OPNb an OPNc splicing isofoms mediate prostate cancer cell pro-survival features by a PI3K/AKT and caspase 3 independent pathway

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BACKGROUND

We previously demonstrated that PC3 cells overexpressing Osteopontin-b (OPNb) and Osteopontin-c (OPNc) splicing isoforms (SI) present higher proliferative rates as compared to OPNa and empty vector (EV) control clones, even under serum starvation. However, no difference on cell death rates were observed among PC3 cells overexpressing the three OPN-SI. Besides, PC3 cells overexpressing OPNc which were treated with an anti-OPNc neutralizing antibody,

OBJECTIVES

The present work aims to investigate the main molecular and signaling pathways by which OPNb and OPNc mediate PCa cell survival and growth.

METHODOLOGY

- PC3 cells were treated with $1x10^{-6}$µg/mL of docetaxel (DXT), which was used as an *in vitro* cell model to induce cell death;
- Cell survival mechanisms have been investigated by cell morphology using phase contrast microscopy, crystal violet and MTT cell growth and viability assays as well as by using specific inhibitors of PI3K (LY294002) and ERK (PD98059) signaling pathways;
- Cell death and survival markers have been analyzed by immunoblot and FACS analysis.

RESULTS

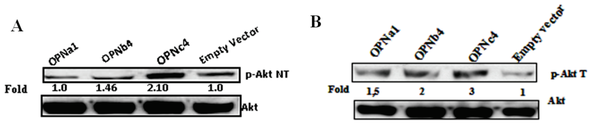


Figure 1: OPNb and OPNc splicing isoforms activate Akt phosphorylation. PC3 cells overexpressing OPNb and OPNc promoted a 1,45 and 2-fold increase on phospho-Akt (Ser473), respectively, before DXT treatment (A). After DXT treatment (B), we observed a 2 and 3-fold increase on Akt phosphorylation, respectively for OPNb and OPNc overexpression clones, as compared to cells overexpressing OPNa and empty vector (EV) control.

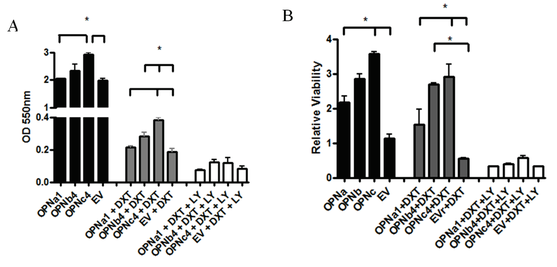


Figure 2: PI3K signaling is possibly mediating OPNb and OPNc pro-survival roles. (A) Cells overexpressing OPNb and OPNc isoforms showed higher cell growth rates, when compared to OPNa and EV control. After DXT treatment all OPN-SI overexpressing clones and EV control decreased cell growth rates, but OPNb and OPNc clones are more resistant to DXT induced cell death, when compared to control clonesclones, as measured by . When PC3 cells overexpressing the three OPN isoforms were simultaneously treated with PI3K inhibitor LY294002 (LY) and DXT, there was a significant inhibition on cell proliferation, as compared to those cells treated only with DXT. Of note, LY treatment reversed OPNb and OPNc differential resistance to DXT induced cell death. (B) Before DXT treatment, cells overexpressing OPNb and OPNc showed higher viability, when compared to OPNa and empty vector (EV) controls, as measured by MTT staining assays. When these PC3 cells clones were treated with DXT, these was no significant effect on cell viability, although OPNb and OPNc clones still presented higher viability than OPNa and EV clones. When these cells were treated with both DXT and LY, there was a significant decrease on the viability of cells overexpressing all three OPN-SI and empty vector control cells. No significant difference on cell viability among cells overexpressing the distinct OPN-SI has been observed. These data indicated that PI3K signaling is mediating OPNb and OPNc pro-survival roles on PC3 cells. Test representative of three independent experiments. * $p < 0.05$. OD = optical density at 550nm of crystal violet. The black and gray bars indicate of the number of proliferating or viable cells not treated or treated with DXT, respectively. The white bars indicate of the number of proliferating or viable cells after simultaneous DXT and LY treatment.

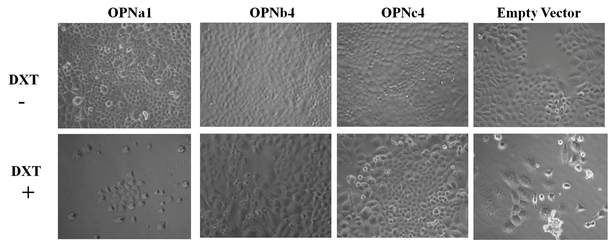
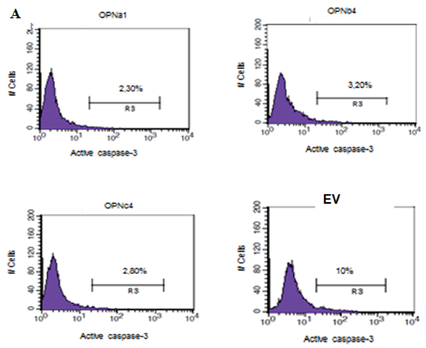


Figura 3: OPNb and OPNc PC3 overexpressing cells present higher resistance to DXT induced cell death. Cells overexpressing OPNb and OPNc isoforms showed resistance to DXT induced cell death, as indicated by higher growth rates in these PC3 cell clones, as compared to OPNa and EV control. as measured by phase contrast microscopy. These data further corroborate to previous indications that OPNb and OPNc splicing isoforms activate cell growth and survival in PC3 cells. The cell morphology was analyzed without DXT (DXT -) or with DXT (DXT +) treatment.



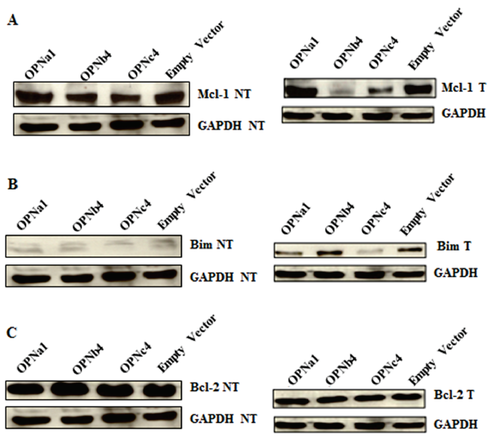


Figure 5: Expression profiling of anti and pro-apoptotic proteins as a result of OPN isoforms overexpression in PC3 cells. As an additonal approach to test whether OPNb and OPNc promote PC3 cell survival by inhibiting apoptosis, we then evaluated the expression patterns of some pro and anti-apoptotic proteins by immunoblot before (NT) and after (T) docetaxel treatment. PC3 cells overexpressing the three OPN-SI and EV control presented similar Mcl-1 expression levels. After DXT treatment, cells overexpressing OPNb and OPNc showed a significant inhibition of Mcl-1 expression, when compared to OPNa and control clones (A). Bim expression was upregulated after DXT treatment in all three OPN-SI and EV clones. However, after DXT treatment, OPNc overexpression clone showed a significant Bim downregulation, when compared to the other OPN-SI and EV clones (B). OPN-SI and EV presented a downregulation of Bcl-2 expression after DXT treatment, but all DXT-treated tested clones showed similar Bcl-2 expression levels (C).

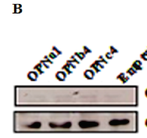


Figure 4: OPN-SI overexpression clones present no difference on caspase 3 acitivity. Considering that apoptosis evasion is one of the main events involved on tumor cell survival, we then investigated some of putative mechanisms by which the isoforms differentially promote the survival of PC3 cells. The apoptotic index was evaluated using an antibody against active caspase-3 by flow cytometry (A) and immunoblot assays (B). After treatment with DXT for 48 hours, PC3 cells overexpressing the three OPN-SI and the EV control presented similar levels of caspase-3 activity, as measured FACS analysis. Similar caspase-3 activity levels for the three OPN-SI and EV control were also observed by immunoblot analysis. These results suggest that OPNb and OPNc mediated cell survival is not due to differential inhibition of caspase-3 induced cell apoptosis. Images are representative of two independent experiments.

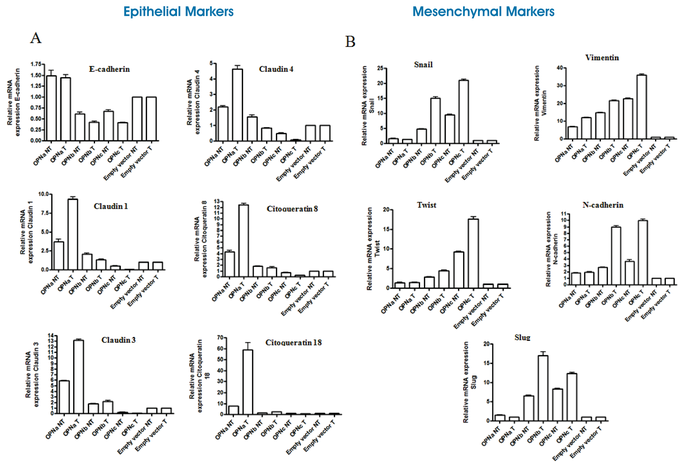


Figure 6: Cells overexpressing OPNb and OPNc isoforms seems to active Epithelial-Mesenchyme Transition (EMT). In order to investigate EMT as one of the cellular and molecular mechanisms by which OPNb and OPNc activate cell survival, we then tested whether classical EMT markers are differentially expressed among OPN-SI overexpressing cells before and after DXT treatment. OPNb and OPNc clones downregulate the expression of epithelial, while upregulate mesenchymal markers, as compared to OPNa and control clones. The GAPDH amplification was used as a control for template amount. Relative mRNA expression represents the increase of expression level of each epithelial and mesenchymal EMT markers in relation to empty vector control. Tests are representative of two independent experiments.

CONCLUSIONS

As a whole, our data indicate that OPNb and OPNc isoforms activate PC3 pro-survival features, which seem to be mediate by PI3K/Akt signaling. In addition, the pro-survival roles seems to be caspase 3 independent, but otherwise favored by pro-proliferative factors, such as those potentially modulated by Mcl-1 and active EMT.

Projeto Gráfico: Serviço de Edição e Informação Técnico-Científica / INCA