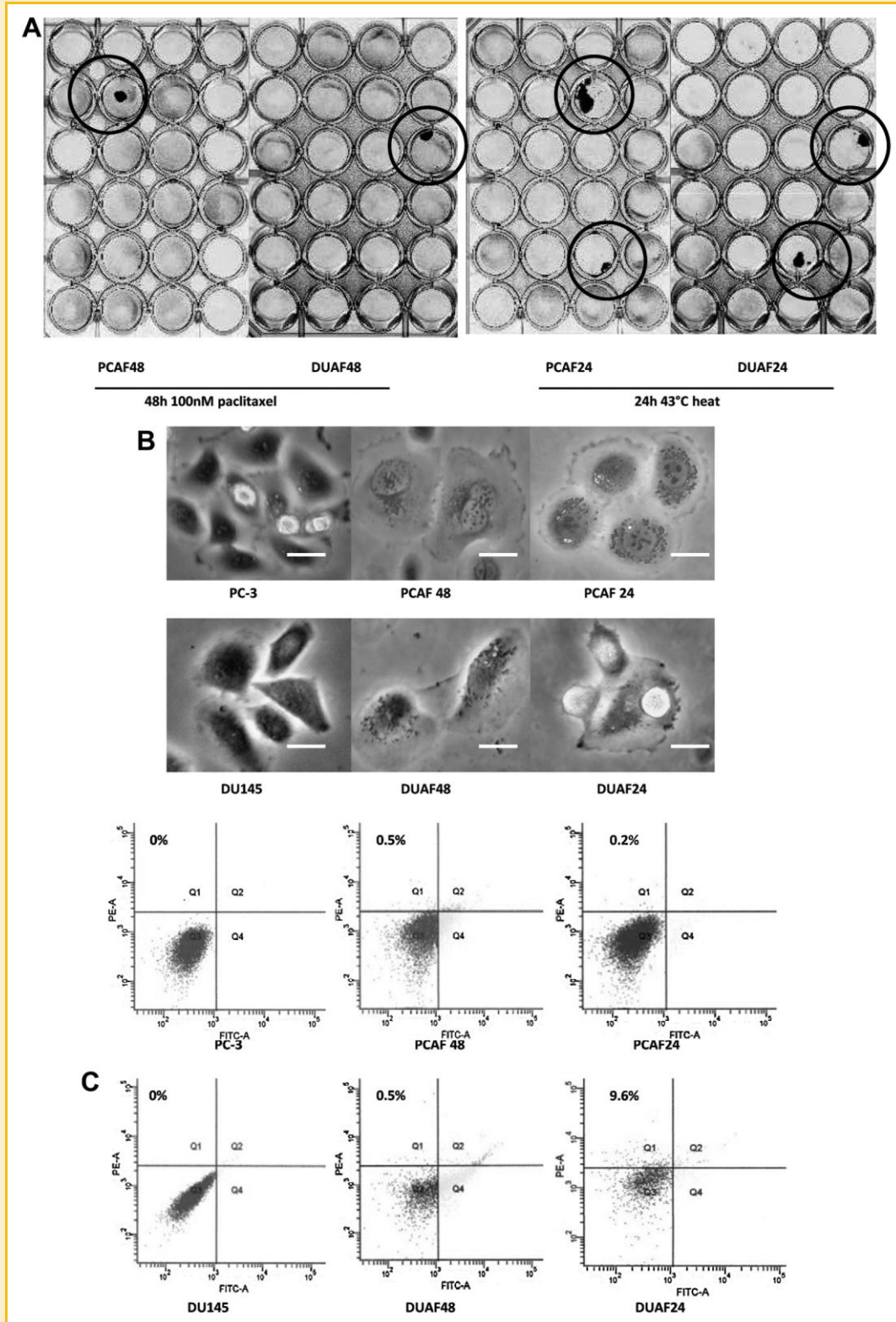
Fig. 5. Cell re-population and modality after acute heat and paclitaxel treatment. A: PC-3 and DU145 cells treated with 24 h heat or 48 h 100 nM paclitaxel, after 30 days recovery, single clones were acquired. B: Microscope picture (×400) of the PC-3, DU145 (third day), and PCAF48, DUAF48, PCAF24 DUAF24 (recovery in 14th day) cells. C: Analysis of the prostate cancer stem cell marker CD133 in PCAF48, DUAF48, PCAF24 DUAF24, and their respective parents cells. Analysis of the CD133 expression on surface of PC-3, DU145, PCAF48, DUAF48, PCAF24 DUAF24 cells. CD133 protein was detected with fluorochrome-conjugated antibody. Flow cytometry was used to sort cells into CD133+ population.
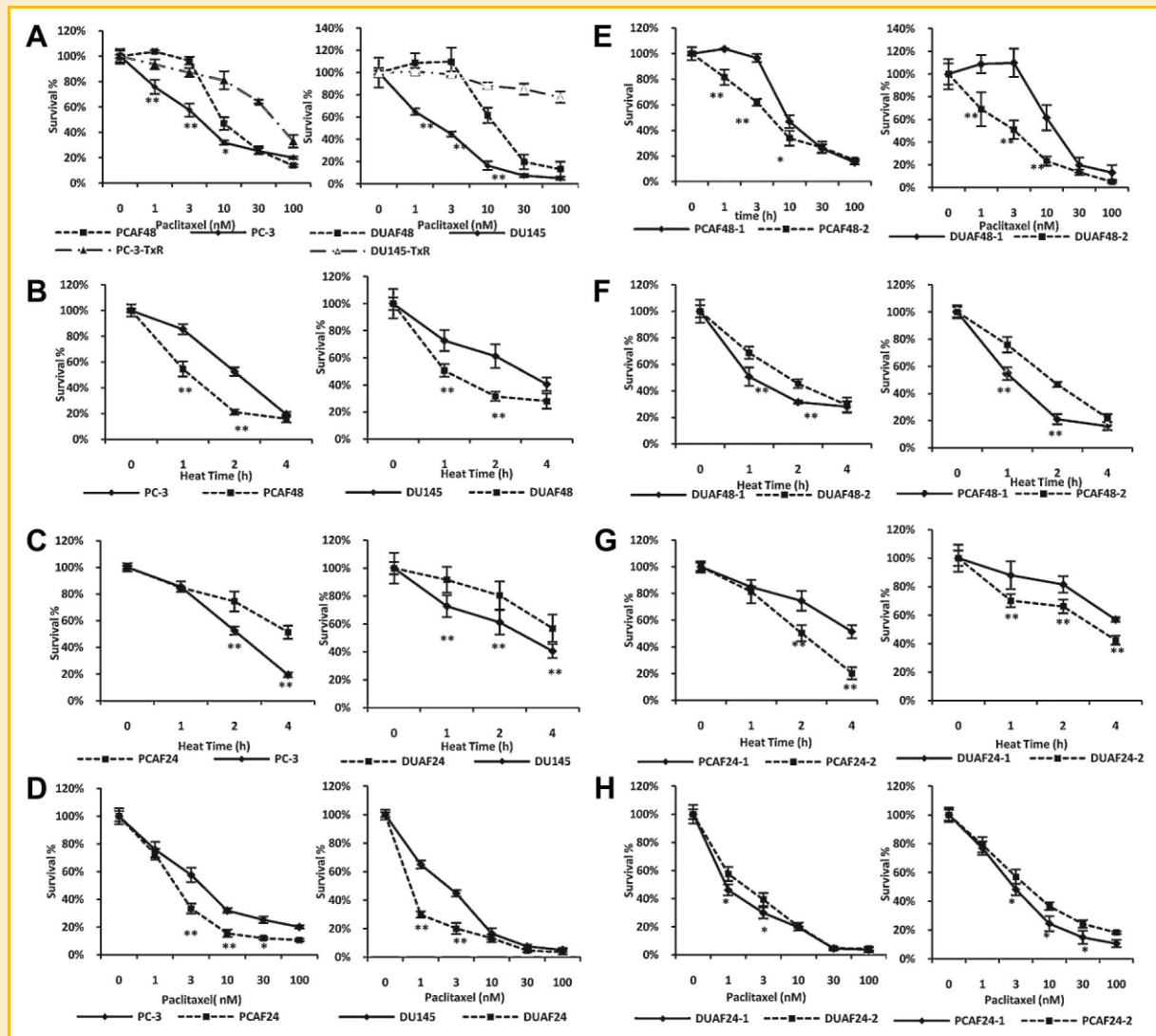
Fig. 6. The re–populated cells developed an inverse response to heat and paclitaxel treatment and the response to heat and paclitaxel in the different recovery times of repopulated cells. A: Determined the paclitaxel resistance in PCAF48 and DUAF48 cell lines by cell survival assay. B: examined the sensitivity to heat treatment in PCAF48 and DUAF48 cells. C: Determined the heat resistance in PCAF24 and DUAF24 cell lines by cell survival assay. D: Examined the sensitivity to paclitaxel treatment in PCAF24 and DUAF24 cells. E,F: After splitting the cells for times, we harvested the PCAF48–2 and DUAF48 cells. We examined the sensitivity to paclitaxel and heat between these paired cell lines as described in Figure 4. G,H: After splitting the PCAF24–1 and DUAF24–1 cells, four times, we harvested the PCAF24–2 and DUAF24–2 cells. We examined the sensitivity to heat and paclitaxel between these paired cell lines as described in Figure 4. $^{*}P < 0.05$, $^{**}P < 0.01$.

pronounced after 4 h of heating (Fig. 7A). In this experiment we also investigated the expression of $NA^+ K^+$-ATPase and CALB1, which were found to be over-expressed in the parental but not in paclitaxel resistant cells after 2 and 4 h of heating (Fig. 7A). This may indicate that these proteins play a role in resistance of the parental cells to heat, a feature that was lost after the cells developed paclitaxel resistance. Protein expression analysis of the cells after treatment with paclitaxel revealed caspase-3-dependent apoptosis in the parental cells but no evidence of apoptosis was present in the resistant cells. The $NA^+ K^+$-ATPase and CALB1 were relatively over-expressed in the parental but down regulated in paclitaxel resistant cell lines (Fig. 5B). Similar protein expression was observed in the

cell lines repopulated after acute stress exposure (Fig. 7C,D). These results suggest the same apoptosis pathways may work in both chronically and acutely resistance developed cell lines.

THE LETHAL EFFECT OF COMBINATION OF HEAT WITH PACLITAXEL IN PROSTATE CANCER CELL LINES

In order to study the synergistic killing effect of heat combined with paclitaxel in PCa cell lines, we treated the cell lines with 43°C for 2 h after exposure to 10 nM paclitaxel for 48 h. When the cells were tested individually with paclitaxel, on the OPCT and LNCap cell lines, it demonstrated the significant killing effects, and no detectable effect on growth in PC-3-TxR, DU145-TxR, PCAF48,
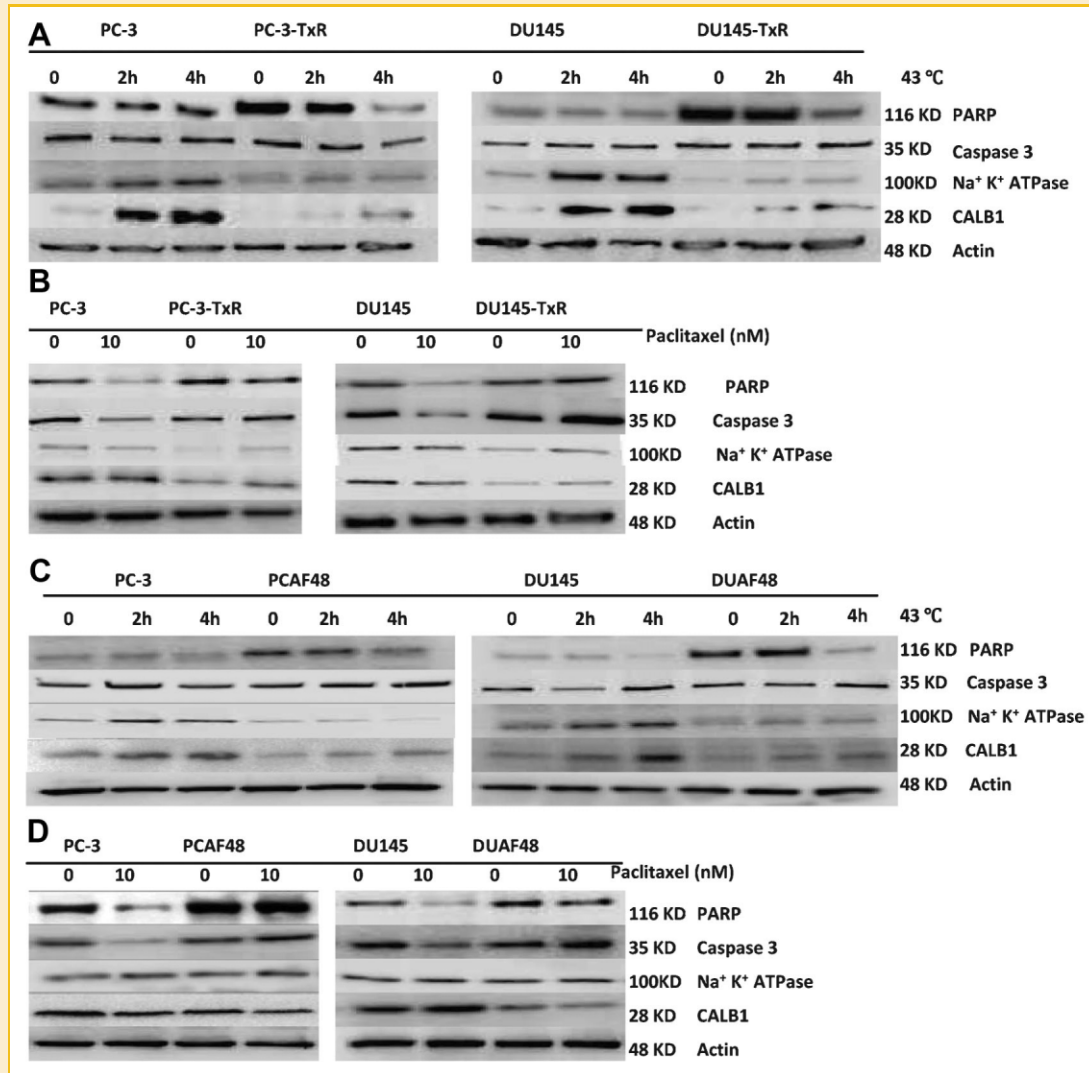
Fig. 7. After heat treatment, Na$^+$ K$^+$–ATPase and calcium binding protein CALB1 are overexpressed in paclitaxel sensitive PC–3 and DU145 but not in paclitaxel resistant PC-3–TxR, DU145-TxR PCAF48, and DUAF48 cell lines. A: Total protein extracted from PC–3, PC–3–TxR, DU145, and DU145–TxR cells after 0, 1, 2, and 4 h exposure to 43°C. B: Total protein extracted from PC–3, PC–3–TxR, DU145, and DU145–TxR cells without treatment or after 10 nM paclitaxel treatment. C: After 0, 2, or 4 h of heating at 43°C. D: Untreated or treated with 10 nM paclitaxel.

and DUAF48 cell lines in the presence of paclitaxel. Importantly the combination of heat with paclitaxel brings out a sensational synergistic lethal effect to all of the PCa cell lines (Fig. 8A).

DISCUSSION

In the current study, we report that resistance to chemotherapy imparts a greater sensitivity to microenvironmental stressors such as heat, basic pH, and low glucose. As a control, normal PrEC were shown to be much less sensitive to these stressful conditions. The collective finding is that the paclitaxel-resistant PCa cell lines, PC-3-TxR, DU145-TxR (stable), and PCAF48, DUAF48 (reversible), have gained resistance to chemotherapy but have in the process become hypersensitive to heat, basic pH, and low glucose. Additionally, there are two types of apoptotic pathways invoked by stress treatments. Paclitaxel and UV stress treatments activated a caspase-3-PARP apoptosis pathway in the paclitaxel sensitive cell lines, PC-3, and DU145, however, the other stresses, induced a caspase-3-independent-PARP apoptotic pathway in the resistant cell lines, PC-3-TxR, DU145-TxR (stable), PCAF48, and DUAF48 (reversible).

As we analyzed the spectrum of PCa as represented by the models used, our findings demonstrate that resistance to one microenvironmental stress appears to result in sensitivity to others. Our study also indicates that PCa cell lines develop resistance to paclitaxel gradually become sensitive to heat treatment. The inverse response between thermal- and chemo-therapies suggest that combining stresses such as heat with currently used therapy may provide an opportunity for more efficacious use of these available approaches.

Since cancers are heterogeneous [Pardal et al., 2003], our analysis implicates two kinds of drug resistance that can be established in PCa cell lines; one is a reversible drug resistance resulting from acute
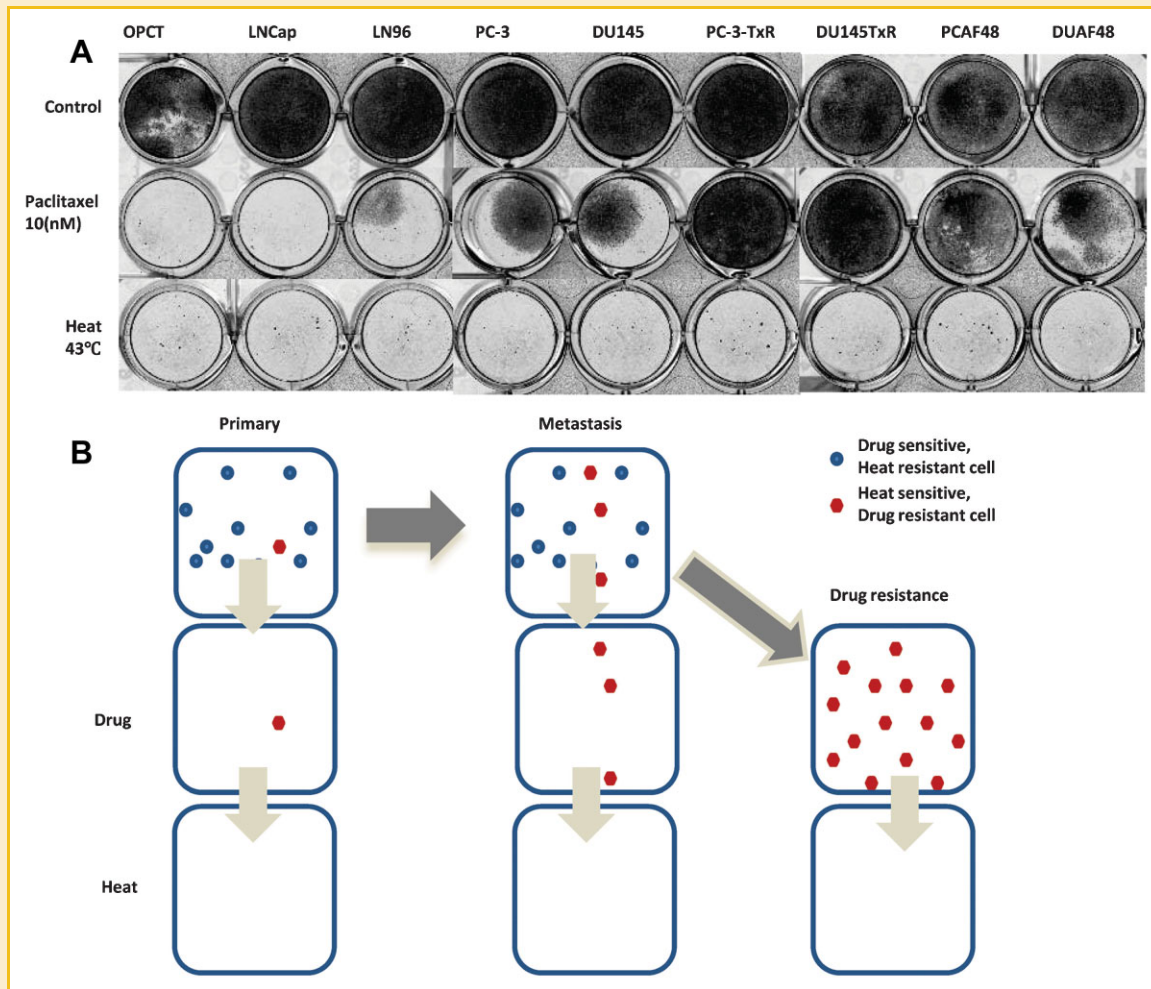
Fig. 8. Combination of paclitaxel with heat activated the cancer killing effect. A: The cell lines were either untreated or treated with paclitaxel (10 nM) for 48 h, then plates had reached confluence and were stained with 0.5% typan blue. The paclitaxel treated plates were treated with 2 h of heat treatment or untreated. After 10 days, the untreated plates had reached confluence and were stained with 0.5% typan blue. The experiment was performed in triplicate and representative stained plates are shown. B: A graphical image of the effect of paclitaxel combined with heat therapy.

paclitaxel treatment, the other is a stable drug resistance caused by chronic paclitaxel treatment. An acute stress might spare tissue stem cells but injure other cell types. It has been demonstrated that in a chronic stress, there appears to be a more pronounced and ongoing influence on cell renewal systems with injury to all kinds of cell types, including stem cells [Johnstone and Baylin, 2010]. Considering reported links between drug resistance and a 'cancer stem cell' population, we examined CSC markers. The putative CSC marker CD133 was not highly expressed after a long recovery period from either acute or chronic exposures. The relationship between the reversibly drug resistant subpopulation and cancer stem cell is potentially complex [Sharma et al., 2010]. In the acute drug treatments, it was shown that drug resistant cancer cells behave similarly and that all of the tumor cells in a population potentially have the ability to stochastically acquire and relinquish this protective phenotype at a low frequency [Sharma et al., 2010]. In the case of resistance after chronic drug treatment, tumor cells surviving have acquired genetic resistance due to secondary mutations

[Dannenberg and Berns, 2010]. In the current study, both drug resistant (reversible and stable) cell lines developed by acute or chronic paclitaxel treatments, respectively, showed an increased sensitivity to heat treatment. These results suggest that the repopulation maybe share a common characteristic by stress-induced, acute and chronic treatment, cellular adaptation. Regardless of which mechanism causes drug-resistance in vivo, thermal therapy may provide an operative way to treat drug-resistant PCa.

Our findings also showed that after heat treatment, NA^+ K^+-ATPase and calcium binding protein CALB1 were up-regulated in both PC-3 and DU145 cell lines, thereby providing them with a mechanism to escape from this apoptotic pathway that is distinct from the PC-3-TxR and DU145-TxR cell lines. Cell ion channel proteins play an important role in cancer apoptosis, intracellular Ca^{2+} and Na^+/K^+ levels are involved in regulating the cell microenvironmental pH value, and in regulating the induction of apoptosis [Furuya et al., 1994; Mijatovic et al., 2009; Simpson et al., 2009]. The NA^+ K^+-ATPase helps maintain resting

potential by active transport, and also functions as a signal transducer/integrator to regulate the MAPK pathway, reactive oxygen species (ROS), as well as intracellular calcium, and regulates cellular volume. For most animal cells it is responsible for 1/3 of the cell's energy expenditure [Wilson, 1954; Leaf, 1956; Civan, 2010]. Furthermore, changes to the microenvironmental levels of ions such as Na^+, K^+, and Ca^{2+}, may alter sensitivity to heat and chemotherapy. Implementation of stressors inducing alternative apoptosis pathways might be beneficial way to prevent the development of therapeutic resistance and post-treatment relapse of PCa.

In summary, the ability of cancer cells to become resistant (reversible and stable) to therapies such as chemotherapy appears to make them vulnerable to stresses in their environment; however, normal cells are much less sensitive to stressful conditions such as heat. In lieu of current therapy, increasing drug resistance causes an increased sensitivity to heat. Therefore combining stresses such as heat with currently used therapies may provide an opportunity for the more efficacious use of these available approaches (Fig. 8B).

Recently new noninvasive nanoparticle-mediated approaches have been reported opening new possibilities of application of different treatments in combination [Coffey et al., 2006; Chopra et al., 2009; Krishnan et al., 2010]. Future therapeutic strategies will undoubtly focus on integrating microenvironmental stresses with current chemotherapy and radiation [O'Neal et al., 2004; McTaggart and Dupuy, 2007]. Thermal therapies offer the advantage of flexible, low cost treatment approaches through percutaneous, laparoscopic, and open surgical access. Thermal therapies are also minimally invasive outpatient procedures with repeatability and reproducibility of results between patients [Carrafiello et al., 2008]. Taken together, thermal therapy may be a cost effective approach for the treatment of chemotherapy resistant PCa. Accumulating evidence indicates that treatment plays a very important role in cancer cell evolution [Collingridge et al., 1999; Becker et al., 2000; Vaupel et al., 2001; Vaupel et al., 2003].

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