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FAST TRACK

Upregulation of manganese superoxide dismutase (SOD2) is a common pathway for neuroendocrine differentiation in prostate cancer cells

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Despite improvements in diagnosis of advanced prostate cancer (PCa), treatment is not efficient and 5-year survival is still low. Initially, the less abundant of cell types, neuroendocrine cells (NE), are involved in regulatory process but their physiological role is not fully understood. Among others, an increase in NE cells along with tumor progression has been commonly reported but their role in tumorigenesis or the molecular mechanisms of transdifferentiation is still a matter of debate. We have used human PCa cells (LNCaP) induced to differentiate to NE cells with several stimuli: androgen withdrawal, cyclic AMP or treatment with the antioxidant pineal hormone melatonin. PCa patients' specimens were also analyzed by western blotting and by immunocytochemistry. NE-like LNCaP cells express high levels of mitochondrial superoxide dismutase (MnSOD/SOD2) in addition to NE markers. MnSOD upregulation is mediated by NFκB transcription factor, mainly through p65 translocation into the nuclei. More importantly, overexpression of MnSOD induces the rise of NE-markers in LNCaP cells, showing that MnSOD upregulation might be instrumental for NE differentiation in PCa cells. Furthermore, MnSOD is highly expressed in advanced tumors of patients' when compared with control, nonpathological samples or with low-grade tumors, along with the presence of synaptophysin, a common NE marker. Also, fluorescence immunohistochemical analysis revealed that MnSOD colocalizes with NE markers in most of NE cells observed in PCa specimens. The present findings indicate that MnSOD is essential for NE transdifferentiation and mediates in part the differentiation process, which appears also to be critical *in vivo*.

Key words: prostate cancer; neuroendocrine differentiation; MnSOD; antioxidant; androgen deprivation

Prostate cancer (PCa) is the most common noncutaneous malignancy and one of the major leading causes of cancer-related deaths among men in the Western world.[1] Although now diagnosed at earlier stages due to improvements in screening methods, PCa is still responsible for more than 27,000 deaths (2007) in the United States. Normal epithelial prostate cells need androgens to regulate an adequate proliferation:death ratio. Thus, commonly the starting therapy in primary PCa includes androgen deprivation, which, initially, halts proliferation and triggers apoptosis in most cancer cells therefore inducing tumor regression.[2] However, with time most patients eventually relapse with a hormone refractory prostate cancer (HRPC). Interestingly, along with the androgen-independent status, an increase in the number of neuroendocrine cells has also been reported in some studies. Tumor cells present at this stage are usually extremely resistant to conventional therapies and finally metastasize.[3] Yet, research efforts during the last 50 years have not improved significantly the life expectancy of PCa patients.[4]

Adult prostate gland possesses basal (proliferating) cells, secretory (luminal) cells and the less abundant (<0.1%) neuroendocrine (NE) cells. NE cells, included in the diffuse endocrine system, share common features with neurons and are present at all the stages from normal benign to prostate adenocarcinomas.[3] They are thought to exert regulatory functions through the production of several substances,[5] although their exact role has not been elucidated yet. A large heterogeneity in NE cells, suggesting the presence of several populations with different functions has been proposed.[6] The presence of NE markers in prostatic tumors is usually associated with poor prognosis and NE differentiation has been related to PCa progression.[7] However, their clinical relevance and their involvement on tumor growth is still a matter of debate. Epithelial PCa cells can differentiate into NE-like cells and this transdifferentiation is hypothesized to be involved in the development of the advanced disease.[8] To date, several signals have been reported to induce NE transdifferentiation in cultured PCa cells, including androgen withdrawal,[8] cAMP, interleukin (IL)-6,[9] antioxidants[10,11] or vasoactive intestinal peptide (VIP).[12] These studies have brought some light about the molecular mechanisms involved in this intriguing process and yet many aspects remain elusive.

The treatment of HRPC has only limited efficacy, mainly due to the resistance to apoptosis.[13] Several factors including bcl-2 overexpression have been implicated as survival factors for PCa.[14] Overexpression of anti-apoptotic Bcl-2 plays an important role in the aggressiveness of HRPC and their resistance to therapy.[11] The acquisition of an androgen-independent growth is associated with an increased expression of bcl-2 and PCa cells overexpressing bcl-2 show a significant growth advantage after castration in nude mice.[15] Transcription factors such as NFκB and AP-1 can also act as survival factors. The activation of NFκB in response to chemotherapy or radiation, as the main pathway involved in inducible resistance to apoptosis, is mainly mediated by an increase of Bcl-2 and Bcl-xL which can suppress the activation of caspases.[15]

Since Harman established the free radical theory of aging,[16] reactive oxygen/nitrogen species (ROS/RNS) have been implicated in several pathologies, including cancer.[17] ROS/RNS are produced during respiratory burst or in pathological conditions. However, cells are armed with a set of enzymes in charge of cleaning up the excess of ROS/RNS. Superoxide dismutases are a family of antioxidant enzymes responsible for the detoxification of superoxide anion ($O_2^{\bullet -}$) free radicals, giving H_2O_2, which is further removed by catalases or glutathione peroxidases. There are 3 major forms of superoxide dismutases (SOD), namely cytoplasmic CuZnSOD (SOD1); mitochondrial, manganase-containg SOD (MnSOD, SOD2); and extracellular SOD (ECSOD, SOD3). Among these, MnSOD is crucial for cellular survival since mitochondria is the major source of $O_2^{\bullet -}$. It has been shown that MnSOD activity was

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diminished in transformed cells when compared with matched normal cells[18] and several groups have reported that antioxidant enzymes are altered in cancer cells.[19] Activities of SOD1/2 or catalase are usually low in cancer cells[20] suggesting a potential role for MnSOD as a tumor suppressor gene.[21] More recently, it has been reported a control of androgen receptor function by $O_2^{\bullet -}$ in PCa cells.[22] The major goal of the work presented here was to study the potential involvement of MnSOD in NE transdifferentiation in PCa cells stimulated with different strategies as well as to investigate the potential colocalization of NE markers and MnSOD in PCa patients' samples with different Gleason score.

Material and methods

Chemicals

Activated charcoal, dextran, dimethyl sulfoxide (DMSO), cytochrome *c*, xanthine, xanthine oxidase, N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate (db-cAMP), phalloidin-TRITC conjugate, kanamycin, ampicillin, antibiotic-antimycotic cocktail and DAPI were purchased from Sigma-Aldrich (St. Louis, MO). Lipofectamine 2000 transfection reagent and FBS were obtained from Invitrogen (Carlsbad, CA). Fluoromount G fluorescent mounting media was provided by Southern Biotech (Birmingham, AL). Melatonin was purchased from Merck KGaA (Darmstadt, Germany). All cell culture media employed were provided by Lonza (Basel, Switzerland).

Cell culture

Androgen-dependent human prostate LNCaP cells and normal immortalized epithelial prostate cells, PNT1A, were purchased from ECACC and cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM pyruvate, 10% FBS, 15 mM HEPES, ampicillin and kanamycin. Androgen-independent human prostatic carcinoma cell line PC-3 was grown in DMEM/F-12 (1:1) culture media supplemented with 2 mM L-glutamine, 10% FBS and 1% antibiotic-antimycotic cocktail containing penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml). Androgen-independent human prostate carcinoma cell line DU-145 were cultured with DMEM supplemented with 2mM L-glutamine, 10% FBS, 15 mM HEPES and 1% antibiotic-antimycotic cocktail. Cell lines were maintained at 37°C in a humidified, 5% CO_2 environment. For experiments, cells were seeded at a initial density of 25,000 cells/ml.

Treatments

Melatonin or db-cAMP were added directly in complete culture medium from a 1,000× stock solution, respectively. Controls received vehicle (0.1% DMSO). For androgen withdrawal, cells were washed twice with Dulbecco's phosphate buffer saline (DPBS) and then charcoal stripped-FBS (cs-FBS) was used to replace complete regular FBS. All treatments were applied 1 day after seeding.

Patients' samples processing

Eleven radical prostatectomy specimens were used in the present study. Specimens were surgically resected at the "Hospital Universitario Central de Asturias" or at the "Hospital de Cabueñes" between 2003 and 2006 with the Institutional Review Board Approval for Guidelines on Ethical Procedures, following the World Medical Association-Declaration of Helsinki protocol. Informed consent was obtained from each patient. Six of the specimens were fixed in formalin and embedded in paraffin following routine histological protocols; for western blotting studies, the other 5 samples were frozen in liquid nitrogen and stored at −80°C. In all the cases except for control sample, due to the high levels of PSA detected during screening, specific biopsies were obtained and adenocarcinomas were detected. In the cases included in the study, prostate tumors were organ-confined and radical prostatectomies were performed before any treatment. According to the clinical records consulted, none of these patients received neoadjuvant hormone therapy. All the samples were classified using the Gleason score by the Pathology Service at the University Hospital. To select tumor samples for western blotting, samples were cryo-preserved by immersion in Tissue-Tek. Twelve micrometer cryosection were obtained using a cryostate; after staining with H&E, section then was observed under the microscope to select and dissect the tumor area, which was exclusively used for blotting assay.

Immunocytochemistry

LNCaP cells were seeded on Thermanox coverslides (Nunc-Thermo Fisher Scientific, Roskilde, DK). After treatment, cells were fixed in 1% formaldehyde in DPBS (pH 7.4), permeabilized with DPBS containing 0.1% triton X-100 and reacted with anti-MnSOD antibody (Calbiochem, Darmstadt, Germany), anti-CuZn-SOD antibody (Calbiochem) or anti-synaptophysin (SYN) antibody (Neomarkers, Fremont, CA, USA) using 1:300, 1:100 and 1:200 dilution respectively. Anti-sheep or anti-rabbit secondary anti-bodies (Calbiochem) were used at a 1:1000 final dilution. 3-3′-Diaminobenzidine was used as chromogen (Dako, Glostrup, Denmark).

PAGE and immunoblotting

After treatment, cells were washed twice with ice-cold DPBS and then, lysed in RIPA lysis buffer containing phosphatases/proteases inhibitors as described. Proteins were transferred to PVDF membranes (Millipore, Billerica, MA) and then incubated overnight at 4°C with anti-MnSOD (Upstate-Millipore, Lake Placid, NY), anti-CuZnSOD (Calbiochem), anti-neural specific enolase (NSE, Santa Cruz Biotechnology, Santa Cruz, CA), anti-SYN (Thermo-Scientific, Cheshire, United Kingdom) or anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were then reacted to the appropriate secondary antibodies and developed using a chemiluminescent reagent (Millipore, Billerica, MA).

For PAGE and western blotting analysis of PCa patients, frozen radical prostatectomy samples were homogenized as detailed above. Lysates were subjected to SDS-PAGE, electrophoretically transferred and membranes were then probed to anti-Bcl-2 (Calbiochem) or the same antibodies mentioned above. Primary antibodies were visualized by binding of horseradish peroxidase-conjugated anti-sheep (Calbiochem), anti-mouse (Calbiochem) or anti-rabbit (Cell Signaling, Beverly, MA) and detected as described above.

Western images were scanned and densitometry was calculated using Scion Image software (downloaded at www.scioncorp.com). Ratios between indicated proteins and GAPDH, used as housekeeping, were calculated and values were standardized *vs*. control samples.

Enzymatic assay

For this purpose, cells were plated in 150 mm plates (3 plates per experimental group). After washing with DPBS, cell lysates were prepared by breaking them in DPBS with 3 freeze–thaw cycles. SOD activity was measured according to the method previously described.[23] One unit of SOD activity is defined as the amount of SOD required to inhibit the rate of cytochrome *c* reduction by 50%.

Transient MnSOD transfection

Transfection was carried out on LNCaP cells when they were at a confluency of 60–70%. Lipofectamine 2000 transfection reagent was maintained according to the manufacturer's instructions for 4 hr. Transfected cells were then collected after 1, 2 or 4 days after transfection. Plasmid expression vector pcDNA3 containing the human MnSOD gene and the corresponding empty vector were kindly provided by Dr. Wenqing Sun (University of Iowa, Iowa City, IA).

Immunofluorescence microscopy

Tissue sections were processed following routine protocols. Heat epitope retrieval was performed in 10 mM citrate buffer (pH 6.0). For double immunostaining, sections were first incubated with anti-MnSOD antibody, anti-CgA (NeoMarkers) or SYN overnight at 4°C and then, incubated with Cy-3-conjugated anti-rabbit and with Cy-2-conjugated anti-sheep secondary antibodies. Confocal immunofluorescence micrographs were captured in a Leica TCS-SP2-AOBS confocal microscope.

For phalloidin staining, cells were grown on Thermanox coverslides, fixed in 70% ethanol for 10 min at −20°C and incubated with phalloidin-TRITC conjugate (1 μg/ml) for 90 min at RT and counterstained with DAPI (10 ng/ml). Cover slides were finally mounted using fluoromount G and observed with a confocal microscopy.

Statistical analysis

Results shown represent the mean ± standard error of the mean (SEM) of 3 samples per group. In the blotting results, a representative of 3 different experiments is shown. Differences among means were calculated using a one-way ANOVA, followed by a Student–Newman–Keuls test. Values were considered statistically significant when $p < 0.05$.

Results

NE-like cells show higher levels of MnSOD

The differentiation process of PCa cells into a NE-like phenotype has been classically studied by using specific cell culture models (*i.e.* LNCaP) which are able to transdifferentiate into NE-like cells under certain treatments. Additionally, it has been previously shown that the endogenous antioxidant melatonin induces LNCaP differentiation.[10,24] To compare the morphological and biochemical features of antioxidant-induced NE-like cells with other classically employed inducers, human androgen-dependent LNCaP cells were cultured for 7 days in the presence of vehicle or with the antioxidant melatonin (MEL), cs-FBS-containing media (cs-FBS) or dibutyryl cyclic AMP (db-cAMP). After treatment, cells were stained with TRITC-conjugated phalloidin (PHA) to visualize cytoskeleton and then counterstained with DAPI for fluorescence confocal microscopy. As it can be observed, antioxidant-induced differentiated LNCaP cells show a similar (but not identical) morphological pattern when compared with NE-differentiated cells induced by other stimuli. Thus, whereas controls (CON) show a spindle-shape, fibroblast-like morphology with wide short projections (usually shorter than the cell body size), all 3 agents used in this study induced both, a reduction in cell proliferation and the display of a NE-like morphology, showing long neurites, frequently with several branches which usually doubles in length the cell body. Morphological changes caused by the antioxidant MEL is accompanied with an overexpression in NE markers such as synaptophysin (SYN), which was confirmed by immunocytochemical studies. Control cells did not show positive staining for SYN, whereas MEL, or db-cAMP treated LNCaP cells show a very high levels of SYN immunostaining. Androgen-deprived cells, cs-FBS, show a lesser increase in SYN levels (Fig. 1*a*).

It is reported that redox may be crucial in the proliferation–differentiation-cell death fate and MnSOD is important in maintaining redox state in mitochondria.[25] Given that melatonin is an antioxidant agent either as a direct scavenger or indirectly by modulating the expression of antioxidant enzymes or glutathione content,[26] we decided to study the levels of MnSOD in melatonin-induced NE-differentiated cells and compared with control, non-differentiated cells. MnSOD but not CuZnSOD immunoreactivity increased in NE-like differentiated cells, either with MEL, cs-FBS or with db-cAMP (Fig. 1*a*). This result was confirmed by western blotting and by SOD activity assay. In both cases, MEL or cs-FBS treatment induced a significant increase in MnSOD protein levels, with a rise of more than 2.5-fold (Fig. 1*b*). Additionally, total SOD activity was also significantly higher in differentiated cells. Interestingly, in db-cAMP treated cells we did not observe any change in total SOD activity, although MnSOD protein levels were higher when compared with control cells, (Fig. 1*c*). As it could be anticipated, no differences in CuZnSOD immunoreactivity (Fig. 1*a*, right panel) were observed among experimental groups and same results were confirmed by western blotting.

MnSOD upregulation during NE differentiation is mediated by NFκB activation

MnSOD is an important enzyme involved in tumorigenesis and it has been related to redox-regulated activity of androgen receptor. This led us to study by western blotting the basal expression of MnSOD in several prostate-derived cell lines, including normal, androgen-dependent or androgen-independent cancer cells. Interestingly, MnSOD levels in PNT1A normal human prostate cells were at least 10-fold higher than in LNCaP cells and between 2- and 5-fold than in androgen-independent cells. PC-3 or DU-145 PCa cells display higher MnSOD protein levels than LNCaP cells (Fig. 2*a*). These results prompted us to study the role of NFκB as a potential transcription factor involved in MnSOD expression during NE differentiation, as androgen-dependent PCa cells usually display low levels of NFκB activation whereas androgen-independent cells, especially PC-3 cells show a constitutive activation of NFκB binding activity. This factor is implicated in the transcriptional control of MnSOD in several cell types. To evaluate NFκB activation after transdifferentiation, we studied the levels of NFκB components, p50 and p65, in nuclei and cytosol from NE-like LNCaP cells. As it is shown, p65 is translocated into the nuclei of NE trans-differentiated cells. All the differentiating agents induced an increase of p65 protein content in nuclei. However, in the case of p50, only androgen withdrawal induced a significant translocation into the nuclei, whereas melatonin and cAMP only induced p50 translocation very slightly (Fig. 2*b*).

Overexpression of MnSOD induces biochemical features of neuroendocrine differentiation

Once we observed that during NE differentiation triggered by any stimuli, MnSOD is upregulated and considering that this enzyme exerts a critical role by metabolizing the excess of superoxide anion formed in mitochondria, and given that $O_2^{\bullet -}/H_2O_2$ are regulating signals in proliferation/differentiation, our next goal was to study whether MnSOD upregulation might be instrumental for NE differentiation. Thus, we studied the impact of MnSOD overexpression in LNCaP cells by transfection with pcDNA containing the human MnSOD sequence. MnSOD overexpression caused an elevation of this enzyme levels by 50% after 1 day of treatment and this increase was maintained for up to 4 days (roughly up to 7-fold increase) after transfection. Interestingly, this elevation in MnSOD was concomitant with a transient increase in NSE (after 1 day) and more significantly with a rise of SYN levels. In fact, cells overexpressing MnSOD showed a very rapid and sustained elevation in SYN. After 4 days of overexpression, SYN levels were over 20 times higher than in control or mock-transfected cells, thus confirming that elevation of this critical antioxidant enzyme is sufficient to trigger at least some of NE features (Fig. 3).

MnSOD and NE markers levels increase with tumor progression and both colocalize in samples from prostate cancer patients

In an attempt to study the potential role of MnSOD in NE differentiation in PCa samples, we used specimens obtained from cancer patients. Samples were classified according to their pathological features based on the Gleason score. As a reference, we used a prostate sample from a non-cancer patient. We used a score of 7 to separate low/medium from high grade prostate tumors. Western blotting results showed that CuZnSOD or Bcl-2 protein levels changed randomly when compared to control sample and showed no correlation with tumor progression (Fig. 4). On the contrary, MnSOD protein levels evidenced a good correlation with

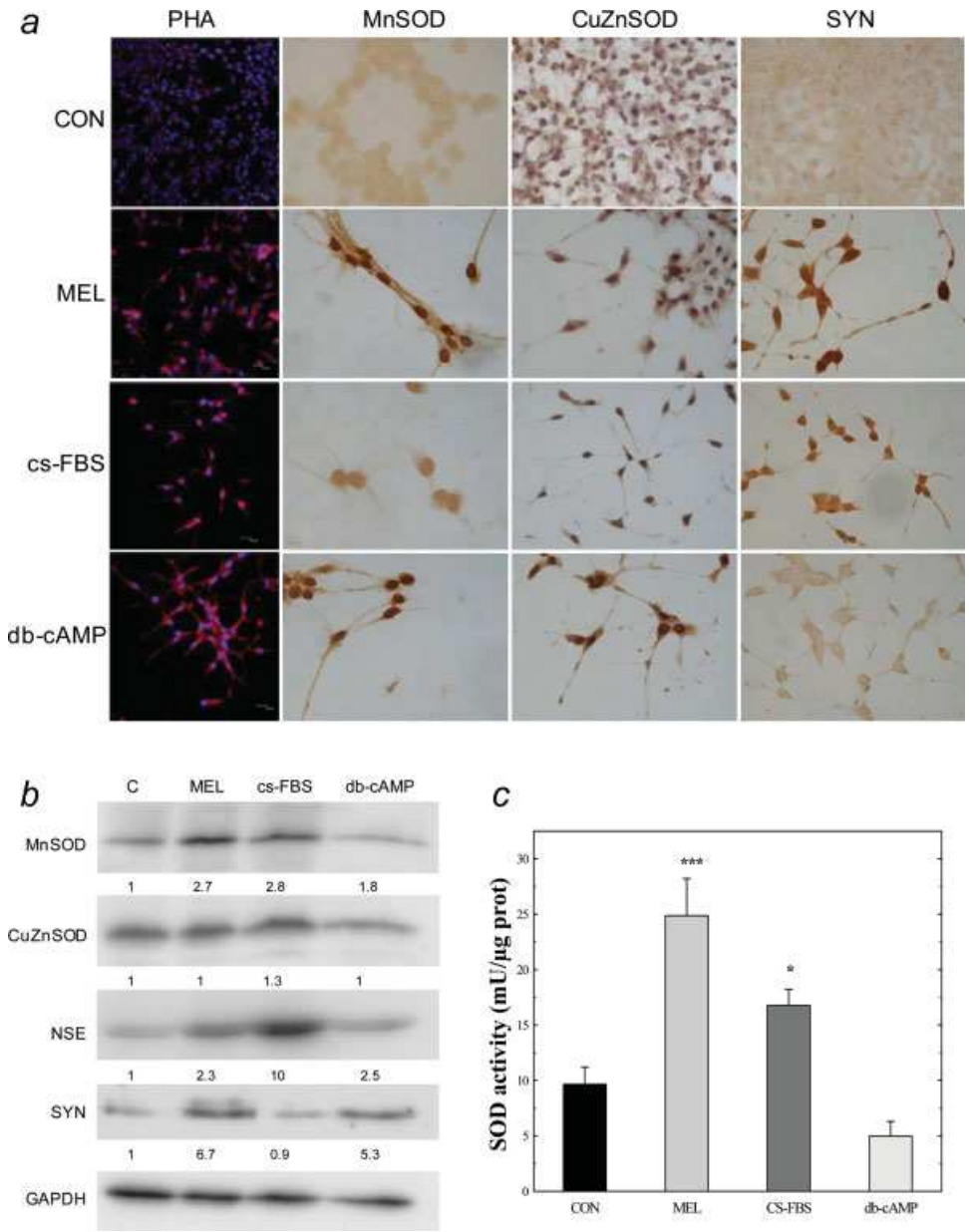

Figure 1 – NE-differentiated cells induced by different stimuli show increased protein levels and activity of MnSOD. Androgen-dependent, human prostate LNCaP cells were induced to differentiate with the antioxidant melatonin (MEL), by androgen withdrawal with cs-FBS or with db-cAMP. (*a*) Cells stained with TRITC-conjugated phalloidin for F-actin visualization (red fluorescence, left panel) and counterstained with DAPI or immunostained with MnSOD, CuZnSOD or the NE marker SYN. Original magnification ×200 (PHA-TRITC) or ×400 (rest). (*b*) Western blot showing protein levels of MnSOD, CuZnSOD, NSE, SYN or GAPDH (house-keeping) in NE-like trans-differentiated LNCaP cells. Cells were treated with vehicle or with MEL, cs-FBS or db-cAMP for 7 days. Numbers represent the fold change observed in experimental group *vs.* control, once standardized with GAPDH protein levels. (*c*) Total SOD activity was measured as described in "Material and method". ***$p < 0.001$ *vs.* CON; *$p < 0.05$ *vs.* CON.

tumor progression as estimated by Gleason score. Therefore, control sample or low-grade tumor (G5) displayed a much lower MnSOD level than medium grade tumors (G7). More importantly, high-grade tumors (>7) showed a much higher MnSOD protein levels when compared with the rest of samples. Surprisingly, results obtained with different NE makers indicate a different pattern. Thus, whereas NSE levels did not increase with tumor progression, higher levels of SYN were clearly associated with tumor progression, although protein levels may vary even among patients with the same Gleason score (Fig. 4).

These results indicate that MnSOD and at least some NE markers (*e.g.* SYN) increased with tumor progression. This encouraged us to our next aim: to study whether MnSOD and NE markers colocalize in the same cellular types. For this purpose, samples from cancer patients were assayed for immunohistochemical localization of both, MnSOD and NE markers and studied with confocal microscopy. As it can be observed in Figure 5 samples from 4 patients showed a strong colocalization between MnSOD (green label, Figs. 5*b*, 5*e*, 5*h* and 5*k*) with classical NE markers used in pathological analysis for diagnostic purpose, namely CgA (Figs. 5*c* and 5*f*) or SYN (Figs. 5*i* and 5*l*) (both, red label). MnSOD was irregularly expressed in some, but not all glands, either in basal or in proliferating epithelial cells and occasionally in surrounding connective tissue. However, NE cells

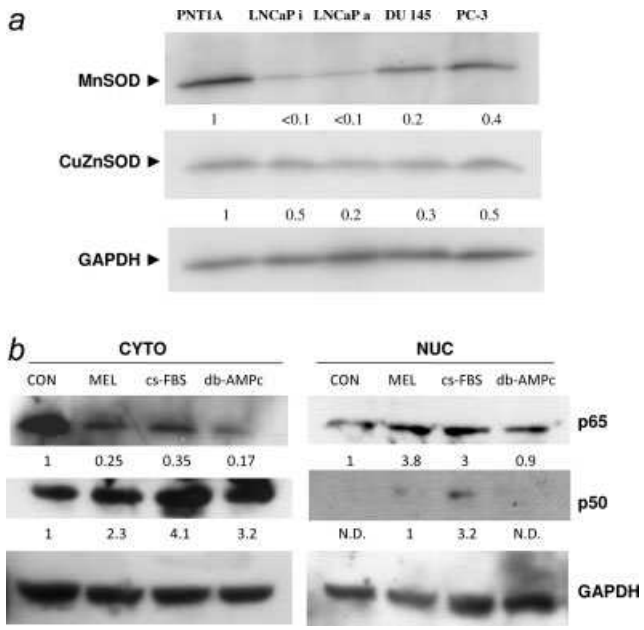

Figure 2 – The rise of MnSOD levels in prostate cancer cells is mediated by NFκB activation. (*a*) Western blot showing MnSOD and CuZnSOD levels in prostate-derived cell lines. Normal transformed human epithelial prostate cells PNT1A, androgen-dependent LNCaP at initial (LNCaPi) or advance (LNCaPa) passages and androgen-independent human prostate cancer cell lines PC-3 or DU-145 were collected in exponentially growing phase and then, total protein was subjected to western blotting for MnSOD, CuZnSOD or GAPDH. Numbers represent the fold change observed in experimental group *vs.* control, once standardized with GAPDH levels. (*b*) Western blotting showing levels of p65 and p50 in cytosolic and nuclei extracts from control or MEL, cs-FBS and db-cAMP-induced NE-like differentiated LNCaP cells. In both, GAPDH was used as a standard protein and numbers represent the fold change *vs.* control.

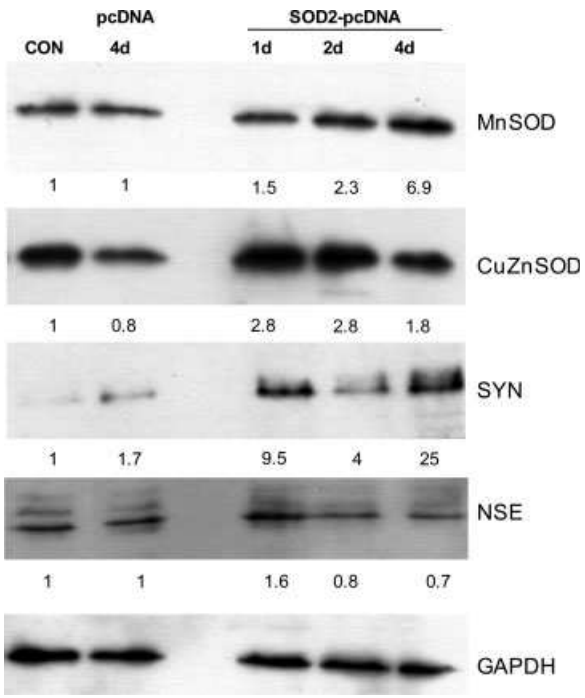

Figure 3 – MnSOD overexpression promote NE-differentiation markers expression. LNCaP cells were left untreated (CON), mock-transfected with the empty vector (pcDNA3) or transfected with a pcDNA3 containing the human MnSOD sequence (SOD2-pcDNA) and then cells were cultured for 1, 2 or 4 days; western blot shows levels of the indicated proteins. GAPDH served as house-keeping protein and numbers reveal the fold change observed *vs.* control after standardization.

always showed a higher MnSOD positive staining, which colocalizes with CgA or SYN, giving a orange-yellowish labeling of these cells when both images are superposed (arrows, left panels Figs. 5*a*, 5*d*, 5*g* and 5*j*). Colocalization was high with CgA (60% samples) and total (100%) with SYN.

Discussion

Data presented here show that increasing MnSOD/SOD2 protein and activity level is a common mechanism in NE differentiation in PCa cells. This was assessed by inducing these morphological and biochemical changes with 3 different stimuli, *i.e.* antioxidants, androgen withdrawal or chronic intracellular cAMP rise. MnSOD scavenges excess of $O_2^{\bullet -}$ mainly generated during electron leakage at the complex I, coenzyme Q or b-type cytochromes at the respiratory electron transport chain.[27] This specific and critical function in the mitochondrial matrix makes this enzyme essential for cell survival of aerobic organisms. In fact, MnSOD knockout mice die within the first days of postnatal development,[28] whereas some microorganisms upregulate MnSOD in the presence of oxygen,[29] pointing out the importance of controlling $O_2^{\bullet -}$ production in cells.

It is widely reported that high concentrations of ROS/RNS either induce cell death or mediates cell transformation and tumor growth.[23] In eukaryotic cells however, low concentrations of $O_2^{\bullet -}/H_2O_2$ have evolutionary acquired further physiological functions regulating cell cycle progression. Thus, low levels of ROS/RNS markedly affect the rate of proliferation influencing G0/G1 or quiescence-growth transition, primarily maintaining cyclin D1 levels.[29] By controlling $O_2^{\bullet -}/H_2O_2$ levels, MnSOD appears to be

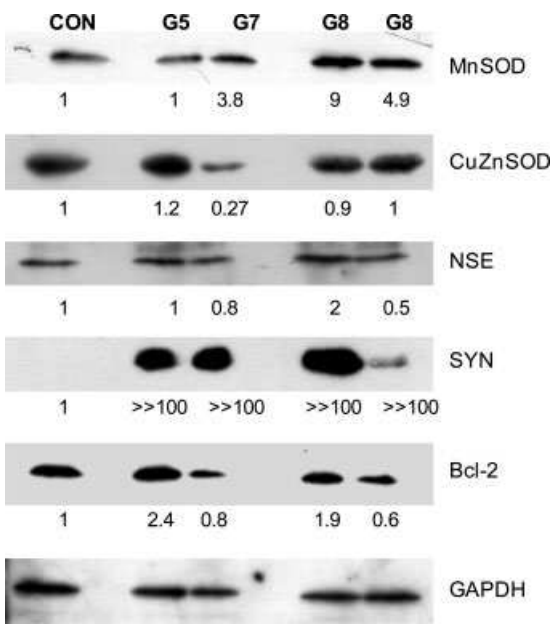

Figure 4 – MnSOD and NE markers protein levels increase with tumor progression in prostate cancer. Frozen prostate cancer specimens were obtained from a tumor bank and classified according to the Gleason score standard. Western blots show levels of the indicated proteins from either a control patient (CON) or from patients' specimens with different Gleason score (G5–G8) as indicative of tumor progression.

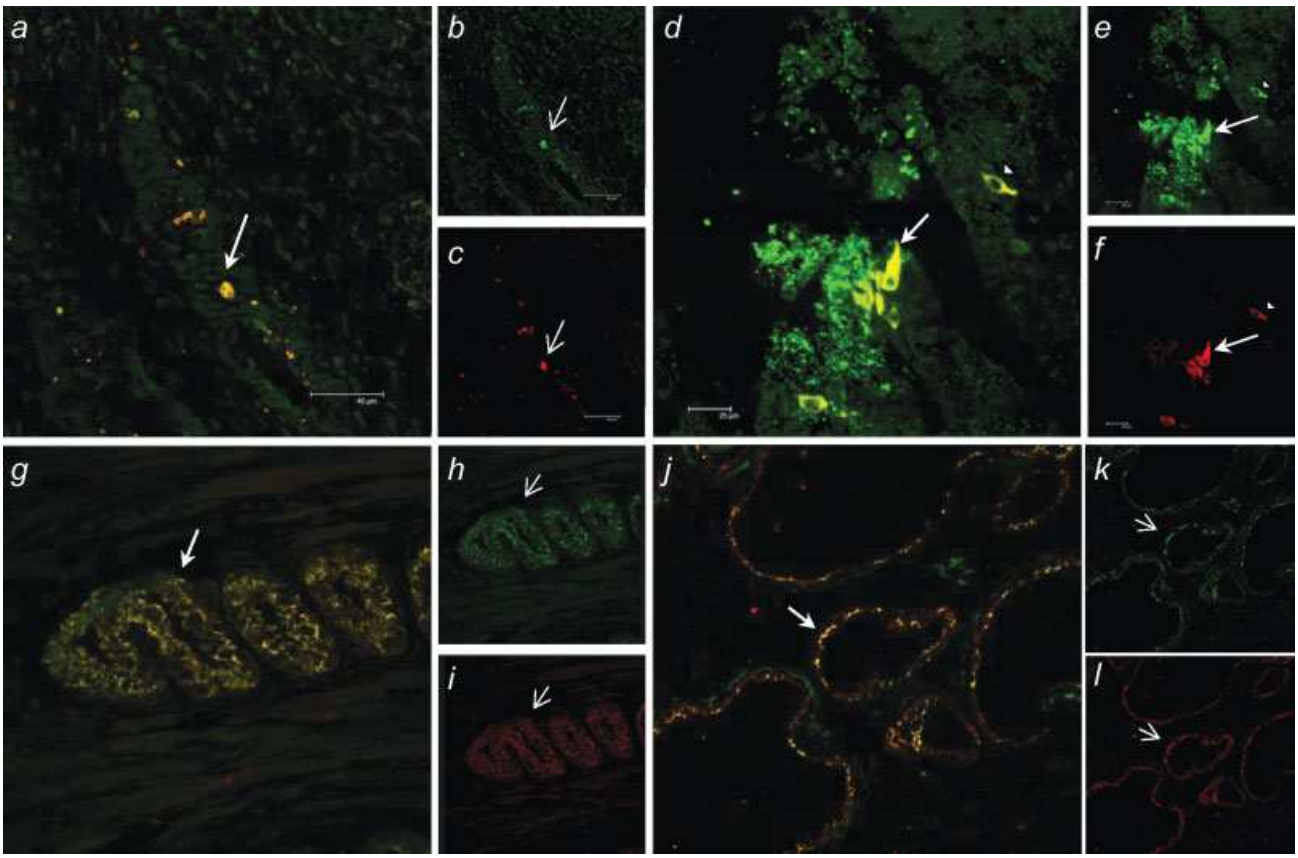

FIGURE 5 – MnSOD and NE markers colocalize in prostate tumor samples. After pathologic diagnosis, paraffin sections selected from advanced Gleason score (G8) specimens were immunostained with anti-MnSOD combined with secondary antibody conjugated with Cy2 (green fluorescence) or with NE markers CgA or SYN, combined with secondary antibody conjugated with Cy3 (red fluorescence). (*a–f*) Immunohistochemical sections stained with anti-MnSOD (*b, e*) or with anti-CgA (*c, f*) and the corresponding superposed images (*a, d*). (*g–l*) Immunohistochemical sections stained with anti-MnSOD (*h, k*) and anti-SYN (*i, l*) and the corresponding superposed images (*g, j*) showing the yellowish/orange cells expressing both proteins. Arrows and arrowheads indicate the same cells stained for anti-MnSOD, anti-CgA or anti-SYN and the corresponding superposed images.

a critical enzyme in cancer progression. Bravard and colleagues first proposed MnSOD as a potential tumor suppressor gene that might also be involved in differentiation.[25] First evidences came from studies in immortal rat embryo fibroblasts that undergo spontaneous differentiation into muscle and adipose cells, process that is accompanied with a significant increase in MnSOD activity.[30] This phenomenon can be extended to some fungi in which MnSOD expression is also associated with both oval and falcate conidia differentiation.[31] All-trans retinoic acid has been shown to induce differentiation in neuroblastoma cells through the activation of NFκB, which in turn induces MnSOD expression.[32] MnSOD expression has also been associated to the developing rat brain and liver.[33] However, those studies only show a temporal relationship between differentiation and MnSOD upregulation, demonstrating that this is an early event in the cell differentiation process. By using DNA transfection methods, St Clair and coworkers were the first group to report the direct role of MnSOD in mouse embryo fibroblasts differentiation and also in the differentiation program of fibrosarcoma cells.[34,35] Accordingly with those results, data presented here indicate that MnSOD is also essential for cell differentiation in PCa. Not only does the antioxidant enzyme level increase during NE transdifferentiation but, reciprocally, when overexpressed it also induces a significant increase in NE markers, indicating that MnSOD is instrumental in prostate NE transdifferentiation. Other intracellular key players may be additionally involved in triggering the differentiation process. Nevertheless, MnSOD overexpression appears to be upstream in the differentiating-signal pathway. All together evidences mentioned above account for MnSOD upregulation as a universal phenomenon in the differentiation process among several cell types.

The mechanism by which MnSOD controls cell differentiation has not been yet elucidated. Some insights about how this mitochondrial enzyme might control cell cycle have recently been provided.[36] Thus, a decrease in MnSOD concomitant with a rise in superoxide levels directs embryonic fibroblasts to proliferation by promoting cell cycle progression to G2+M, whereas an increase in the mitochondrial enzyme facilitates proliferating cells' transition to quiescence and increases the number of cells in G1. Therefore, higher activity of MnSOD indirectly moves the balance $O_2^{\bullet -}/H_2O_2$ toward the latter, keeping cells in G0/G1. Our data would confirm that MnSOD increase is an essential step not only for quiescence but to further proceed to NE-like transdifferentiation. Furthermore, differentiated cells display a higher resistance to pro-oxidants and to radiotherapy/chemotherapy treatments.[37] In addition, it is well documented that MnSOD upregulation provides protection against radiotherapy and chemotherapy,[38,39] which matches up the explanation for the resistance of NE cells. Accordingly, it has been reported that ionizing radiation triggers NE differentiation process in PCa cells.[39]

It had been previously reported that some antioxidants like silibinin or melatonin reduce cell proliferation.[24] Polyphenolic flavonoid silibinin induces G1 arrest by decreasing CDK and cyclin D1 and inducing p21 and p27 which is essential for its anti-proliferative

action in PCa.[40,41] Similarly, the indole melatonin reduces cell proliferation by increasing p21 and p53.[24] To some extent, both antioxidants seem to work similarly; they share p21 induction and prevention of NFκB activation induced by TNF, sensitizing PCa cell to apoptosis induced by this cytokine. Additionally, melatonin seems to regulate glutathione pool, therefore, controlling intracellular redox state. Interestingly, neither melatonin nor silibinin are able to induce cell death which makes a difference in the outcome triggered by other antioxidants.[42,43] On the contrary, both antioxidants redirect cells toward a transdifferentiation process that matches a NE-like phenotype.

Evidences obtained from PCa patients' specimens correlates with data obtained from cell culture studies, showing a strong colocalization between MnSOD and NE markers, *i.e.* CgA or SYN. In our study, stromal (in a minor extent) or epithelial other than NE cells show different degrees of MnSOD positive staining. However, most cells expressing NE markers show a strong coimmunoreactivity with MnSOD. This is reinforced by data obtained from immunoblots where it is shown how SYN levels, almost undetectable in the control, increase massively in samples diagnosed with a high Gleason score. The role of NE cells in normal or in pathologic prostate is not fully understood and their participation in PCa progression is a matter of debate. Although some authors associate the presence of an increased number of NE cells with tumor progression and consequently with a poor prognosis,[44] other suggest a more complex role for NE cells and PCa.[45] Pure NE prostate tumors are very rare (<0.1%) and highly aggressive but tumors with focal NE differentiation are more common. In the latter, the increased number of NE cells in advanced prostate tumors are frequently observed in HRPC after long-term androgen deprivation therapy.[46] It has been reported that several factors secreted from NE cells induce cell proliferation and the expression of oncogenes but cell culture studies have shown no effect or even an inhibitory effect on cell growth and a lack of aggressiveness in normal epithelial cells. This has led to some investigators to suggest that these NE cells observed in PCa may have a different origin and should be considered as NE-like PCa cells and not NE cells. Most transdifferentiation observed in PCa cells like models reported here are reversible, showing a difference with the NE-like PCa cells *in vivo*. Further details of a possible heterogeneity in these NE-like cells induced by different agents are needed.

All together, the evidences reported here demonstrate a reciprocal relationship between MnSOD upregulation and NE differentiation, which may explain the high resistance of these cells to conventional therapies. Additionally, a possible critical role of MnSOD in tumor progression in PCa deserves further studies.

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