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Mitochondrial DNA depletion reduces PARP-1 levels and promotes progression of the neoplastic phenotype in prostate carcinoma

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Abstract. Mitochondrial dysfunction resulting from mitochondrial DNA (mtDNA) mutations and/or depletion has been correlated with cancer progression and drug resistance. To investigate the role of mtDNA in prostate cancer progression, we used LNCaP and PC-3 prostate carcinoma cells as experimental model. Compared to minimally invasive androgen-dependent LNCaP cells, highly invasive androgen-independent PC-3 cells, as well as androgen-independent DU145 and C4-2 cells, exhibited significantly reduced mtDNA content. In PC-3 cells, reduction of mtDNA was accompanied by decreased mitochondrial membrane potential ($\Delta\Psi_m$), increased migration onto the basement membrane protein laminin-1, reduced chemosensitivity to paclitaxel (IC_{50} = 110 nM vs. 22 nM) and decreased expression of poly(ADP-ribose) polymerase (PARP)-1. To investigate the relationship between mtDNA depletion and these phenotypic characteristics, we established mtDNA-depleted LNCaP cells [Rho(−)] by long-term exposure to ethidium bromide or treated wild-type LNCaP cells with a mitochondrial ionophore, carbonyl cyanide m-chlorophenylhydrazone. Both manipulations resulted in $\Delta\Psi_m$ loss, acquisition of invasive cytology, increased motility onto laminin-1, reduced sensitivity to paclitaxel (IC_{50} = ∼100 nM) and ∼75% reduction in PARP-1 protein levels, resembling PC-3 cells. Overall, these results provide novel evidence demonstrating that mtDNA depletion in early prostate carcinoma may contribute to the acquisition of a more invasive phenotype that is less sensitive to paclitaxel-induced apoptosis.

Keywords: Prostate cancer, mitochondria, PARP-1, invasion, paclitaxel

1. Introduction

Prostate cancer is a leading cause of morbidity and mortality among men in the Western world. Despite increased awareness and improved methods for early detection, a large proportion of patients succumb to disseminated cancer that is resistant to conventional therapies [67]. Therapeutic approaches are mostly palliative and involve androgen ablation, which prevents androgen-dependent cancer cell growth by predisposing cancer cells to enter an apoptotic pathway [37]. However, tumor clones that evade apoptotic death and thrive in absence of androgens (androgen independency) normally arise after a 2–3 year period [67]. Furthermore, the biological processes resulting in androgen-independent tumor growth also reduce the effectiveness of conventional chemotherapeutic agents, at least partly explained by the fact that apoptosis, the predominant form of cell death triggered by many chemotherapeutic agents, is decreased in advanced disease [66,67].

Paclitaxel, a natural antitumoral product obtained from the bark of *Taxus brevifolia* [69], is currently used in combination with other chemotherapeutic agents for the treatment of numerous types of tumors, including hormone-refractory prostate cancer [17,45]. Its antineoplastic effect is significant at nanomolar concentrations at which few molecules bound to β-tubulin in the microtubules are sufficient to suppress their dynamics thus interfering with cell mitosis [34], ultimately inducing Bcl-2 phosphorylation at the inner mitochondrial membrane [24] and cell death by apoptosis

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through release of cytochrome c and caspase activation [57]. The effector caspases (caspase-3, -6 and -7) carry out the apoptotic process by mediating the enzymatic cleavage of a wide range of substrates including poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) [18]. PARP-1 is a nuclear enzyme that is induced in response to DNA damage whose main role is to catalyze NAD^+-dependent synthesis of ADP-ribose polymers onto several DNA-binding proteins to facilitate base excision repair of DNA single-strand breaks [20]. ADP-ribose polymerization seems to facilitate the recruitment of repair enzymes to damage sites, as well as eliciting structural chromatin changes required for the repair to occur [20]. PARP-1 cleavage by caspase-3 occurs at an early stage during apoptosis. Hence, the detection of an 85-kDa-degradation product is widely used as a marker of apoptosis induced by several agents, including paclitaxel [31]. Despite recent promising data on the use of combined paclitaxel therapy for prostate cancer treatment [17,41] androgen-independent disease and drug tolerance remain the main obstacles to improving the survival and quality of life in patients with advanced prostate cancer and elucidation of the molecular mechanisms contributing to prostate cancer progression and apoptosis resistance to rationally develop more effective strategies for treating advanced disease is required.

Growing evidence suggests that mitochondrial defects may play an important role in drug chemosensitivity and metastatic progression of a variety of cancers, including prostate carcinoma [49,56]. Mitochondria are complex cellular organelles involved in vital cellular tasks such as energy production, generation of reactive oxygen species (ROS) and apoptosis, all of which have been postulated to play a role in the initiation and/or perpetuation of the malignant phenotype [19]. They are endowed with their own genome consisting of a 16.6 kb circular DNA (mtDNA) that encodes for 13 of the 87 polypeptides of the respiratory chain, 22 tRNAs and 2 rRNAs. Mutations of mtDNA have been reported in various types of cancer including breast cancer, colon carcinoma, pancreatic cancer and prostate carcinoma [16,32,33,58]. In prostate cancer, Chen et al. [16] found an extremely high incidence of somatic mutations (90% of prostatectomy cancer specimens) in the control region (the displacement loop, D-loop) of mtDNA. In addition, accumulation of large-deletion mutant mtDNA has been reported in the malignant prostate of 34/34 patients analyzed, with the number of deletions directly correlated with the age of the patients [33]. Reduced mtDNA content in renal cancer [61], hepatocellular carcinoma [40,74], gastric cancer [71] and breast carcinoma [44] has also been reported. Recently, Booker et al. [10] have demonstrated that the inheritance of mitochondrial haplotype U is associated with increased risk of prostate and renal cancer in white North America individuals, thus definitely implicating mtDNA in cancer etiology.

Two experimental strategies have been employed to study the contribution of mitochondria in cancer initiation and progression. The first is based on the generation of mitochondrial genetic stress by depleting cultured cells of mtDNA. mtDNA-less [Rho(0)] cells, i.e., cells devoid of mtDNA molecules, or mtDNA-depleted [Rho($-$)] cells are developed following long-term treatment with ethidium bromide (EtBr), a well-known inhibitor of mtDNA replication and transcription [54]. EtBr intercalates into mitochondrial DNA and RNA and nucleolar ribosomal RNA but insignificantly within the chromatin network [21]. In turn, depletion of mtDNA induces mitochondrial dysfunction resulting in disruption of the mitochondrial membrane potential ($\Delta\Psi_m$) and reduced ATP levels [6]. The second approach is based on the direct generation of mitochondrial metabolic stress, by treating cultured cells with mitochondrial ionophores, such as CCCP, which collapse the $\Delta\Psi_m$ [3,4,6]. Avadhani and colleagues [4,8] have demonstrated that disruption of $\Delta\Psi_m$ due to either genetic or metabolic mitochondrial stress caused a sustained increase in cytosolic free Ca^{2+} and activation of Ca^{2+}-sensitive transcription factors in murine skeletal myoblasts C2C12 cells and A549 lung carcinoma cells, leading to acquisition of invasive phenotypes and overexpression of the tumor-specific markers cathepsin L and transforming growth factor β, indicating that the loss of mtDNA could contribute to tumor progression and metastasis. mtDNA depletion induced also resistance to apoptosis in C2C12 and A549 cells [3,4], 143B osteosarcoma cells [22], SK-Hep1 cells [36], ML-1a myelogenous leukemia cells [26], HeLa cells [65] and intestinal epithelial cells [59]. In addition, a recent study has demonstrated that mtDNA depletion in the LNCaP androgen-dependent prostate carcinoma cells promotes resistance to apoptosis induced by androgen ablation resulting in acquisition of an androgen-independent phenotype [27]. In mtDNA-depleted C2C12 and A549 cells, resistance to apoptosis was associated with higher expression of the antiapoptotic protein Bcl-2 [7]. It is thus likely that mtDNA mutations or depletion could promote progression and metastasis of cancer cells by preventing apop-

tosis and modulating the expression of cancer-related proteins.

In this study, we investigated the role that mtDNA may play upon the transition from low metastatic/drug sensitive androgen-dependent to highly metastatic/drug tolerant androgen-independent prostate cancer cells. Our results demonstrate that mtDNA depletion in early prostate carcinoma contributes to the acquisition of a more invasive phenotype that is less sensitive to paclitaxel-induced apoptosis, at least in part through down-regulation of PARP-1 protein levels.

2. Material and methods

2.1. Cell lines and culture

LNCaP (clone FGC) is an androgen-dependent, low metastatic prostate carcinoma cell line [30]. PC-3 cells derive from an androgen-independent, highly invasive carcinoma metastatic to the bone [35]. They were both grown in DMEM-high glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 1 mM pyruvate. DU145 cells, derived from a brain lesion of a patient with metastatic prostate carcinoma (kind gift of Dr. J.T. Hsieh, UT Southwestern Medical Center, Dallas, TX), were grown in RPMI supplemented with 10% FBS, 2 mM glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 1 mM pyruvate. C4-2 (kind gift of Dr. J.T. Hsieh) is an androgen-independent, highly tumorigenic and metastatic prostate cancer cell line established by inoculating the androgen-dependent LNCaP cells into castrated mice [72]. They were grown in T-medium supplemented with 5% FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin. To induce genetic mitochondrial defects, mtDNA was depleted from LNCaP cells using an established method with EtBr [38]. After 20 days of growth in the presence of EtBr (100 ng/ml), cells were screened for mtDNA content by PCR and maintained in the growth medium supplemented with 50 μg/ml uridine. To ensure steady mtDNA levels, aliquots from the same cell isolate were used in all experiments. To induce metabolic mitochondrial defects, wild-type LNCaP cells were treated with the mitochondrial ionophore CCCP (25 μM, Sigma) for 12 h.

2.2. PCR analysis of mtDNA

Total DNA containing nuclear and mitochondrial DNA was isolated from 1×10^6 cells using the NucleoSpin kit (Macherey-Nagel Gmb, Germany), according to the manufacturer's instructions. Different regions of the mitochondrial genome were amplified under standard polymerase chain reaction (PCR) conditions using mtDNA-specific primers (Fig. 1(a)). Amplifications were performed in 50 μl reactions comprising of 50 ng DNA, 0.1 mM dNTPs (Roche, Indianapolis, IN, USA), 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.5 μM each primer (Operon Biotechnologies GmbH, Cologne, Germany), 2.5 U Taq polymerase (Roche) and nuclease-free H_2O (Roche). Primer sequences were as follows: 5′CACCCTATTAACCACTCACG3′ sense and 5′TGAGATTAGTAGTATGGGAG3′ antisense for D-loop (# 15-484), 5′TGGAGCCTCCGTAGACCTAA3′ sense and 5′TCCGAAGCCTGGTAGGATAA3′ antisense for cytochrome oxidase I (# 6321-6660; Cox I), 5′ATCCTACCTCCATCGCTAAC3′ sense and 5′AGCCTTCTCCTATTTATGGG3′ antisense for NADH dehydrogenase subunit 6 (# 14371-14610; ND6). Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were previously described [50]. PCR reactions were amplified in a Perkin Elmer CETUS PCR System (Perkin Elmer Life Sciences) using the following cycling protocol, where X represents the annealing temperature: 94°C for 3 min, X°C for 3 min, 72°C for 1 min (first cycle) and 94°C for 1 min, X°C for 1 min, 72°C for 1 min for 35 cycles. The final cycle was modified to allow for a 10 min extension at 72°C. Annealing temperatures used were as follows: 46°C for D-loop, 55°C for Cox I, ND6 and GAPDH. The PCR products were electrophoresed on a 1.5% agarose gel stained with EtBr and photographed under UV light.

2.3. Real-time PCR

Real-time PCR was carried out in an Applied Biosystems Inc. (ABI) Prism 7000 Sequence Detection using the SYBR-Green PCR Master Mix (Qiagen, Valencia, CA, USA). Primers for two mtDNA markers (D-loop and Cox II) and a nuclear DNA marker (β-actin) were used as described by Chen et al. [15]. Amplifications were performed in 25 μl reactions comprising of 15 ng total genomic DNA, 1X SYBR-Green PCR Master Mix (Qiagen) with 3.5 mM $MgCl_2$ and 0.3 μM each primer (Operon Biotechnologies GmbH). Triplicate reactions were performed for each marker in a 96-well plate using a two-step amplification program of initial denaturation at 95°C for 15 min, followed by 35 cycles of 94°C for 20 s and 61°C for 30 s. Standard curves were generated from each experimental plate

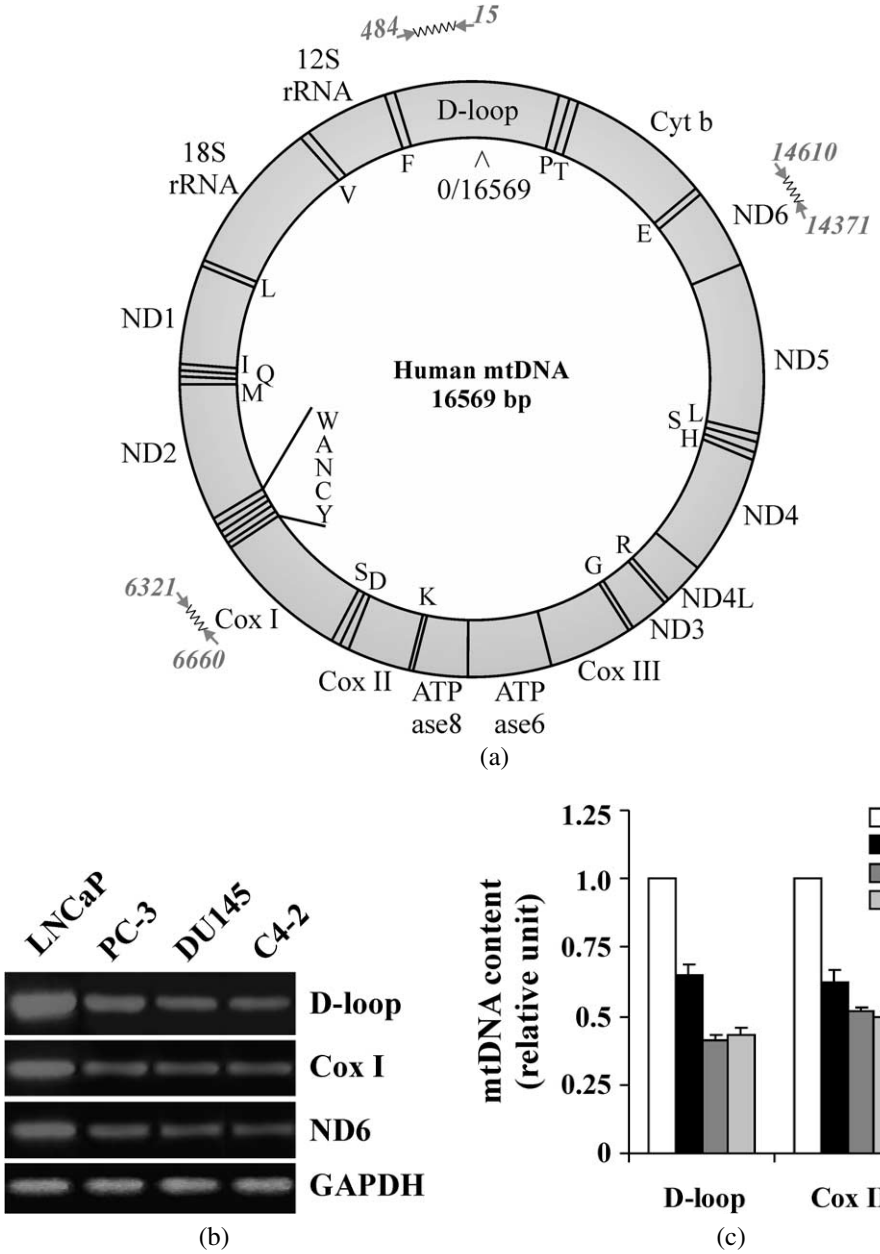
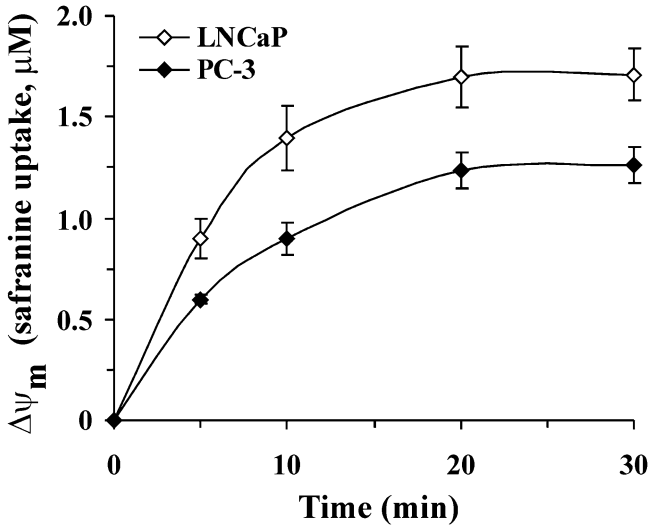


Fig. 1. mtDNA content and $\Delta\Psi_m$ in LNCaP and PC-3 cells. (a) Human mtDNA (16569 bp) is a double-stranded circular genome that encodes for 13 proteins of the respiratory chain, 2 rRNAs (12S and 16S) and 22 tRNAs required for intramitochondrial protein synthesis. ND1–ND6 and ND4L encode seven complex I (NADH–ubiquinone oxidoreductase) subunits, Cyt b encodes one subunit of complex III (ubiquinol:cytochrome c oxidoreductase), Cox I–Cox III encode the three major catalytic subunits of complex IV and ATPase6 and ATPase8 encode two subunits of complex V (ATP synthase). The D-loop (or control region) contains regulatory elements for mtDNA transcription and replication. Arrows indicate the primers for Cox I, ND6 and D-loop regions used to analyze the mtDNA content by PCR. (b) To analyze the mtDNA content in LNCaP, PC-3, DU145 and C4-2 cells, 10 ng total DNA/sample were subjected to PCR using primers for three different mtDNA regions, i.e. D-loop, ND6 and Cox I. Amplification products of the nuclear-encoded gene GAPDH were used as control. PCR products were analysed by 1.5% agarose gel stained with EtBr. (c) The mtDNA content was quantitated using real-time PCR. Two mtDNA markers (D-loop and Cox II) and a nuclear DNA marker (β-actin) were used. The relative amplification of mtDNA markers in PC-3, DU145 and C4-2 versus LNCaP cells was calculated upon normalization to the reference β-actin as described in Section 2. Values are the mean $\pm$ S.E. of three independent experiments.



(d)

Fig. 1. (*Continued*). (d) To evaluate changes in $\Delta\Psi_m$, 1×10^6 LNCaP and PC-3 cells were incubated for 30 min at 37°C. At time zero, 20 μM safranine was added and the safranine concentration in the cells was measured following the absorbance decrease of the outer medium at 524 nm. Data are reported as mean ± S.E. of three different experiments.

using serial 5-fold dilutions of genomic DNA. The authenticity of the PCR products was verified by agarose gel electrophoresis. The comparative cycle threshold (C_t) method was used to analyze the data by generating relative values of the amount of mtDNA. Relative quantitation (R_c) of mtDNA was calculated after determination of the difference between C_t of the given mtDNA marker A and that of the calibrator nuclear marker B (β-actin) in PC-3, DU145, C4-2 cells or EtBr-treated LNCaP cells ($\Delta C_{t1} = C_{t1A} - C_{tB}$) and control LNCaP cells ($\Delta C_{t0} = C_{t0A} - C_{tB}$), using the $R_c = 2^{-\Delta\Delta Ct(1-0)}$ formula [28]. Experiments were repeated three times.

2.4. Analysis of mitochondrial membrane potential ($\Delta\Psi_m$)

Changes in $\Delta\Psi_m$ in prostate cancer cells were monitored spectrophotometrically, using the cationic probe safranine as previously described [23]. Briefly, cells (1×10^6 cell/ml) were suspended in a medium containing 140 mM NaCl, 6 mM $MgCl_2$, 20 mM Tris, 10 mM HEPES, pH 7.4, added with 20 μM safranine and incubated at 37°C for 30 minutes. Aliquots (200 μl) were withdrawn and centrifuged at 13,000*g* for 1 minute. Safranine uptake by mitochondria within the cells was measured by following the absorbance decrease in the supernatant at 524 nm by means of a Perkin–Elmer λ3B UV/VIS Spectrophotometer.

2.5. Cell migration assay

Cells (1×10^5) were seeded on 8-μm (PC-3) or 12-μm (LNCaP) pore-sized transwell Boyden chamber (Corning Costar) coated overnight at 4°C with 10 μg/ml laminin-1 (LN-1). After 12 h at 37°C, migrated cells were fixed with 3% paraformaldehyde, stained with crystal violet and the number of cells per square millimeter on the bottom was counted (average and S.E. of 10 random fields). Each experiment was performed in duplicate.

2.6. Growth inhibition assays

Cells were plated at a density of 1×10^4/well in 96-well plates and cultured for 24 h in presence or absence of 25 μM CCCP in the last 12 h. Media were then changed to growth media containing paclitaxel (1×10^{-9} to 1×10^{-7} M; Sigma). Cells from three wells at each time point were washed once with PBS and analyzed by either crystal violet (Sigma) or 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) staining. Viable cells normally uptake and reduce MTT in the mitochondria to a magenta derivative, which is readily measured by light absorbance yielding an accurate estimation of viability and cell proliferation. For crystal violet assay, cells were washed twice with PBS and then stained with 0.2% crystal violet in 20% ethanol for

5 min. Wells were then rinsed with PBS and 0.1 ml of H_2O:methanol:ethanol (5:1:4) solution was added to each well to solubilize the cells. The absorbance of each well, which is proportional to the number of attached cells, was recorded at 630 nm in an ELISA reader. For the MTT assay, 10 µl of MTT (5 mg/ml) were added to each well and plates were incubated at 37°C for 2 hours. The absorbance of each well was recorded at 450 nm in an ELISA reader. IC_{50} was defined as the paclitaxel concentration that inhibited cell growth by 50% compared with control cells.

2.7. Western blotting

To analyze PARP-1 expression and cleavage, cells were washed twice in cold PBS and lysed in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Triton X100, 1% Na deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1 mM dithiothreitol, 5 mM Na orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 38 µg/ml aprotinin, frozen at −80°C for 12 h, then sonicated for 10 seconds and centrifuged at 14,000 × g for 20 min at 4°C. The protein content of each lysate was quantified using the Bio-Rad Dc protein assay reagent according to the manufacturer's protocol. Protein extracts (100 µg) were electrophoresed on 10% SDS-polyacrylamide gel under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Immunoblotting was performed as previously described [52] using a 1:500 dilution of monoclonal antibody to PARP-1 (Ab-2, Calbiochem) or to tubulin (Sigma).

2.8. Statistical analysis

Data are reported as the mean ± S.E. Statistical analysis was performed by the Student's t test. All experiments were repeated at least twice.

3. Results

3.1. mtDNA content and $\Delta\Psi_m$ in LNCaP and PC-3 cells

Depletion of mtDNA has been implicated in the acquisition of androgen-independency in prostate cancer [27]. We thus hypothesized that in comparison with LNCaP androgen-independent PC-3, DU145 and C4-2 cells must have suffered pronounced reduction of the mtDNA content as part of the natural history to becoming poorly differentiated androgen-independent carcinoma cells. Hence, we analyzed the mtDNA content of these cell lines by PCR, using primers specific for three different regions of the mtDNA (Fig. 1(a)). As shown in Fig. 1(b), in comparison with LNCaP, the signal for all three mtDNA regions in PC-3, DU145 and C4-2 cells was noticeably reduced, index of reduced mtDNA content. We then performed real-time PCR analysis to quantify the differences in mtDNA content. As shown in Fig. 1(c), the mtDNA content was reduced by 35–38% in PC-3 ($p < 0.01$), 48–59% in DU145 ($p < 0.001$) and 40–47% in C4-2 cells ($p < 0.001$).

To explore the role of mtDNA depletion in prostate cancer progression, we focused our attention on PC-3 cells as model of advanced androgen-independent prostate cancer. In PC-3 cells mtDNA depletion resulted in mitochondrial dysfunction as revealed by measurement of $\Delta\Psi_m$ using the safranine method [2]. As shown in Fig. 1(d), PC-3 cells exhibited reduced safranine uptake as compared with LNCaP cells, demonstrating reduced $\Delta\Psi_m$.

3.2. Migration and sensitivity to paclitaxel in LNCaP and PC-3 cells

To investigate whether mtDNA depletion was associated with the metastatic potential of the two cell lines, we measured their migratory properties onto LN-1, an extracellular matrix protein enriched at the basement membranes [51]. As depicted in Fig. 2(a), PC-3 cells migrated more efficiently onto LN-1 than LNCaP cells, in agreement with their higher metastatic capability. We then analyzed the cellular chemosensitivity to the anticancer drug paclitaxel. LNCaP and PC-3 cells were treated with increasing concentrations of paclitaxel and cell viability was measured using the MTT assay (Fig. 2(b)–(c)). Both cell lines were inhibited in a dose- and time-dependent manner. However, when compared to LNCaP, PC-3 cells required significantly higher concentrations (IC_{50} of 110 nM vs. 22 nM, Fig. 2(b)) or longer exposure (∼55 h vs. ∼26 h, Fig. 2(c)) to achieve similar levels of inhibition. Measurements of cell viability using crystal violet assays gave similar results (data not shown).

3.3. PARP-1 expression is reduced in PC-3 cells

Paclitaxel acts as antineoplastic drug by promoting apoptosis [57]. Production of an 85 kDa PARP-1 degradation product is considered a good indicator of

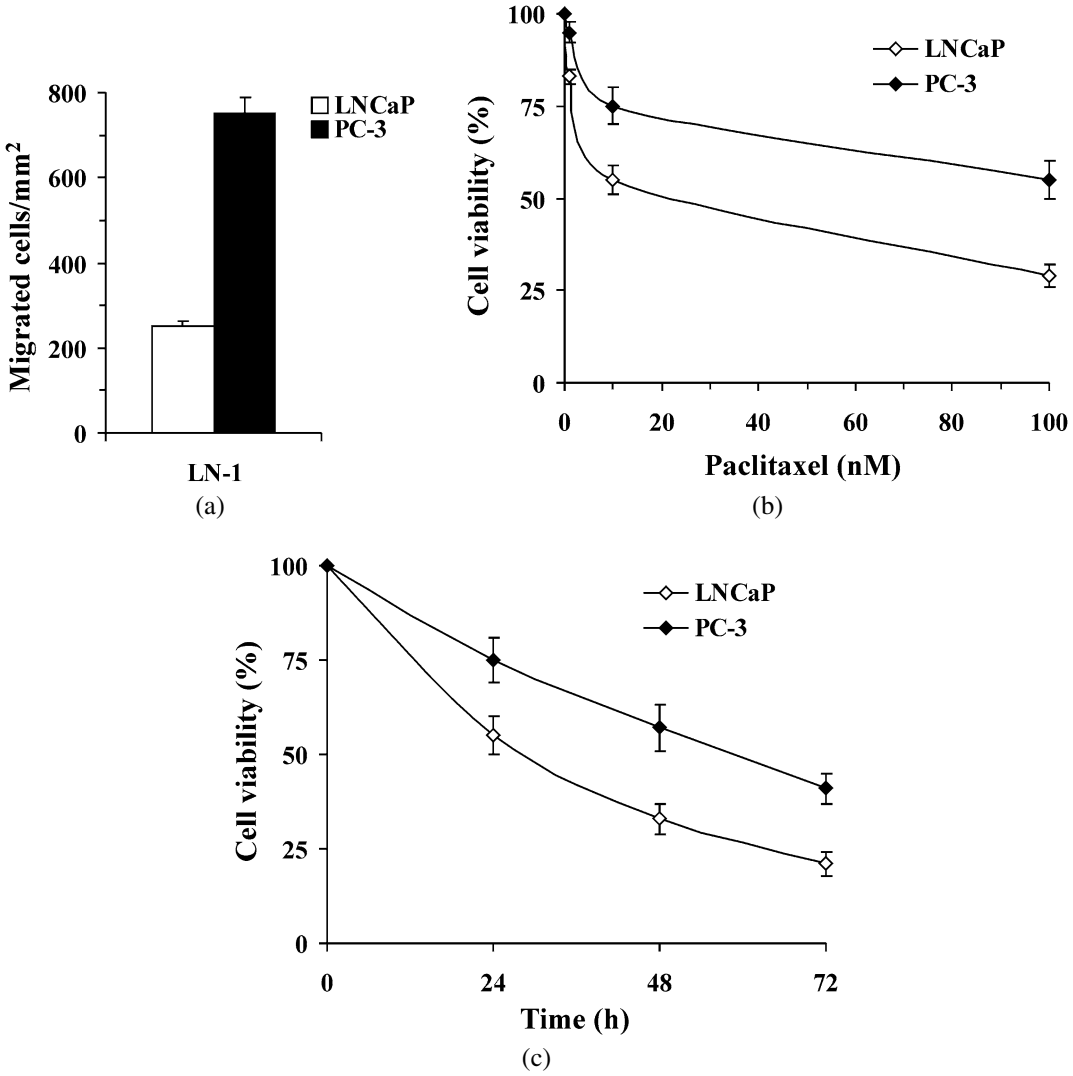


Fig. 2. Migration and chemosensitivity to paclitaxel in LNCaP and PC-3 cells. (a) LNCaP and PC-3 cell migration was assessed by plating 1.0×10^5 cells onto transwell insert filters coated with 10 μg/ml LN-1. After 12 h, cells on the bottom of the filter were fixed, stained with crystal violet and counted. Measurements represent mean ± S.E. of 10 random fields. A representative experiment of two is shown. (b) Shows the dose-response curves of LNCaP and PC-3 cells after exposure to paclitaxel at three different concentrations (1 nM, 10 nM and 100 nM) for 24 h. Viable cells were measured by MTT assay and expressed as a percentage of the controls. (c) Shows the time-response curves of LNCaP and PC-3 cell lines treated with 10 nM paclitaxel for 24, 48 and 72 h. Data represent mean ± S.E. of three independent experiments.

apoptosis onset [11], thus we also analyzed the effect of paclitaxel treatment by Western blotting using an antibody that recognized both the intact molecule (116 kDa) and the cleaved fragment of PARP-1. As shown in Fig. 3, the 85-kDa fragment was present in LNCaP and PC-3 cells after 24 h treatment with paclitaxel, confirming occurrence of apoptosis in both cell types, but it was approximately 5-fold less in PC-3 than in LNCaP cells. We noticed that also the 116 kDa full-length PARP-1 was reduced in PC-3 vs. LNCaP cells. Thus, we measured the relative amount of cleaved vs. total PARP-1 and found it was decreased in PC-3 as compared with LNCaP cells, being 29% ± 2 in the first and 40% ± 3 in the latter ($p < 0.01$), confirming reduced drug-induced apoptosis in PC-3 cells. The lower expression of PARP-1 in PC-3 cells was not due to a differential effect of paclitaxel in the two cell lines since in the absence of apoptotic stimuli PARP-1 baseline levels were also reduced by 71% ± 3 in PC-3 vs. LNCaP cells ($p < 0.0001$; Fig. 3).

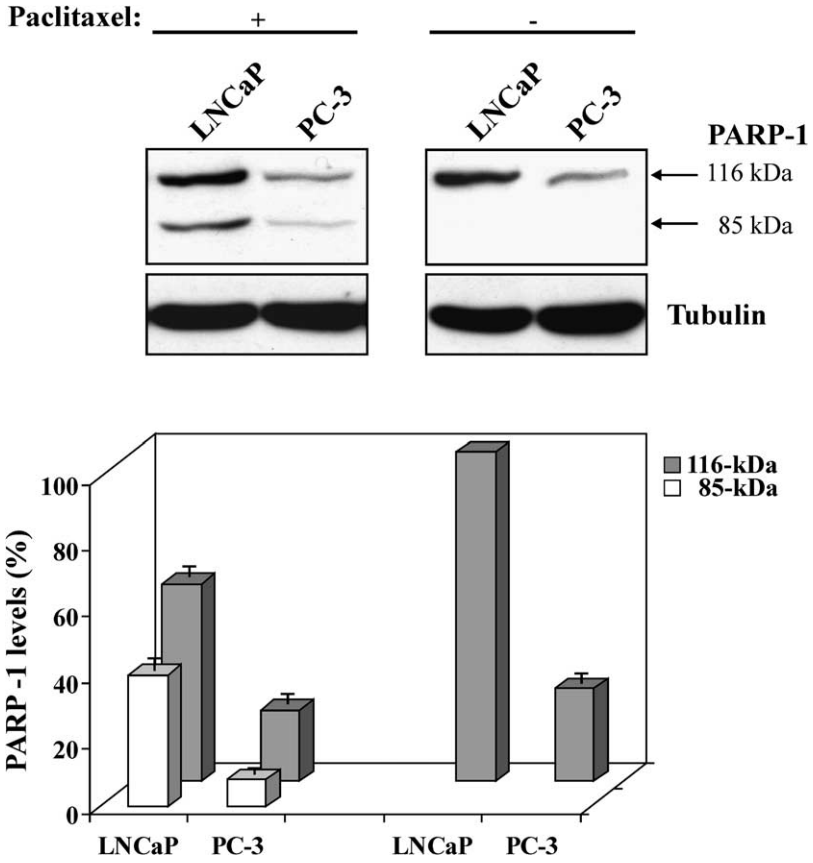


Fig. 3. PARP-1 expression is reduced in androgen-independent PC-3 prostate cancer cells. PARP-1 cleavage in LNCaP and PC-3 cells treated for 24 h with 10 nM paclitaxel was measured by Western blotting using a monoclonal antibody to PARP-1 that recognizes both the uncut form (116 kDa) and the apoptosis-specific cleaved fragment (85 kDa) of PARP-1. Baseline PARP-1 (116 kDa) expression levels were measured in LNCaP and PC-3 cells grown for 24 h on plastic plates in the absence of paclitaxel (−). Blots are representative of three independent experiments. At the bottom, PARP-1 protein levels in PC-3 (mean ± S.E.) are reported as percentage of LNCaP cells, where 100% represents the sum of the 116 and 85 kDa signals in LNCaP cells in presence or absence of paclitaxel. The relative amount of the 116 and 85 kDa bands is shown.

3.4. MtDNA depletion confers an invasive phenotype to LNCaP prostate cancer cells

To investigate the role of mtDNA depletion upon cancer cell phenotype, we established mtDNA-depleted LNCaP cells [Rho(−)] by long-term exposure to EtBr [38]. Low concentrations of EtBr inhibit mtDNA synthesis but not host DNA synthesis, reduce mtDNA and thus reduce mitochondrial functionality [38]. MtDNA was not detectable in EtBr-treated LNCaP cells by PCR (Fig. 4(a)) and, unlike wild-type cells, EtBr-treated LNCaP cells could not survive in the absence of high glucose concentrations (4500 mg/l) and uridine, likely related to the impaired respiratory function (data not shown). Quantitative PCR showed negligible amounts of mtDNA at less than 0.5% that of wild-type cells (Fig. 4(b)) and a very slow recovery in the absence of EtBr (negligible after 6 days, ∼4% after 10 days in culture). The variability of mtDNA content was minimized by using cells from the same frozen stock containing less than 0.5% mtDNA content for all the experiments. Thawed Rho(−) LNCaP cells were kept in culture for no more than 6 days. We also induced a mitochondrial metabolic stress by treating LNCaP cells for 12 h with the protonophore CCCP, a mitochondrial uncoupler that abates $\Delta\Psi_m$. To confirm that both mtDNA depletion and CCCP treatment resulted in mitochondrial dysfunction, we analyzed $\Delta\Psi_m$. As shown in Fig. 4(c), CCCP prevented any safranine uptake by treated cells. LNCaP Rho(−) cells also showed reduced $\Delta\Psi_m$, in agreement with previous findings in other cell types following mtDNA depletion [3,4,6,8].

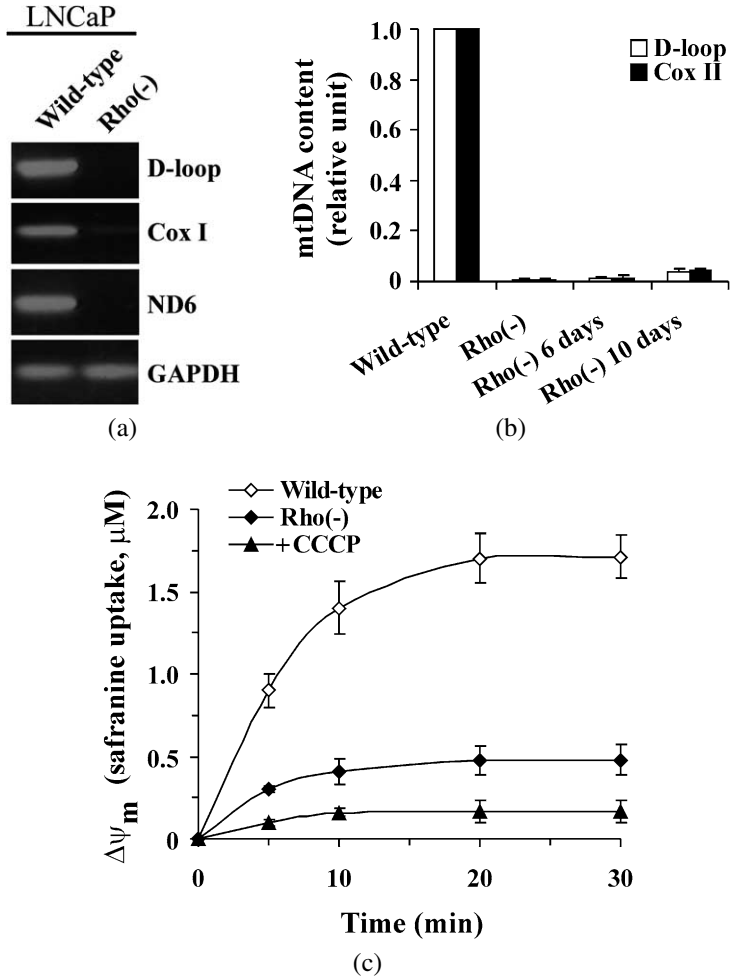
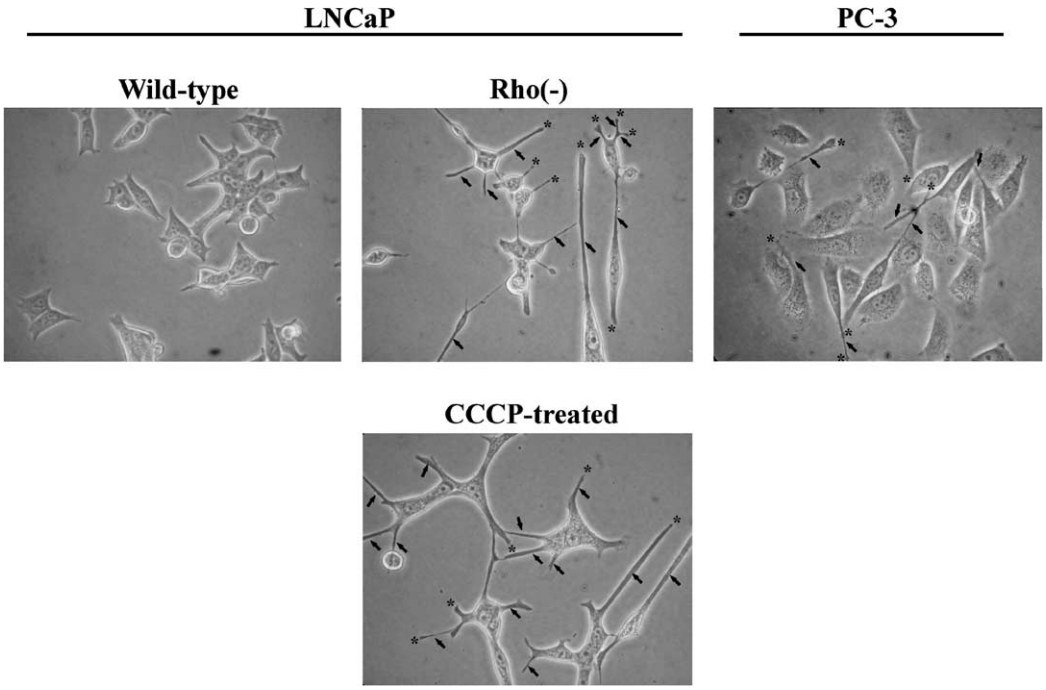
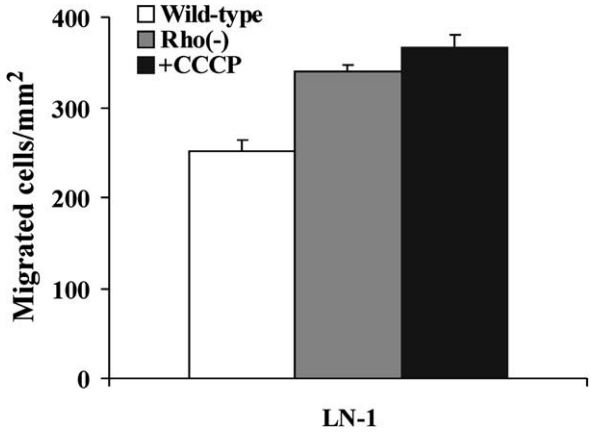


Fig. 4. Generation of Rho(−) LNCaP cells. (a) LNCaP cells were treated with EtBr (100 ng/ml) and, after 20 days, the mtDNA content was analysed by PCR. Total DNA (10 ng/sample) was subjected to PCR using primers for D-loop, ND6 and Cox I. Signals of the nuclear-encoded gene GAPDH were used as control. PCR products were analysed by 1.5% agarose gel stained with EtBr. (b) The mtDNA content was quantitated by real-time PCR in wild-type and EtBr-treated LNCaP cells [Rho(−)] and in Rho(−) LNCaP cells after 6 or 10 days in culture in absence of EtBr. Two mtDNA markers (D-loop and Cox II) and a nuclear DNA marker (β-actin) were used. The relative amplification of mtDNA markers in Rho(−) versus wild-type LNCaP cells was calculated upon normalization to the reference β-actin as described in Section 2. Values are the mean ± S.E. of three independent experiments. (c) To evaluate changes in $\Delta\Psi_m$, 1×10^6 wild-type, Rho(−) and CCCP-treated (25 μM for 12 h) LNCaP cells were incubated for 30 min at 37°C. At time zero, 20 μM safranine was added and the safranine concentration in the cells was measured following the absorbance decrease of the outer medium at 524 nm. Data are reported as mean ± S.E. of three different experiments.

It has been recently reported that mtDNA depletion in LNCaP cells induces androgen-independent cell growth [27]. Hence, we investigated whether mitochondrial dysfunction resulting from mtDNA depletion had a role in the development of the phenotypical features typical of highly invasive androgen-independent tumor cells. We first analyzed the morphology of Rho(−) and CCCP-treated LNCaP cells by light microscopy. As shown in Fig. 5(a), both Rho(−) and CCCP-treated cells exhibited conspicuous pseudopodia and invadopodia along the cell surface or at the end of a dominant leading edge pseudopod, phenotypic characteristics underlining motility and invasive ability [12], features that are common to highly metastatic PC-3 cells. To confirm the acquisition of increased motility ability upon mtDNA depletion, we analyzed the cell migratory properties onto LN-1. As depicted in Fig. 5(b), LNCaP cell migration was increased by 35% in Rho(−) cells ($p < 0.01$) and by 46% after CCCP treatment ($p < 0.003$).



(a)



(b)

Fig. 5. MtDNA depletion confers an invasive phenotype to LNCaP prostate cancer cells. (a) Morphological changes induced by either genetic or metabolic mitochondrial defects were noticeable by microscopic analysis. Representative pictures of PC-3 cells and wild-type, mtDNA-depleted [Rho(−)] and CCCP-treated (25 μM for 12 h) LNCaP cells are shown (100×). Pseudopodia are indicated by arrows and invadopodia along the cell surface or at the end of a dominant leading edge pseudopod are indicated by asterisks. These phenotypic characteristics underline motility and invasive ability. (b) Wild-type, Rho(−) and CCCP-treated LNCaP cell migration was assessed by plating 1.0×10^5 cells onto transwell insert filters coated with 10 μg/ml LN-1. After 12 h, cells on the bottom of the filter were fixed, stained with crystal violet and counted. Measurements represent mean ± S.E. of 10 random fields. A representative experiment of two is shown.

3.5. MtDNA depletion reduces chemosensitivity to paclitaxel and PARP-1 protein levels in LNCaP prostate cancer cells

In the natural history of prostate carcinoma the development of invasive metastatic disease is accompanied by a decreased response to therapeutic agents [47]. Hence, we hypothesized that mtDNA depletion would have a similar effect upon chemosensitivity to paclitaxel. Indeed, as depicted by dose-response curves in Fig. 6, CCCP-treated and Rho(−)

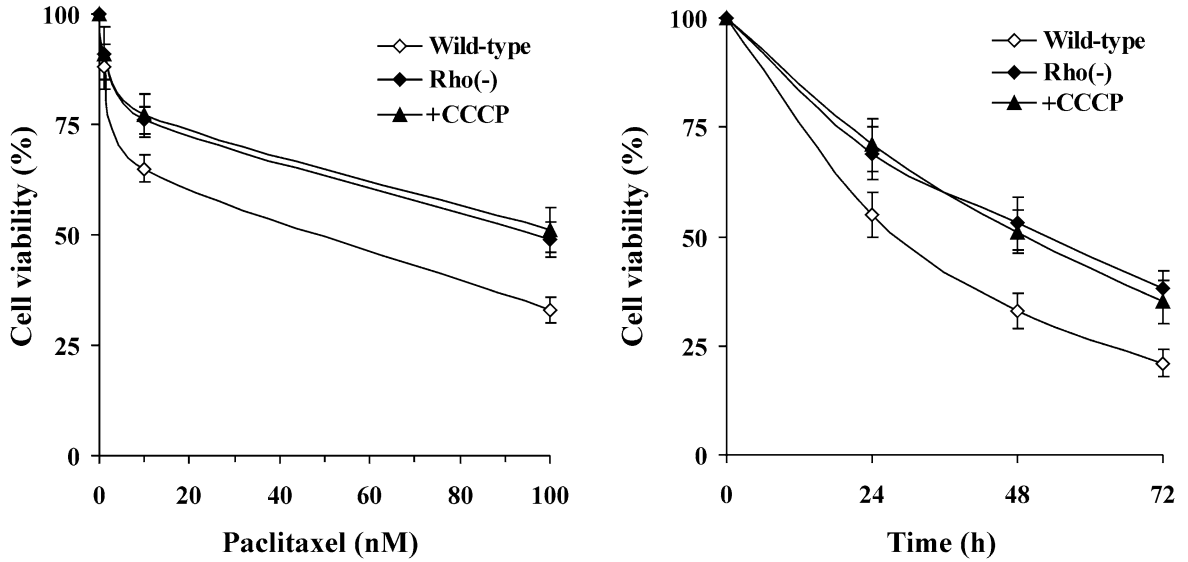


Fig. 6. MtDNA depletion reduces chemosensitivity to paclitaxel and PARP-1 protein levels in LNCaP prostate cancer cells. (a) Shows the dose-response curves of wild-type, Rho(−) and CCCP-treated LNCaP cells after exposure to paclitaxel at three different concentrations (1 nM, 10 nM and 100 nM) for 24 h. Viable cells were measured by MTT assay and expressed as a percentage of the controls. (b) Shows the time-response curves of wild-type, Rho(−) and CCCP-treated LNCaP cells in presence of 10 nM paclitaxel for 24, 48 and 72 h. Data represent mean ± S.E. of three independent experiments.

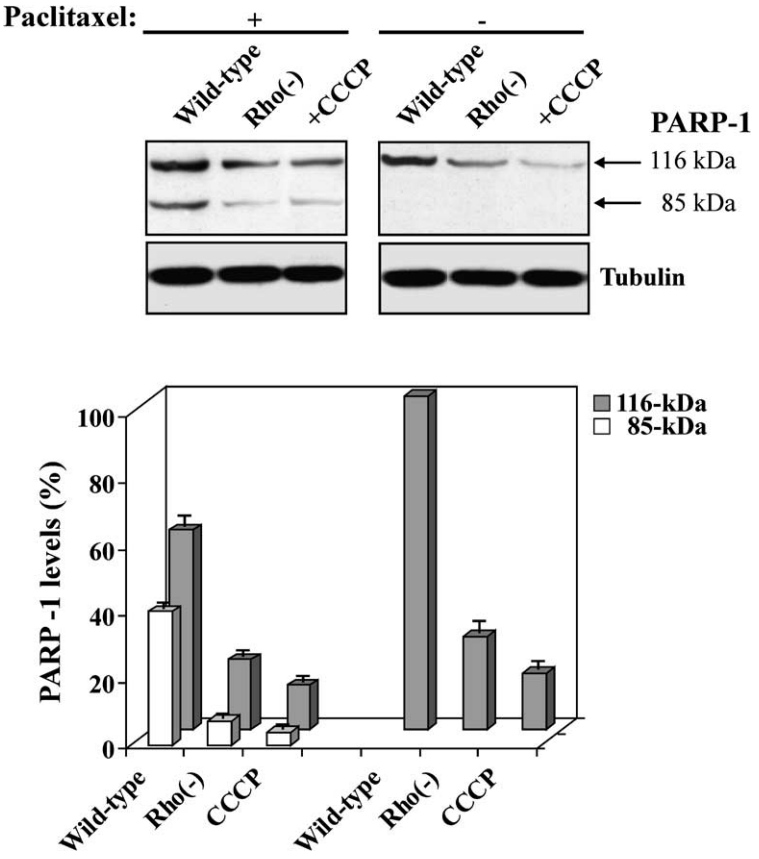
cells displayed a significantly higher IC_{50} (100 nM and 93 nM, respectively, vs. 22 nM, Fig. 6(a)) and required longer exposure to achieve 50% decrease in cell viability (∼48 h, Fig. 6(b)) than control cells (∼26 h).

We had previously noticed that PARP-1 expression in PC-3 cells is significantly reduced in comparison to LNCaP cells (Fig. 3). Hence, we investigated whether mtDNA depletion or chemical mitochondrial uncoupling would affect PARP-1 levels and/or evidence of paclitaxel-induced apoptosis. As shown in Fig. 6(c), after 24 h treatment with paclitaxel, the 85 kDa cleaved fragment of PARP-1 was approximately 6- and 7-folds less in Rho(−) and CCCP-treated cells vs. wild-type LNCaP cells, respectively. Levels of the 116-kDa full-length PARP-1 were also reduced. Nonetheless, the relative amount of cleaved PARP-1 was significantly decreased in Rho(−) and CCCP-treated cells (25% ± 3 and 22% ± 2, respectively) when compared with wild-type cells (40% ± 3; $p < 0.005$), confirming a reduction in drug chemosensitivity upon mtDNA defects. In addition, in the absence of apoptotic stimuli, the levels of full-length PARP-1 were decreased by 72% ± 7 and 83% ± 6 in Rho(−) and CCCP-treated cells, respectively (Fig. 6(c); $p < 0.001$), resembling PC-3 cells (Fig. 3).

4. Discussion

The development of androgen-independent cancer cell growth remains the main obstacle to improving the quality of life and survival of patients with advanced prostate cancer. Hence, a profound understanding of the molecular events that mediate the switch to androgen independence is pivotal in the design of novel therapeutic strategies aimed at preventing the progression to an ultimately fatal neoplastic disease. During that last few years there has been growing evidence implicating mtDNA defects in different aspects of cancer biology [25,49,56]. In this study, we demonstrate that mtDNA depletion is an important event intimately related to androgen independence and consequently to a more invasive and drug-resistant prostate cancer.

We show here that several androgen-independent prostate cancer cell lines, such as C4-2, PC-3 and DU145, exhibit significantly reduced amounts of mtDNA when compared to the androgen-dependent LNCaP cells. In addition, depletion of mtDNA in LNCaP cells or their treatment with the mitochondrial uncoupler CCCP, that mimics the cellular effects of mtDNA defects by abating $\Delta\Psi_m$, resulted in increased resistance to paclitaxel-induced apoptosis and the appearance of a more invasive phenotype as suggested by changes in cell morphology as well as



(c)

Fig. 6. (*Continued*). (c) PARP-1 protein expression and cleavage were analysed by Western blotting in wild-type, Rho(−) and CCCP-treated LNCaP cells in presence (+) or absence (−) of 10 nM paclitaxel for 24 h using a monoclonal antibody to PARP that recognizes both the uncut form (116 kDa) and the apoptosis-specific cleaved fragment (85 kDa) of PARP-1. Blots are representative of three independent experiments. At the bottom, PARP-1 protein levels in Rho(−) and CCCP-treated cells are reported as percentage (mean ± S.E.) of wild-type LNCaP cells, where 100% represents the sum of the 116 and 85 kDa signals in LNCaP cells in presence or absence of paclitaxel. The relative amount of the 116 and 85 kDa bands is shown.

by increased migration onto LN-1. These phenotypic changes are reminiscent of those associated with the development of androgen independence [1,55] and indeed were detected in PC-3 cells. The relationship between mtDNA defects and androgen-independent cancer cell growth has been demonstrated in a recent study showing that depletion of mtDNA in LNCAP cells triggered a shift to androgen-independent growth and that reconstitution of the mtDNA pool was sufficient to restore androgen sensitivity [27]. The authors also showed that the androgen-independent C4-2 cells, derived by inoculation of LNCaP cells into castrated mice, exhibited reduction of mtDNA content, consistently with our results and suggested that androgen ablation may induce mtDNA defects [27]. However, the precise chain of events is still poorly understood since progression to androgen independence may occur *in vivo* either spontaneously or after androgen withdrawal therapy [67], suggesting a more complex scenario than a mere cause-effect relationship among androgen ablation, mtDNA defects and androgen independence. Overall, our results provide compelling evidence for a pivotal role of mtDNA defects in triggering a cascade of events that ultimately mediate the acquisition of an invasive, apoptosis-resistant phenotype in prostate cancer cells. Recent studies performed in other cell types suggest that the involvement of mtDNA defects in tumor progression is far from being a phenomenon unique to prostate cancer and androgen-dependent cells. Indeed, there is a growing number of studies demonstrating that mtDNA depletion may confer resistance to apoptosis-inducing agents, such as tumor necrosis factor α in myelogenous leukemia cells [26], adriamycin and photodynamic therapy in HeLa

cells [65], cisplatin in intestinal epithelial cells [59], etoposide in myoblasts and lung carcinoma cells [7,9], antiestrogen in breast cancer cells [53] as well as promoting an invasive phenotype in myoblasts and lung carcinoma cells [3,4]. Our studies also confirm the notion that the increased invasiveness of prostate cancer cells is closely associated with a desensitization to chemotherapeutic agents, findings that are in line with a previous report demonstrating that cell resistance to apoptosis increases in parallel with the metastatic potential of prostate carcinoma cells [47]. However, the potential role of mtDNA as a central player in the acquisition of apoptosis resistance and tumor progression has only been recognized recently. Indeed, accumulation of point mutations and large deletions of mtDNA in prostate cancer specimens *in vivo* has been recently described [16,32,33].

The mechanisms involved and to what extent a reduction in mtDNA content and/or accumulation of mutant mtDNA may contribute to prostate cancer progression and loss of androgen dependence are poorly understood. However, some of the biological events involved in the transition to androgen independence have been recognized. For example, it has been reported previously that cells derived from androgen-independent prostate cancer express high levels of the anti-apoptotic protein Bcl-2 [48] and that transition from androgen-dependent to androgen-independent LNCaP cells is accompanied by changes in the expression of cyclin-dependent kinase inhibitors, ultimately mediating resistance to TPA-induced apoptosis [43]. There is also strong evidence demonstrating that genetic and/or metabolic mitochondrial stress are able to trigger changes in the expression of nuclear genes involved in cell invasion, such as cathepsin L and transforming growth factor-β [6–9], DNA repair, such as APE1 [64] and apoptosis, including members of the Bcl-2 family [7,9,26]. Thus, it is plausible that mtDNA defects may induce resistance to androgen ablation through activation of anti-apoptotic and/or pro-survival pathways.

Another factor that may provide a link between androgen independence and mitochondrial dysfunction is PARP-1, a protein involved in DNA repair. We show here for the first time that PARP-1 is among the proteins whose expression is regulated by mitochondrial signaling. Indeed, mitochondrial dysfunction in LNCaP cells, generated by either mtDNA depletion or treatment with CCCP, resulted in down-regulation of PARP-1 protein expression to levels comparable to those detected in androgen-independent PC-3 cells. It has been proposed that deficiency in PARP-1 might contribute to malignant progression through induction of genomic instability, alteration of transcriptional regulation and differentiation (reviewed in [46]). We observed that a decreased baseline expression of PARP-1 occurred in concomitance with reduced chemosensitivity to paclitaxel and increased invasiveness of prostate cancer cells. It should be noted that in comparison with wild-type LNCaP cells the relative amount of the 85-kDa apoptosis-specific fragment detected after paclitaxel treatment was further reduced in Rho(−) and CCCP-treated LNCaP cells and in PC-3 cells, likely reflecting that a decreased sensitivity to apoptosis related to mtDNA depletion may occur independently of upstream events controlling PARP-1 expression. Various explanations have been proposed for the requirement of PARP-1 during the apoptotic process, including depletion of NAD^+ and ATP [13], modulation of expression and activity of proteins involved in apoptosis like p53 [68,70], promotion of oligonucleosomal DNA fragmentation [62] and up-regulation of the multidrug resistance gene product P-glycoprotein in PARP-depleted cells [73]. Other studies have also reported that depletion of PARP-1, either by gene disruption [63,73], antisense RNA [39,63], or pharmacological inhibitors [14,60,63], results in decreased drug-induced apoptosis. In addition, *in vivo* studies have shown that low PARP-1 expression levels in breast cancer correlate with increased genomic instability and resistance to chemotherapeutic drugs [5]. More recently, Holleman et al. [29] found that low or absent PARP-1 baseline expression in childhood acute lymphoblastic leukemia *in vivo* is associated with cellular drug resistance. Our findings demonstrating that reduction in PARP-1 protein occurs in concomitance with an increase in cancer cell invasiveness and tolerance to chemotherapeutic agents further support the potential role of decreased PARP-1 expression in cancer progression and suggest that it could represent a novel biomarker for advancing prostate cancer. Further studies investigating the effect of ectopic expression of PARP-1 on drug chemosensitivity and migration in prostate cancer cells are being devised. Nonetheless, the recent finding from Lockett et al. [42], demonstrating that a mutation in the PARP-1 gene contributes to prostate cancer susceptibility, offers circumstantial evidence supporting an important role for PARP-1 in the biology of prostate cancer.

In summary, we have presented strong evidence supporting the contention that mtDNA defects are a pivotal event in the natural evolution of prostate cancer

into a highly malignant neoplasm. The precise nature and temporal relationship of the chain of events that include down-regulation in PARP-1 expression and decreased sensitivity to apoptosis-inducing agent such as paclitaxel is not well understood and requires further investigation. However, immediate clinical benefit may be available by screening for mtDNA mutations/content in prostate cancer samples as it may represent a valuable tool in predicting the efficacy of therapy with paclitaxel and similar anti-apoptotic agents.

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