



## Nonylphenol effects on human prostate non tumorigenic cells



Maurizio Forte<sup>a,b</sup>, Mariana Di Lorenzo<sup>a</sup>, Albino Carrizzo<sup>c</sup>, Salvatore Valiante<sup>a</sup>, Carmine Vecchione<sup>c,d</sup>, Vincenza Laforgia<sup>a,b</sup>, Maria De Falco<sup>a,b,\*</sup>

<sup>a</sup> Department of Biology, University of Naples, "Federico II", Naples, Italy

<sup>b</sup> National Laboratory on Endocrine Disruptors of Interuniversity Consortium INBB, Institute of Genetic and Biophysics (IGB) "ABT", CNR, Naples, Italy

<sup>c</sup> IRCCS Neuromed, Department of Angio-Cardio-Neurology, Pozzilli (Isernia) 86077, Italy

<sup>d</sup> Department of Medicine and Surgery, University of Salerno, Baronissi 84081, Italy

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### ABSTRACT

Nonylphenol (NP) is an industrial chemical with estrogenic activity both *in vivo* and *in vitro*; estrogens play a critical role in the development of prostate and may be the cause of some pathological states, including cancer. In this study we examined the effects of NP on human prostate non tumorigenic epithelial cells (PNT1A) investigating on cell proliferation, interaction with estrogen receptors (ERs) and gene expression of genes involved in prostate diseases. We found that NP affects cell proliferation at  $10^{-6}$  M, promoting a cytoplasm-nucleus translocation of ER $\alpha$  and not ER $\beta$ , like the natural estrogen 17 $\beta$ -estradiol (E2). Moreover, we showed that NP enhances gene expression of key regulators of cell cycle. Estrogen selective antagonist ICI182780 in part reverted the observed effects of NP. These results confirm the estrogenic activity of NP and suggest that other transduction pathways may be involved in NP action on prostate.

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### 1. Introduction

Nonylphenol (NP) is generated by the degradation of nonylphenol ethoxilates (NPEs). NPEs are chemicals widely used as non ionic surfactants in the manufacture of rubber and plastic for domestic, industrial and agricultural products (Fiege et al., 2000; Langford, 2002; Vazquez-Duhalt et al., 2005). Due to its high hydrophobicity and low solubility NP accumulates in several environmental matrices, such as seas, rivers, soils, groundwaters and sediments, in a range between  $10^{-13}$  to  $10^{-6}$  M (Berryman et al., 2004; Careghini et al., 2015; Vazquez-Duhalt et al., 2005). It was also found as a contaminant of food and drinking water (Gyllenhammar et al., 2012; Maggioni et al., 2013; Soares et al., 2008). Human exposure to NP may occur by inhalation, cutaneous absorption and ingestion of contaminated food or water (Guenther et al., 2002; Soto et al., 1991). In this regard, NP was found in human amniotic fluid, urine and plasma samples, breast milk, fetal cord serum, placenta and maternal blood, with levels in these tissues generally varying from  $10^{-10}$  to  $10^{-9}$  M (Calafat et al., 2005; Huang et al., 2014). However, in some cases, concentrations of NP have

been reported to be much higher in human samples. In this regards in breast milk of healthy Italian women, Ademollo et al. (Ademollo et al., 2008) detected about  $10^{-7}$  M of NP as well as in urine and in plasma of textile and housekeeping workers were found the same NP levels (Chen et al., 2005). Instead, in maternal cord blood, Chen et al. (Chen et al., 2008) found a concentration of NP of about  $10^{-6}$  M.

NP belongs to the subclass of endocrine disrupting chemicals (EDCs) that mimic the endogenous estrogens, called xenoestrogens (Falconer et al., 2006; Wozniak et al., 2005), that also includes dioxins, polychlorinated biphenyls, hexachlorocyclohexane, octylphenol and bisphenol A (Kuo et al., 2012; Forte et al., 2016). Estrogenic activity of NP has been reported both *in vitro* (de Weert et al., 2008; Soto et al., 1991; White et al., 1994) and *in vivo*, in reproductive and in non reproductive tissues, such as brain (Blom et al., 1998; Laws et al., 2000; Nagel et al., 1999; ter Veld et al., 2008; Xia et al., 2008; De Falco et al., 2014, 2015) and it has been shown that NP interacts with estrogen receptors (ERs), competing with the natural estrogen 17 $\beta$ -estradiol (E2) (Bechi et al., 2006; Kwack et al., 2002; White et al., 1994), although with less specificity (Bechi et al., 2010; Blom et al., 1998; Nagel et al., 1999).

Estrogens predominantly bind two nuclear receptors: the estrogen receptor alpha (ER $\alpha$ ) and the estrogen receptor beta

\* Corresponding author at: Department of Biology, University of Naples, "Federico II" Via Mezzocannone 8, Naples, 80134, Italy.  
E-mail address: [madefalco@unina.it](mailto:madefalco@unina.it) (M. De Falco).

(ER $\beta$ ). Both ER $\alpha$  and ER $\beta$  bind to the active form of estrogen E2, with similar affinities (Siewit et al., 2010). ERs mediated estrogen signaling in reproductive tissues but also in non-reproductive tissues as the brain, lungs, colon, prostate and cardiovascular system (Shanle and Xu, 2011). In the cell, E2 is able to activate both genomic and non-genomic responses. In the genomic pathway, E2 mediates target gene regulation through binding directly DNA at estrogen response elements (EREs) or indirectly through transcription factors like Sp1 or AP-1 (Kushner et al., 2000; Saville et al., 2000); the non-genomic pathway is not mediated by ER $\alpha$  or ER $\beta$  but through the G-protein coupled receptor, GPR30, that localizes in the plasma membrane activating rapid responses such as increased levels of c-AMP (Filardo et al., 2007; Lin et al., 2009; Wang et al., 2010).

Several studies have suggested the role of estrogens in normal and aberrant growth of prostate, alone or in synergy with androgens (Ho et al., 2011) and epidemiological and experimental studies underline a relationship between estrogens/xenoestrogens and pathogenesis of prostate cancer (PCa) (Bostwick et al., 2004; Ho et al., 2006). Neonatal treatment with Bisphenol A (BPA), a well-known xenoestrogen, was reported to induce high-grade prostatic intraepithelial neoplasia in Sprague-Dawley rats (Ho et al., 2006) and to increase cell proliferation of urogenital sinus epithelium (UGE) in the primary prostatic ducts of CD1 mice (Timms et al., 2005). BPA was also found to increase the number of basal epithelial cells in the adult prostate of BALB/c mice (Ogura et al., 2007). Recently, Tarapore et al. (2014) found in prostate cancer patients high BPA urinary levels compared to non prostate cancer patients.

Despite the relationship between estrogen and prostate, the precise functions of the two ER subtypes in this gland remain unclear; several authors have reported differential expression patterns of the two receptors between the epithelial and stromal compartment of the prostate, with ER $\alpha$  localized predominantly in the stroma and ER $\beta$  in the epithelium (Fixemer et al., 2003; Leav et al., 2001; Tsurusaki et al., 2003; Weihua and Warner, 2002).

Considering this background and given the human exposure to EDCs, the estrogen-like action of NP is conceivable to influence the normal growth of prostate and to be the cause of some pathological states of this gland, affecting the male reproductive functions. Thus, in this study we evaluated the effects of NP on the proliferation of human non tumorigenic prostate cells (PNT1A), which is responsive to sex hormones (Stephen et al., 2004), the cellular localization of ER $\alpha$  and ER $\beta$  after exposure to NP and gene expression of genes involved in pathological states of the prostate such as *cyclin D*, *Ki67*, *p53* and *IL1- $\beta$* . We performed the same experiments treating cells with the natural estrogen E2 and with the selective antagonist of estrogen receptors ICI 182,780 (Osborne et al., 2004). This study aims to facilitate the understanding of the mechanisms by which xenoestrogens and estrogens may exert their activity on prostate.

## 2. Material and methods

### 2.1. Cell culture

PNT1A cells (a human prostate cell line established by immortalization of adult prostate epithelial cells) were obtained from the European Collection of Cell Culture (ECACC Salisbury, UK). PNT1A cells were grown in red phenol free RPMI-1640 medium (LONZA, Basel, Switzerland), supplemented with 10% dextran-coated charcoal fetal bovine serum (FBS) (GIBCO, Grand Island, NY), 2 mM L-glutamine and antibiotics (100 U/mL penicillin/streptomycin, 10  $\mu$ g/mL gentamicin) in a humidified incubator at 37 °C and 5% CO<sub>2</sub>. When confluent, the cells were detached

enzymatically with trypsin-ethylenediamine tetra-acetic acid and subcultured into a new cell culture flasks. The medium was replaced every 2 days. Cells were used for experiments between passages 5–20.

### 2.2. Chemicals

Nonylphenol (NP), 17 $\beta$ -Estradiol (E2) and selective estrogen antagonist ICI 182,780 (ICI) were purchased from Sigma-Aldrich (Sigma Aldrich, St. Louis, MO) and were dissolved in DMSO (Invitrogen Carlsbad, CA). NP, E2 and ICI were diluted with culture medium at final concentrations from 10<sup>-12</sup> to 10<sup>-6</sup> M for NP and E2 and 10<sup>-5</sup> M for ICI. In all the experiments with the inhibitor, ICI was added 1 h prior to start treatments. Final concentration of DMSO in the medium did not exceed 0.01%.

### 2.3. MTT assay

The effects of NP or E2 on PNT1A cells proliferation was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-3,5 diphenyl tetrazolium bromide (MTT) test (Sigma Aldrich, St. Louis, MO). Cells were seeded in 200  $\mu$ L of growth medium (5  $\times$  10<sup>4</sup> cells/well) in 96-well plates and hormone deprived (1% FBS) for 24 h. Then, NP or E2 was added after dilution to an appropriate concentration (from 10<sup>-12</sup> M to 10<sup>-6</sup> M), with or without 10<sup>-5</sup> M ICI. Control cells were treated with vehicle (DMSO 0.01%). The test was performed for 24 h of incubation. After the incubation period, 10  $\mu$ L of a MTT solution was added to each well. After 4 h of 37 °C incubation, the culture medium was gently aspirated and replaced by 100  $\mu$ L of DMSO/isopropanol (1:1) in order to dissolve the formazan crystals. The absorbance of the solubilized dye, which correlates with the number of living cells, was measured with a microplate reader at 570 nm. The test was performed in triplicate.

### 2.4. Fluorescence microscopy

PNT1A cells were seeded in 4-well chamber slide (Sarstedt, Nürnberg, Germany) overnight at a density of 5  $\times$  10<sup>4</sup>/well. After 24 h serum starvation (1% FBS), cells were incubated with 10<sup>-6</sup> M NP or 10<sup>-6</sup> M E2, with or without 10<sup>-5</sup> M ICI for four different times: 15 min, 1 h, 2 h and 6 h. Control group was treated only with vehicle (DMSO 0.01%). Control and treated cells were fixed with methanol for 10 min at RT, permeabilized with 0.25% Triton X-100 for 10 min, washed in PBS, and blocked in 5% normal goat serum (NGS) for 1 h at RT. Then cells were subjected to immunofluorescence protocol using a mouse monoclonal anti-human ER $\alpha$  (Santa Cruz Biotechnology, Santa Cruz, CA, Cat. sc-8005) and a mouse monoclonal anti-human ER $\beta$  antibodies (Santa Cruz, Cat. sc-373853), diluted 1:100 in 1% NGS for 24 h at 4 °C. For detection of ER $\alpha$  and ER $\beta$ , secondary goat anti-mouse Alex Fluor 488 (Cat. A11001, Invitrogen, Carlsbad, CA), dilution 1:200 in 1% NGS was used. Cell nuclei were counterstained with 0.1  $\mu$ g/mL Hoechst (Invitrogen, Carlsbad, CA, Cat. H3570). Negative control for ER $\alpha$  and ER $\beta$  was performed by avoiding incubation with the primary antibodies (Supplementary data Fig. S1). Fluorescent images were taken on an Axioskop (Carl Zeiss, Milano, Italy) epifluorescence microscope using a 40 $\times$  objective. AxioCam MRC5 and the acquisition software Axiovision 4.7 (Carl Zeiss) were used to capture the images in different channels (Alexa Fluor 488, Hoechst 33258). Three independent immunofluorescence experiments were performed for each experimental conditions and different fields were randomly chosen for data analysis. Then, images were processed with the Image J software (developed by Wayne Rasband, National Institutes of Health, USA).

## 2.5. Protein extraction and separation

Nuclear and cytoplasmic proteins were extracted from PNT1A cells after two and six hours of treatment with  $10^{-6}$  M NP,  $10^{-6}$  M E2, with or without  $10^{-5}$  M ICI. Control cells were treated with 0.01% of DMSO and western blot was performed for detection of ER $\alpha$  and ER $\beta$ . Different buffers were prepared to isolate cytoplasmic/membrane and nuclear proteins: harvest buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA, 0.5% Triton X-100 and freshly added 1 mM DTT, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 1 mM PMSF, 4 mg/mL Aprotinin and 2 mg/mL Pepstatin A), buffer A (10 mM HEPES pH 7–9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and freshly added 1 mM DTT, 1 mM PMSF, 4 mg/mL Aprotinin and 2 mg/mL Pepstatin A) and buffer B (10 mM HEPES pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA 0.1% NP-40 and freshly added 1 mM DTT, 1 mM PMSF, 4 mg/mL Aprotinin and 2 mg/mL Pepstatin A). 10 cm cell dishes with confluent PNT1A cells were placed on ice for 10 min and washed twice with ice cold PBS. Then 100  $\mu$ L of PBS-EDTA 1 mM was added and cells were scraped and transferred to a microcentrifuge tube. The collected cells were then centrifuged at 3000 rpm for 5 min at 4 °C and then resuspended in cold harvest buffer, incubated on ice for 5 min and subsequently centrifuged at 1000 rpm for 10 min to pellet nuclei. Then the supernatant was transferred into a new tube and centrifuged at 14000 rpm for 15 min, in order to clear the supernatant. This latter contains the cytoplasmic and membrane proteins. Nuclear pellet was then resuspended in buffer A, centrifuged at 1000 rpm, the supernatant was discarded. Then 4 vol of buffer B were added and the tubes were vortex for 15 min at 4 °C to loosen pellet. Finally, a centrifugation for 10 min at 14,000 rpm at 4 °C was performed and the supernatant that contain nuclear extract was transferred into a new tube. Protein concentration was determined with Bradford assay (Biorad).

## 2.6. Western blot

50  $\mu$ g of protein extracts for each sample was separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were blocked with 5% milk in TBS-Tween for 2 h at room temperature and then incubated with primary antibodies in TBS-Tween and 5% milk overnight. Blocked membranes were then incubated with anti-ER $\alpha$  (1:500) or anti-ER $\beta$  (1:500) and with mouse anti-human  $\beta$ -tubulin (1:2000) (Santa Cruz, Cat. sc-5274) or mouse anti-human HDAC2 (1:2000) (Santa Cruz, Cat. sc-55542) overnight and then detected using appropriate horseradish peroxidase-coupled secondary antibody (Santa Cruz, Cat. sc-2005) and visualized with enhanced chemiluminescence (Amersham, Thermo Fisher Scientific, Milano, Italy). The purity of nuclear and cytoplasmic fractions was confirmed using anti-HDAC-2 and anti- $\beta$ -tubulin, respectively. All antibodies have been used to probe the same experimental membrane. In detail, before incubation with another primary antibody, the membranes have been stripped with the stripping solution: 100 mM 2-Mercaptoethanol, 1% SDS, 62.5 mM Tris-HCl pH 6.7 and incubated at 50 °C for 30 min with agitation and, subsequently, the membrane have been re-equilibrated in TBS and then blocked with 5% milk in TBS-Tween for 2 h at room temperature. The rendering of stripping has been tested by evaluating the ECL- signal after treatment with the stripping solution. Only when the signal of the previous antibody was absent, the membrane was incubated with a new antibody. Immunoblotting data were analyzed using ImageJ software to determine optical density (OD) of the bands. The OD reading was normalized on anti- $\beta$ -tubulin and anti-HDAC2 to account for variations in loading. For each time of treatment (i.e. 2 h, 6 h, 2 h

with ICI) were analyzed data of three independent western blotting.

## 2.7. RNA extraction and RT-qPCR

mRNA expression levels of estrogen target genes were analyzed using real-time PCR. Total RNA from PNT1A control cells and treated for 24 h with  $10^{-6}$  M E2 or  $10^{-6}$  M NP, with or without  $10^{-5}$  M ICI was extracted using Trizol (Life Technologies, Carlsbad, CA). After purification from genomic DNA with TURBO DNA-free™ Kit (Ambion, Life Technologies), the total amount of RNA was quantified with a NanoDrop spectrophotometer. cDNAs were synthesized from 1  $\mu$ g RNA using the High Capacity cDNA Reverse Transcriptase (Life Technologies) and quantitative PCR (RT-qPCR) was performed by using the 7500 Real-Time PCR System and SYBR<sup>®</sup> Select Master Mix 2X assay (Applied Biosystem, Foster City, CA, USA). All primers used (Table 1) were designed according to the sequences published on GenBank using Primer Express software version 3.0 and primer efficiencies were tested prior to perform qPCR. The amount of target cDNA was calculated by comparative threshold (Ct) method and expressed by means of the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001) using the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene, which expression was not affected by the treatment. Three separate experiments (n = 3) were performed for RT-qPCR and each sample was tested in triplicate.

## 2.8. Statistical analysis

Data reported in graphs are expressed as mean values  $\pm$ SEM for the indicated number of independent determinations. The statistical significance was calculated by the one way ANOVA with Bonferroni's multiple comparison test, and differences were considered statistically significant when the P value was at least <0.05.

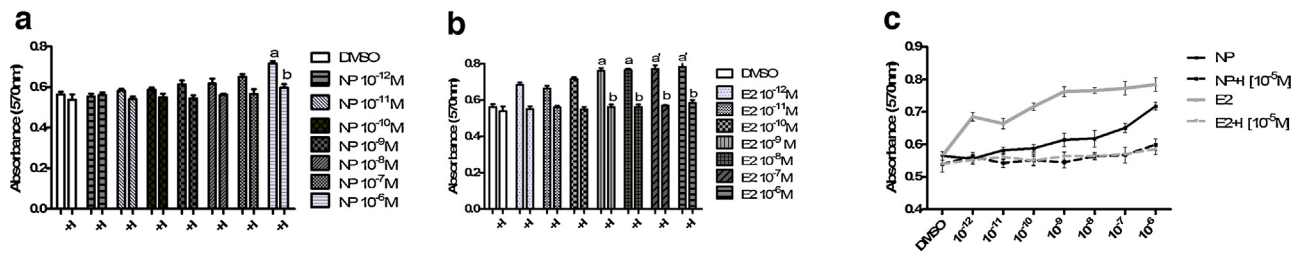
## 3. Results

### 3.1. Cell proliferation assay

To determine if NP affects cellular proliferation, PNT1A cells were treated with increasing concentration of NP (from  $10^{-12}$  M to  $10^{-6}$  M) for 24 h of exposure; to assess any similarities, treatment was performed also exposing cells with E2; the same experiments were also carried out in presence of  $10^{-5}$  M ICI. NP stimulated PNT1A cells proliferation at the highest concentration we used ( $10^{-6}$  M) (Fig. 1a). At lower concentrations, we did not observe any significant effects when compared to control group.  $10^{-5}$  M ICI inhibited the proliferation induced by  $10^{-6}$  M NP. Similarly, treatment with E2 stimulated PNT1A cells proliferation from  $10^{-9}$  M to  $10^{-6}$  M, with the greatest effect showed at  $10^{-6}$  M (Fig. 1b). E2 induced proliferation is strongly inhibited by adding ICI. Fig. 1c shows as E2 has a greater effect compared to NP on PNT1A cells proliferation.

**Table 1**  
Primers used in qPCR.

Gene	5'-Forward-3'	5'-Reverse-3'
Cyclin D	CGTGGCCTCTAAGATGAAGGA	CGGTGTAGATGCACAGCTTCTC
Cyclin E	GATGACCGGGTTTACCCAAA	CCTCTGGATGGTGAATAATCC
Ki67	CCCGTGGGAGACGTGGTA	TTCCCGTGACCGCTTCCA
p53	TCTGTCCCTTCCCAGAAAACC	CAAGAAGCCCAGACGGAAAC
IL1- $\beta$	ACGATGCACTGTACCATCACT	CACCAAGCTTTTGTCTGTGAGT
GAPDH	CAAGGCTGTGGCAAGGT	GGAAGCCATGCCAGTGA



**Fig. 1.** MTT assay after 24 h of exposure to nonylphenol (NP) and 17 $\beta$ - estradiol (E2) alone or in combination with ICI 182, 780 (+). NP stimulates PNT1A cells proliferation at 10<sup>-6</sup> M (a) while E2 at 10<sup>-9</sup> M, 10<sup>-8</sup> M, 10<sup>-7</sup> M and 10<sup>-6</sup> M (b). 10<sup>-5</sup> M ICI (I) reverts this effect. E2 shows the greatest effect on proliferation if compared to nonylphenol (c). In graph is reported the absorbance measured at 570 nm which correlates with the number of living cells. a, response significantly different than the vehicle control ( $P < 0.05$ ); a', response significantly different than the vehicle control ( $P < 0.01$ ); b, response significantly different than cells without ICI ( $P < 0.05$ ).

### 3.2. Fluorescence microscopy

#### 3.2.1. Localization of ER $\alpha$

We investigated on estrogen receptors ER $\alpha$  localization after 10<sup>-6</sup> M NP and 10<sup>-6</sup> M E2 treatment, in order to evaluate the interaction between NP and ERs. After 15 min and 1 h ER $\alpha$  remained localized in the cytoplasm after both NP and E2 treatments (data not shown). After 2 h of treatment, NP did not affect ER $\alpha$  cellular localization, that was localized in the cytoplasm, as in control. On the contrary, in E2 treated cells for 2 h, ER $\alpha$  localized predominantly in the nucleus (Fig. 2).

After 6 h of exposure, both in PNT1A cells treated with NP and E2, ER $\alpha$  shifted from the cytoplasm to the nucleus that appeared to be strongly positive, with a weak cytoplasmic fluorescence if compared to control (Fig. 2).

In PNT1A cells pre-treated with ICI ER $\alpha$  was found in the cytoplasm both after 2 and 6 h of treatment with nuclei completely negatives (Fig. 3).

#### 3.2.2. Localization of ER $\beta$

ER $\beta$  localization after treatment with 10<sup>-6</sup> M NP and 10<sup>-6</sup> M E2 was also investigated; data after 15 min and 1 h (data not shown) as well as after 2 h and 6 h of exposure did not reveal any differences between control and exposed cells. ER $\beta$  was localized in the cytoplasm of PNT1A cells and cell nuclei appeared with a weak signal (Fig. 4).

#### 3.3. Western blot analysis

After separation of cytoplasmic and nuclear proteins we performed a translocation study of ER $\alpha$  and ER $\beta$  with a western blot analysis, in order to confirm microscopy results after 10<sup>-6</sup> M NP and E2 exposure. Densitometric analyses were normalized for cytoplasmic and nuclear extracts with  $\beta$ -tubulin (55 Kda) and HDAC2 (55 KDa), respectively. After 2 h of exposure (Fig. 5) we found ER $\alpha$  protein (molecular weight 66 KDa) in the cytoplasm of NP treated and non treated PNT1A cells whereas optical density values were significantly lower in E2 treated cells (Fig. 5a,b). Moreover, after 2 h nuclear proteins revealed a signal only in E2 treated cells (Fig. 5a). ER $\beta$  (56 KDa) after 2 h of treatment was found only in cytoplasmic fractions (Fig. 5a,c). No signal for HDAC2 and  $\beta$ -tubulin in the cytoplasm and nucleus proteins, respectively, suggest that protein separation was performed correctly.

After 6 h of exposure (Fig. 6) we observed a nuclear translocation of ER $\alpha$  both in NP and E2 treated cells (Fig. 6a,b). However, densitometry did not reveal significant differences in nuclear extracts between NP and E2 treated cells (Fig. 6b). In contrast, values were significantly lower in the cytoplasm in treated cells compared to control (Fig. 6b). ER $\beta$  was found only in cytoplasmic

fractions (Fig. 6a,c) and there were not significant differences in optic density between treated and non treated cells (Fig. 6c).

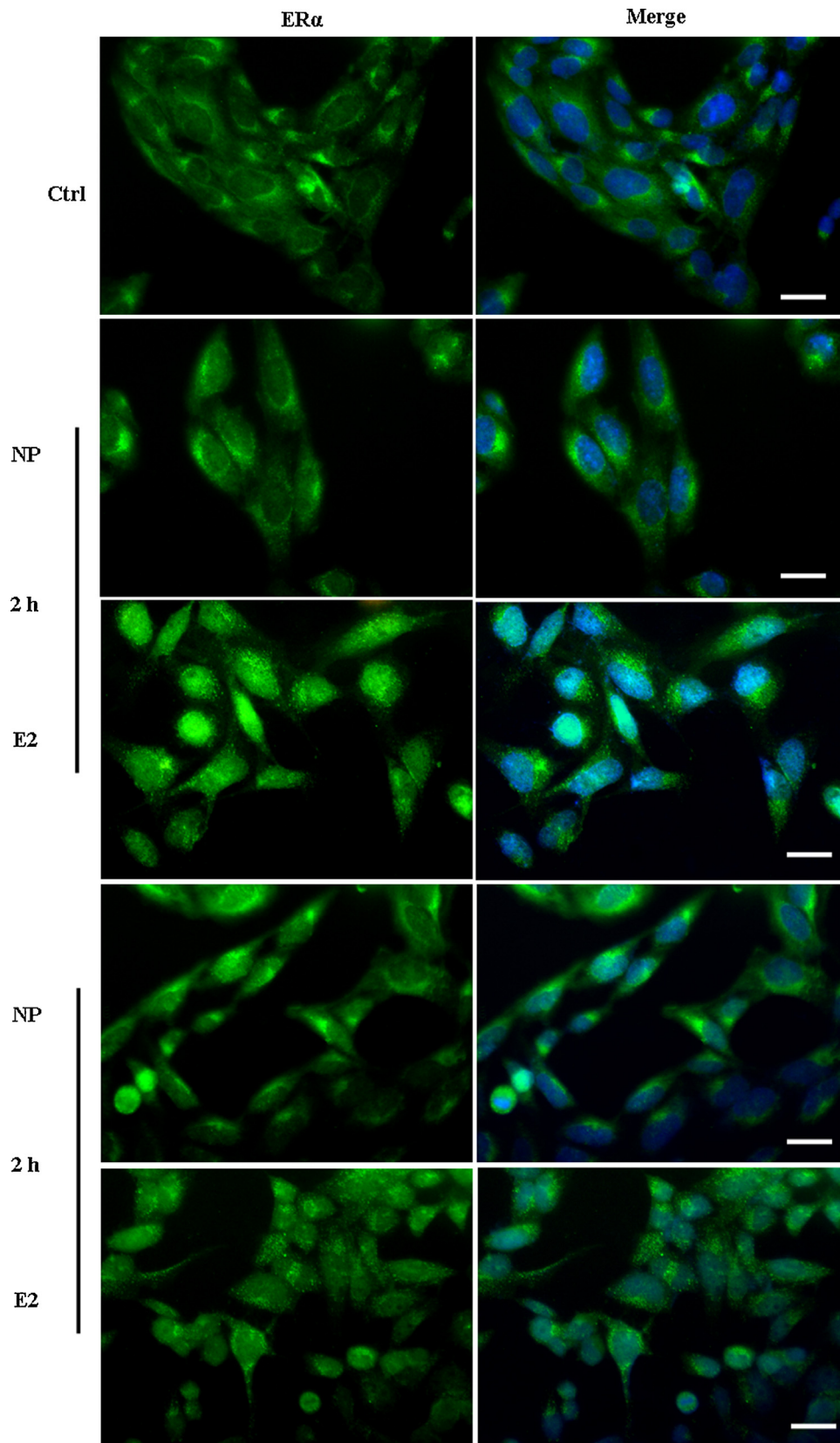
Western blot for ER $\alpha$  localization performed in presence of 10<sup>-5</sup> M ICI after two (Fig. 7a,b) and 6 h of treatment (Fig. 7c,d) revealed ER $\alpha$  exclusively in cytoplasm proteins, with a weak signal in the nuclear extracts after 6 h of exposure (Fig. 7c). No statistically significant differences were showed comparing non treated and treated cells (Fig. 7b,d).

#### 3.4. RT- qPCR analysis

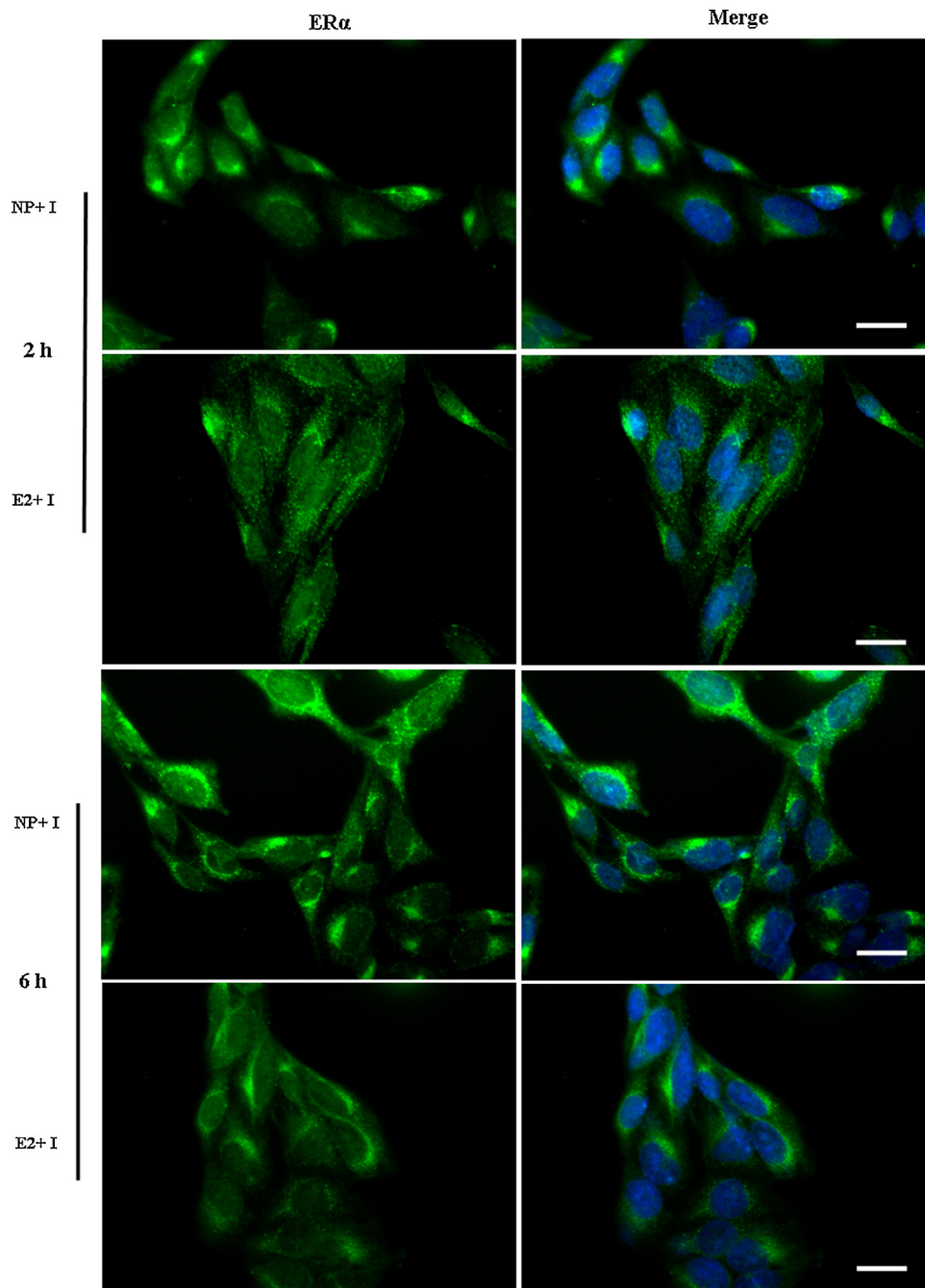
In order to investigate if NP is able to affect gene expression, RT-qPCR analysis of genes involved in cell cycle regulation and in pathological states of prostate were investigated after 24 h of exposure. NP enhanced mRNA levels of *Cyclin D* (Fig. 8a), *Cyclin E* (Fig. 8b), *Ki67* (Fig. 8c) and *IL1- $\beta$*  (Fig. 8e) while it did not affect *p53* expression (Fig. 8d). Interestingly, ICI reduced gene expression of *Cyclin D* (Fig. 8a) and *Ki67* (Fig. 8c) in PNT1A cells treated with NP, while it did not inhibit gene expression of *Cyclin E* (Fig. 8b) or *IL1- $\beta$*  (Fig. 8e). E2 significantly affected gene expression up-regulating *Cyclin D* (Fig. 8a) and *Ki67* (Fig. 8c). This induction was strongly inhibited by ICI (Fig. 8a,c).

### 4. Discussion

EDCs are receiving more and more attention by scientific community, due to their ability to mimic endogenous hormones and altering the metabolism of organisms (De Falco et al., 2014). NP belongs to the family of xenoestrogens and its estrogenic activity is well documented both with *in vitro* (de Weert et al., 2008; Soto et al., 1991; White et al., 1994) and *in vivo* studies (Laws et al., 2000; ter Veld et al., 2008). In this work we seek to investigate the effects of NP on human prostate cells PNT1A, precisely evaluating its estrogenic action in terms of proliferation, interaction with ERs and gene analysis of genes involved in cell cycle regulation and aberrant physiology of prostate. Few studies investigated the effects of NP on prostate, both on cellular and animal models. These findings did not characterize any molecular mechanisms and results often appear to be in conflict. For example, Lee et al. showed that NP is able to reduce the weight of the prostate in rats, in a dose dependent manner (Lee, 1998). In the same study, authors demonstrated that ICI reverted this effect. Similarly, Who et al. obtained the same result but with higher concentration of NP (Woo et al., 2007). Moreover, gestational exposure to NP was reported to affect prostate morphology in F1 rats (Jie et al., 2010). In contrast, other authors failed to demonstrate any adverse effects on rat prostate caused by NP (Inaguma et al., 2004; Odum and Ashby, 2000). These contrasting data may be explained by the time and the way of NP dosage, as well as by the duration of treatment. We conducted our experiments also testing the effects of E2 and ICI.



**Fig. 2.** Localization of ER $\alpha$  after 2 and 6 h of exposure to NP and E2. E2 promotes translocation of ER $\alpha$  to the nucleus at both time of treatment, while NP at 6 h. PNT1A cells were plated in chamber slide under hormone deprived conditions. 24 h later, cells were treated with  $10^{-6}$  M NP or  $10^{-6}$  M E2. ER $\alpha$  (Alexa Fluor 488) and nuclear staining (Höchst) were analyzed by immunofluorescence. Scale bar 10  $\mu$ m.



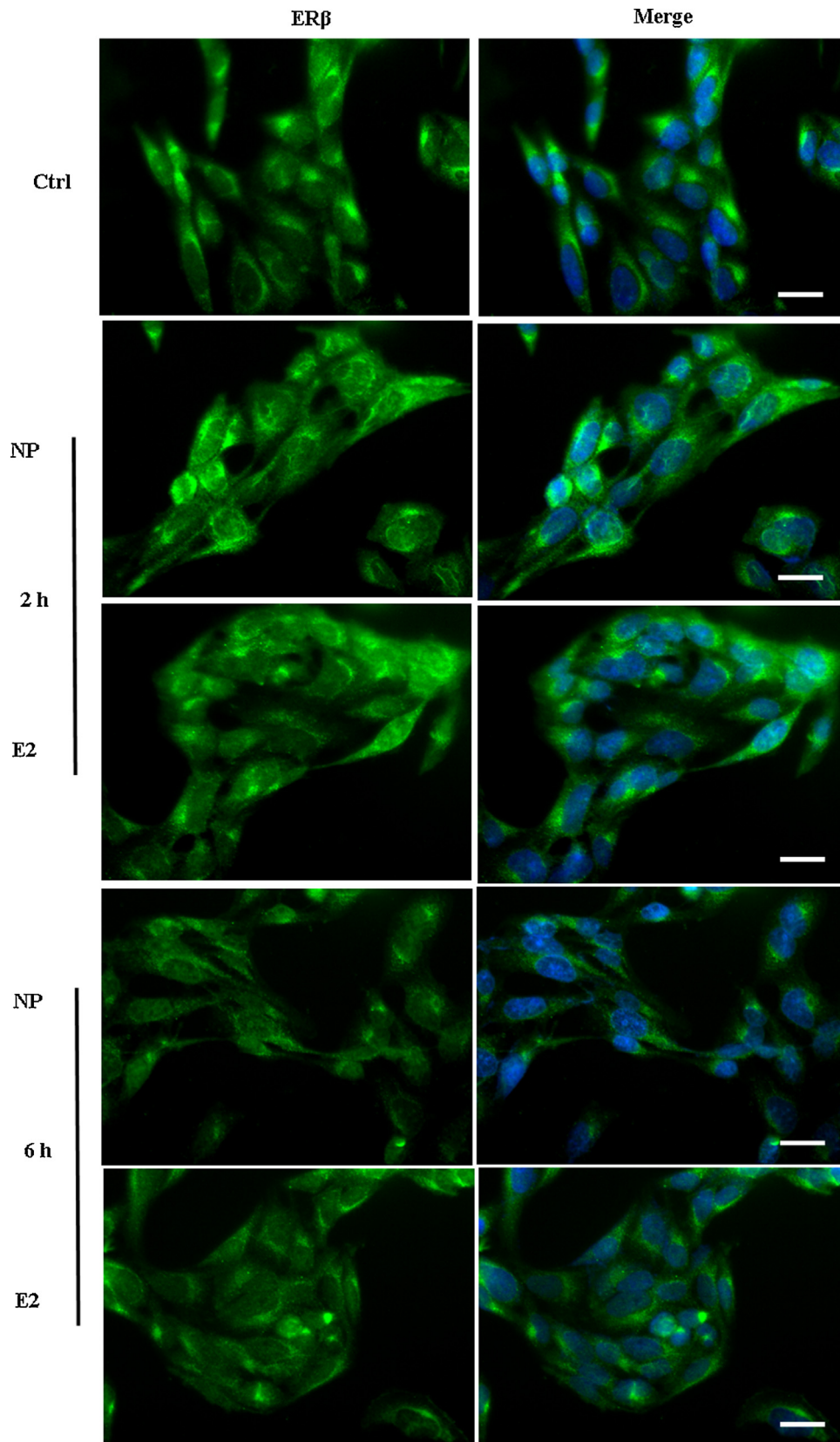
**Fig. 3.** Localization of ER $\alpha$  after 2 and 6 h of exposure to NP and E2 combined with ICI (+). ICI inhibits ER $\alpha$  nucleus translocation at both time considered. Cells were pre-treated for 1 h with  $10^{-5}$  M ICI and then treated with  $10^{-6}$  M NP or  $10^{-6}$  M E2. ER $\alpha$  (Alexa Fluor 488) and nuclear staining (Höchst) were analyzed by immunofluorescence. Scale bar 10  $\mu$ m.

We showed that NP stimulated PNT1A cells proliferation after 24 h of exposure at  $10^{-6}$  M as well as E2 did. However, E2 affected PNT1A cells proliferation also at lower concentrations. Interestingly, ICI reverted NP and E2 proliferative stimuli. These results suggest that NP may interfere with normal cell cycle of PNT1A cells as reported by other authors in different cell lines (Choi et al., 2011; Manente et al., 2011). Recently, Gan et al. (Gan et al., 2015) in human prostate epithelial cell line RPWE-1 showed a reduction in cell viability after 24 h exposure to NP. The incongruity between our results and those of Gan et al. can be explained by the different concentrations used. In this study, no effects were reported at  $10^{-6}$

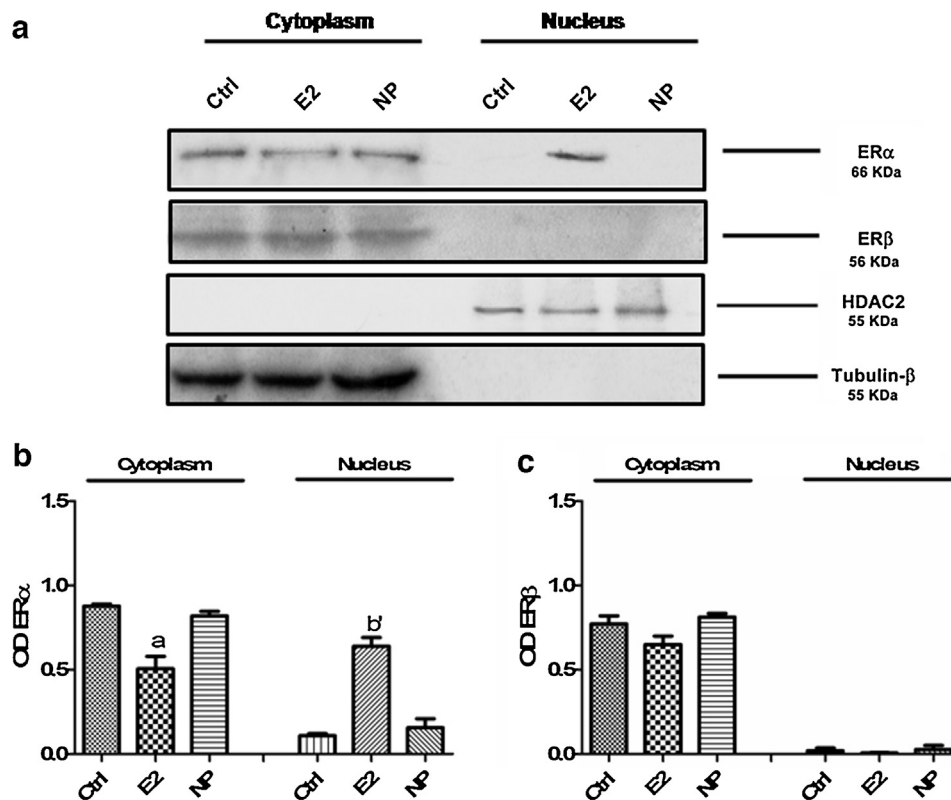
NP and the decrease in cell proliferation appeared to be evident only at high concentration ( $10^{-5}$ – $10^{-4}$  M).

With two different approaches, we studied the localization of ER $\alpha$  and ER $\beta$  in a time course analysis testing the concentrations ( $10^{-6}$  M) that showed the greatest effect on cell proliferation. We demonstrated that E2 induced cytoplasm-nucleus translocation of ER $\alpha$  at both time tested, while NP only after six hour of exposure. Surprisingly, both E2 and NP did not affect ER $\beta$  localization. ICI inhibited the ER $\alpha$  translocation observed with NP and E2 alone.

Both proliferation and localization data confirm the estrogenic activity of NP. However, the greatest biological responses showed



**Fig. 4.** Localization of ER $\beta$  after 2 and 6 h of exposure to NP and E2. In all the images ER $\beta$  is localized in cellular cytoplasm. PNT1A cells were plated in chamber slide under hormone deprived conditions. 24 h later, cells were treated with  $10^{-6}$  M NP or  $10^{-6}$  M E2. ER $\beta$  (Alexa Fluor 488) and nuclear staining (Höchst) were analyzed by immunofluorescence. Scale bar 10  $\mu$ m.



**Fig. 5.** Western blot analysis and nuclear and cytoplasmic quantification of ER $\alpha$  and ER $\beta$  after 2 h of exposure to  $10^{-6}$  M NP and E2. E2 induces nucleus translocation of ER $\alpha$  while ER $\beta$  was found only in the cytoplasmic proteins (a). The graphs represented the Optical density (OD) ratio of ER $\alpha$  and ER $\beta$  normalized to the OD of Tubulin  $\beta$  for cytoplasmic proteins and to the OD of HDAC2 for nuclear proteins (b). (N=3 separate experiments) a, response significantly different than the cytoplasmic control ( $P < 0.05$ ); b', response significantly different than the nuclear control ( $P < 0.01$ )

by E2 can be explained by its best binding affinity with ERs compared to NP (Laws, 2000). Notwithstanding the lowest responses to NP, we can speculate that its proliferative effects, as for E2, is mediated by the interaction with ER $\alpha$ . On this issue, it is well known the role of ER $\alpha$  in cellular proliferation process and carcinogenesis of prostate, while some authors suggested that ER $\beta$  seems to be involved in apoptosis of prostate cells (Hartman et al., 2012). For example, it has been reported in knockout ER $\beta$  mice prostatic hyperplasia and cancer (Weihua et al., 2001). Moreover, in mice and rats prostate, like in human, it has been shown that ligands that interact with ER $\beta$  may reduce proliferation (Ellem and Risbridger, 2009; Omoto et al., 2005; Prins and Korach, 2008).

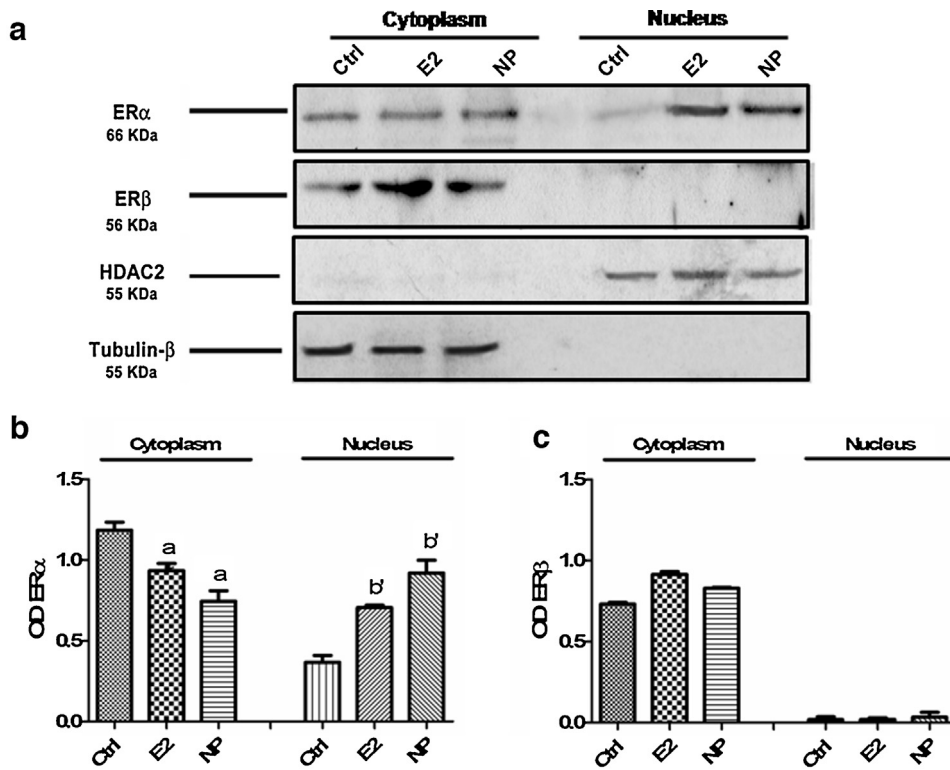
To assess if the presence of ER $\alpha$  in the nucleus led to the activation of transcription, we analyzed gene expression of E2 gene targets also known to be deregulated in pathological state of the prostate. We demonstrated that NP was able to upregulate *Cyclin D*, *Cyclin E*, *Ki67* and *IL1- $\beta$*  gene expression whereas E2 induced upregulation only of *Cyclin D* and *Ki-67*. Moreover, we showed that up-regulation of *Cyclin D* and *Ki67* is mediated by estrogen signaling pathways, while the induction of *Cyclin E* and *IL1- $\beta$*  involved an estrogen independent pathways, since ICI did not revert this induction. These results of gene expression are in agreement with the showed induced proliferation caused by NP and E2. In this regard, it is well known that *Cyclin D* and *Cyclin E* promoting G1/S phase transition of cell cycle (Kastan and Bartek, 2004) and are often used to screening the carcinogenic potential of EDCs (Diamanti-Kandarakis et al., 2009). Moreover, it has been reported that overexpression of *Cyclin D*, *Ki67*, *Cyclin E* and *IL1 $\beta$*  are a prognostic factors prostate cancer (Aaltomaa et al., 2006; De

et al., 2013; Sfanos and De Marzo, 2012). In particular, down-regulation of *Cyclin D* and *Cyclin E* has been shown to inhibit tumor progression in different prostate cell lines (Alagbala et al., 2006; Chinni et al., 2001; Lin et al., 2015). In addition, the null effect showed for *p53*, the hallmark of apoptosis, reinforce the idea that NP has a role in promoting prostate cells survival (Gan et al., 2011; Gumulec et al., 2014).

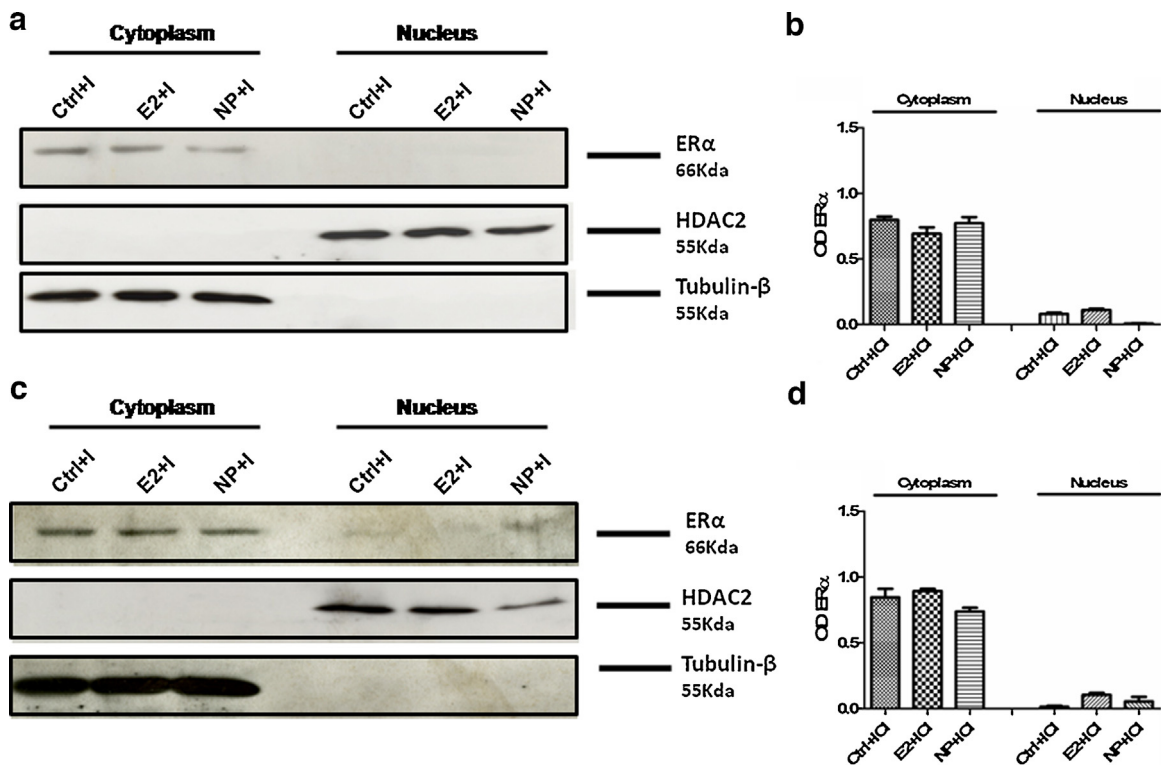
This data, together with the less estrogenic activity of NP in terms of proliferation and interaction with ER $\alpha$  strongly suggest that NP may activate also other transduction pathways, such as the G-protein coupled estrogen receptor GPR30 (Filardo et al., 2007) or the androgen receptor (ARs) (Wang et al., 2010). In this regard, in a recent study, Kim et al. showed that NP induced human tumorigenic prostate cells (LNCaP) proliferation in a pathway mediated by ARs (Kim et al., 2016). In addition to ARs mediate pathways, in epithelial non tumorigenic cells DU145, Gan et al. (Gan et al., 2014) provided evidences about the involvement of GPR30 in NP induced proliferation, when used at concentration from  $10^{-8}$  to  $10^{-6}$  M. Interestingly, according with our data and despite the different prostate model used, in both studies, the concentration that showed the best effects was  $10^{-6}$  M in both the studies.

Considering the exposure level of NP,  $10^{-6}$  M represents a high dose of NP, found rarely in human samples, such as in breast milk and in umbilical cord blood (Chen et al., 2005). Furthermore, it is far from the tolerable daily intake limit values for NP (Woo et al., 2007). Despite these considerations, it should not be underestimated the adverse effects that NP may exert when combined with circulating estrogens or with other EDCs which we are simultaneously exposed. This phenomena, commonly known as

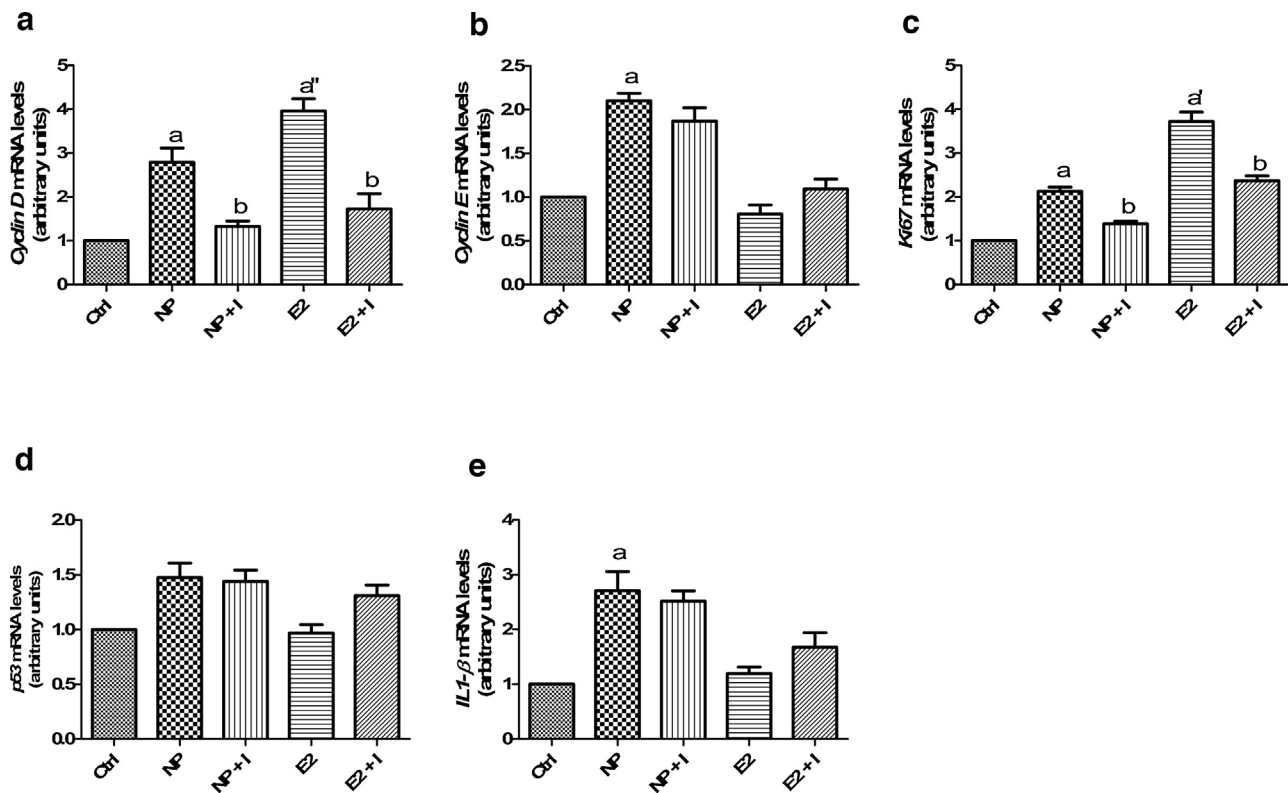




**Fig. 6.** Western blot analysis and nuclear and cytoplasmic quantification of ERα and ERβ after 6 h of exposure to  $10^{-6}$  M NP and E2. ERα was found in nuclear proteins after E2 and NP treatment while ERβ was found only in the cytoplasmic proteins (a). The graphs represented the Optical density (OD) ratio of ERα and ERβ normalized to the OD of Tubulin β for cytoplasmic proteins and to the OD of HDAC2 for nuclear proteins (b). (N = 3 separate experiments) a, response significantly different than the cytoplasmic control ( $P < 0.05$ ); b', response significantly different than the nuclear control ( $P < 0.01$ ).



**Fig. 7.** Western blot analysis and nuclear and cytoplasmic quantification of ERα after pre-treatment with  $10^{-5}$  M ICI 182,780 (+I). ICI inhibits cytoplasm-nucleus translocation of ERα after 2 h of exposure to  $10^{-6}$  M E2 (a–b) and after 6 h of exposure to  $10^{-6}$  M E2 and NP (c–d). The graphs represented the Optical density (OD) ratio of ERα normalized to the OD of Tubulin β for cytoplasmic proteins and to the OD of HDAC2 for nuclear proteins (b, d). (N = 3 separate experiments) no significant differences.



**Fig. 8.** RT-qPCR analysis after 24 h of exposure to  $10^{-6}$  M NP and E2, alone or in combination with  $10^{-5}$  M ICI (+I). *Cyclin D* (a), *Cyclin E* (b), *Ki67* (c), *p53* (d) and *IL1β* (e) relative mRNA levels were normalized using *GAPDH* as housekeeping gene. (N=3 separate experiments) a, response significantly different than the vehicle control ( $P < 0.05$ ); a', response significantly different than the vehicle control ( $P < 0.01$ ); a'', response significantly different than the vehicle control ( $P < 0.001$ ) b, response significantly different than cells without ICI ( $P < 0.05$ ); b', response significantly different than cells without ICI ( $P < 0.01$ ).

“cocktail effect” is a feature widely accepted for EDCs risk management (Bergman et al., 2012).

In conclusion, we demonstrated that NP acts on PNT1A cells with similar effects if compared to E2, probably mediated by ER $\alpha$  and it may be involved in a deregulation of cell cycle, leading to aberrant proliferation of prostate epithelial cells, which in turn may contribute to pathological states, including cancer. We are also providing data on the dual role of ERs in prostate cells.

Notwithstanding the findings of this study, further evidences remain to be investigated in order to best characterize the risk of NP exposure for prostate diseases. In addition, more cellular and *in vivo* models will be needed. However, our data may help epidemiologists to consider and monitoring the association between NP and prostate pathologies.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tox.2016.05.024>.

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