INVESTIGATION OF MOLECULAR MECHANISMS UNDERLYING NUCLEAR XIAP FUNCTIONS IN BREAST CANCER GROWTH AND CHEMORESISTANCE

Bruna dos Santos Mendonça[1], Deborah Delbue[1], Marcela Cristina da Silva Robaina[1], Pedro Ivo Lucena[2], Renata Ramalho Oliveira[2], Joao Paulo de Biaso Viola[2], Caio Almeida Batista de Oliveira[3], Felipe Roberti Teixeira[3], Raquel Ciuvalschi Maia[1], Gabriela Nestal de Moraes[1]

[1]Laboratório de Hemato-Oncologia Celular e Molecular; Programa de Hemato-Oncologia Molecular, INCA, RJ, Brazil.

[2]Programa de Imunologia e Biologia Tumoral, INCA, RJ, Brazil.

[3]Departamento de Genética e Evolução, Universidade Federal de São Carlos (UFSCar), SP, Brazil.

Introduction and objective: Evasion from apoptosis is one of the hallmarks of cancer. X-linked inhibitor of apoptosis protein (XIAP) is known to modulate the apoptotic balance by binding to and inhibiting caspases as well as ubiquitinating target proteins. XIAP is mainly found at the cytoplasm, but recent data link nuclear XIAP to poor prognosis in breast cancer. A previous study from our group demonstrated that nuclear expression of XIAP induced resistance and increased proliferative capacity of breast cancer cells, in a RING-dependent manner. Interestingly, nuclear expression of XIAP identified a subset of hormone receptor-negative breast cancer patients with an even shorter survival **(Figure 1)**. Here, we aimed to explore the mechanisms underlying nuclear XIAP role in cell proliferation and resistance, trying to identify possible partners that might cooperate to these oncogenic functions.

Material and method: MCF-7 human breast-derived cells were transfected with wild-type and mutant forms of XIAP: $XIAP^{H467A}$ (lack of ubiquitin ligase activity), $XIAP^{\Delta RING}$ (RING deletion) and $XIAP^{NLS\ C\text{-}term}$ (Insertion of a Nuclear Localization Signal at the C-terminal end) **(Figure 2)**. MCF-7 cells stably overexpressing XIAP variants were generated by doxycycline-inducible Tet-On system **(Figure 7)**. The expression and localization of XIAP and potential interaction partners were investigated by subcellular fractionation, Western blotting and immunofluorescence (confocal analysis). XIAP activity on NF-KB promoter was investigate in HEK293T cells by the luciferin-luciferase assay.

Results and conclusion: Initially, we investigated the mechanisms mediating the effect of XIAP at the nucleus by looking for proteins known to interact with XIAP. Our results show that Survivin, c-IAP1 and c-Myc expression were not similarly increased in $XIAP^{NLSC\text{-}term}$ overexpressing cells **(Figure 3)**. Although c-Myc global expression remained unaffected, confocal analysis revealed that XIAP overexpression, at both nucleus and cytoplasm, led to nuclear exclusion of c-Myc in a RING-independent manner **(Figure 4)**. We also found that p50 subunit of NFkB had expression increased in $XIAP^{NLS\ C\text{-}term}$ overexpressing cells, however these effects were not followed by an induction in NFkB transcriptional activity **(Figure 5)**. Interestingly, the pattern of ubiquitination in K63, but not K48 ubiquitin chains, was increased following overexpression of $XIAP^{NLSC\text{-}term}$, pointing to nuclear signaling transduction **(Figure 6)**.

In conclusion, our data suggests that the oncogenic effects mediated by nuclear XIAP are not associated with Survivin, cIAP-1, NFkB and c-Myc expression and might trigger nuclear signaling instead of degradation of target proteins. Experiments involving XIAP stable transfectants are ongoing **(Figure 8)** and will enable a better understanding of nuclear XIAP mechanisms in breast cancer growth and chemoresistance.

Keywords: Breast cancer; XIAP subcellular localization; Drug resistance; Prognosis

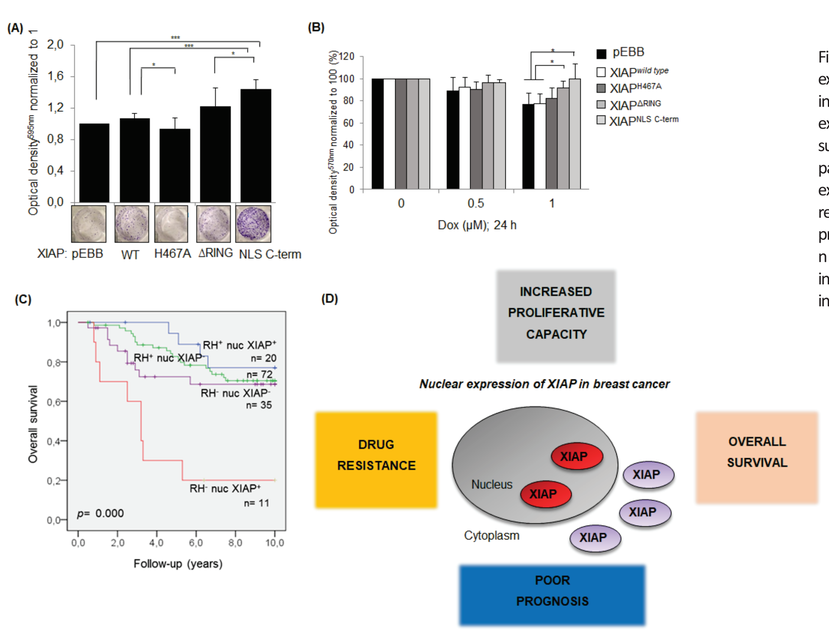

Figure 1: Association between nuclear expression of XIAP and malignant features in breast cancer. (A, B, C and D) XIAP expression can be found in different subcellular compartments in cell lines and patient samples. In the nucleus, XIAP expression is associated with a drug resistance phenotype and a increased proliferative capacity in vitro. Additionally, nuclear XIAP expression is an independent predictor of poor prognosis in breast cancer patients.

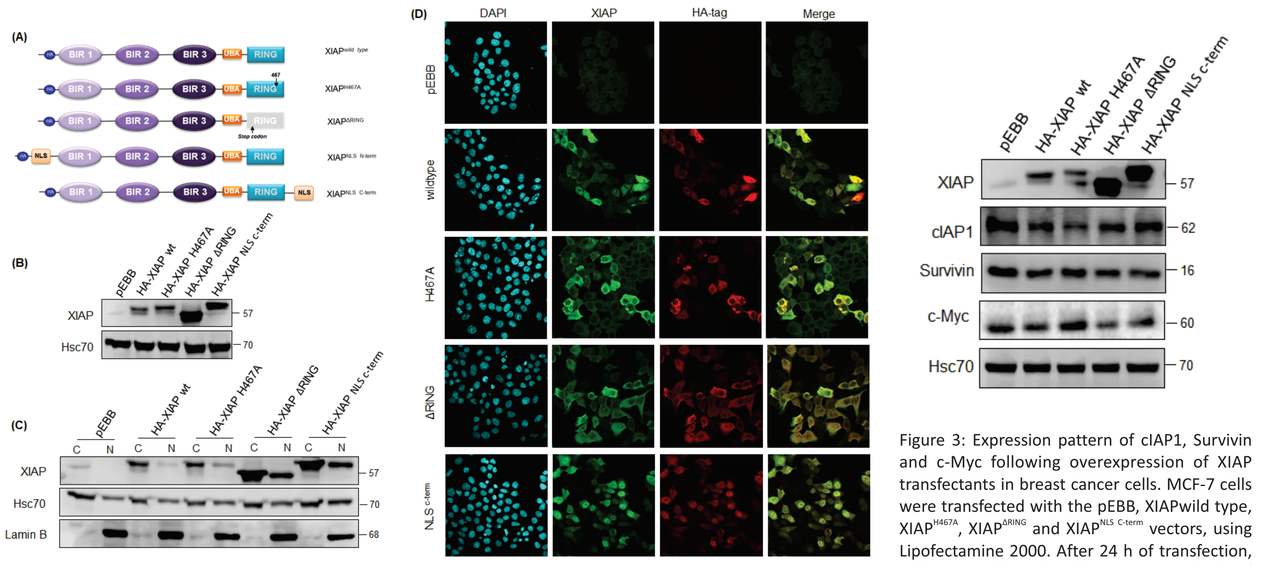

Figure 2: XIAP expression and subcellular localization in MCF-7 cells overexpressing the vectors pEBB, $XIAP^{wild\ type}$, $XIAP^{H467A}$, $XIAP^{\Delta RING}$ and $XIAP^{NLS\ C\text{-}term}$. MCF-7 cells were left to adhere in petri dishes (10cm) for 24 h and thereafter, were transfected with the pEBB, $XIAP^{wild\ type}$, $XIAP^{H467A}$, $XIAP^{\Delta RING}$ and $XIAP^{NLS\ C\text{-}term}$ vectors (A), using Lipofectamine 2000. (B) The levels of XIAP expression in XIAP-transfected cells were examined by Western blotting . (C) MCF7 cells had their cytoplasmic and nuclear fractions separated by NE-PER kit (Thermoscientific). (D) Immunofluorescence analysis indicates the subcellular localization in XIAP-transfected cells. Lamin B was used as a nuclear constitutive control, while Hsc70, as cytoplasmic constitutive control. C: cytoplasm; N: nucleus.

Figure 3: Expression pattern of cIAP1, Survivin and c-Myc following overexpression of XIAP transfectants in breast cancer cells. MCF-7 cells were transfected with the pEBB, XIAPwild type, $XIAP^{H467A}$, $XIAP^{\Delta RING}$ and $XIAP^{NLS\ C\text{-}term}$ vectors, using Lipofectamine 2000. After 24 h of transfection, cells were collected and the expression levels of XIAP, cIAP1, Survivin and c-Myc in XIAP-transfected cells in whole cell lysates were examined by Western blotting . Hsc70 was used as a constitutive control.

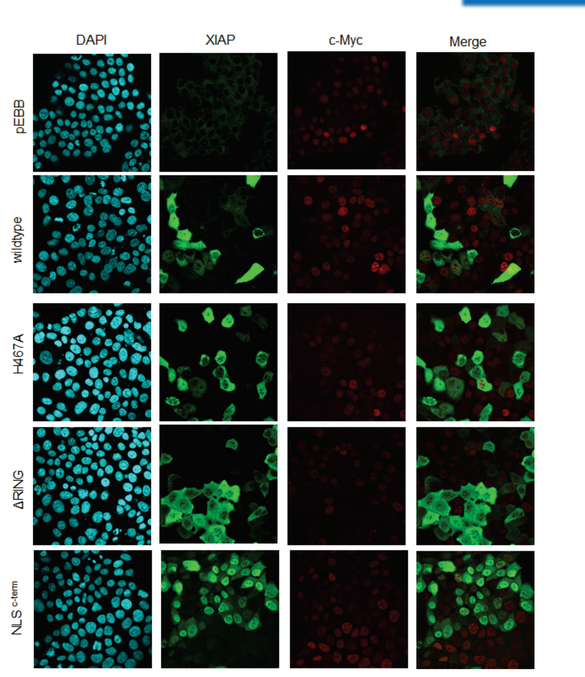

Figure 4: Analysis of c-Myc subcellular localization in XIAP-transfected cells. MCF-7 cells were transfected with the pEBB, $XIAP^{wild\ type}$, $XIAP^{H467A}$, $XIAP^{\Delta RING}$ and $XIAP^{NLSC\text{-}term}$ vectors (A), using Lipofectamine 2000. Cells were fixed and submitted to XIAP and c-Myc staining by immunofluorescence.

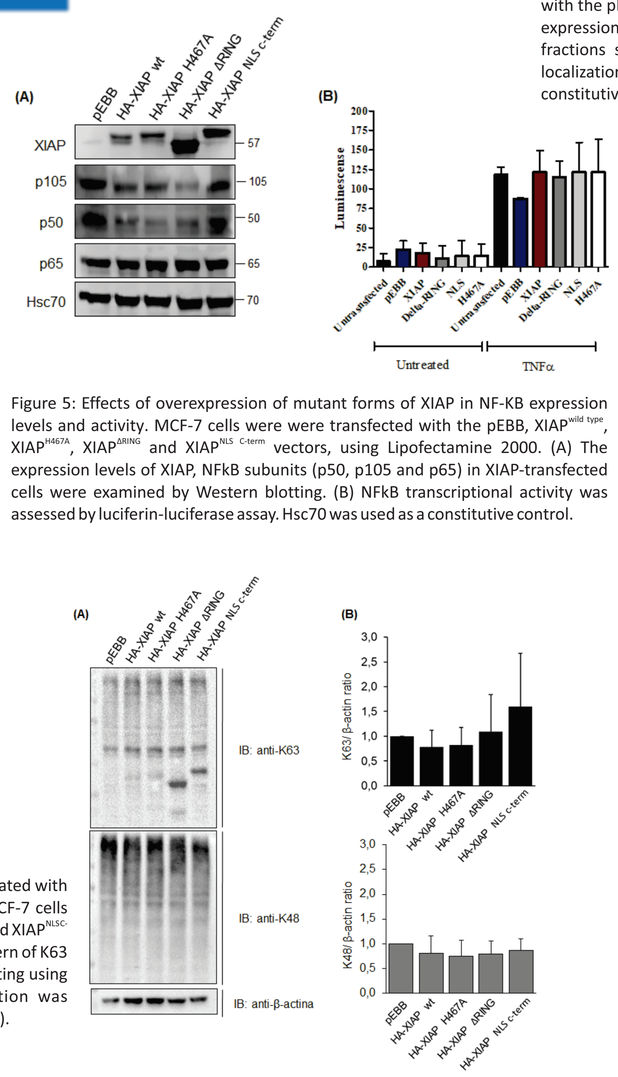

Figure 5: Effects of overexpression of mutant forms of XIAP in NF-KB expression levels and activity. MCF-7 cells were were transfected with the pEBB, $XIAP^{wild\ type}$, $XIAP^{H467A}$, $XIAP^{\Delta RING}$ and $XIAP^{NLS\ C\text{-}term}$ vectors, using Lipofectamine 2000. (A) The expression levels of XIAP, NFkB subunits (p50, p105 and p65) in XIAP-transfected cells were examined by Western blotting. (B) NFkB transcriptional activity was assessed by luciferin-luciferase assay. Hsc70 was used as a constitutive control.

Figure 6: Overexpression of mutant forms of XIAP is associated with an increase in K63, but not K48-linked ubiquitination. MCF-7 cells were transfected with pEBB, $XIAP^{wildtype}$, $XIAP^{H467A}$, $XIAP^{\Delta RING}$ and $XIAP^{NLSC\text{-}term}$ vectors, using Lipofectamine 2000. The expression pattern of K63 and K48 ubiquitin chains were measured by Western blotting using K63 and K48-specific antibodies (A) and quantification was performed following normalization against β-actin levels (B).

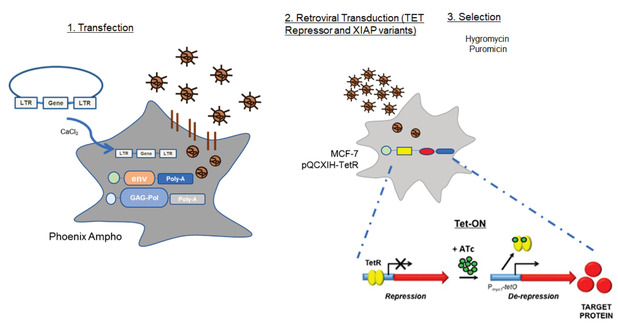

Figure 7: The schematic representation of the generation of MCF-7 cells stably expressing XIAP transfectants by doxycycline-inducible Tet-On system. 1- Vírus packaging cells Phoenix Ampho were transfected with calcium cloride to produce retroviral particles containing vectors for TET repressor or XIAP variants. 2- After, the supernatant was collected and retroviruses were added to MCF-7 cells to retroviral transduction. 3- Clones stably expressing XIAP and the TET repressor were selected with puromycin and hygromycin, prior to doxycycline treatment.

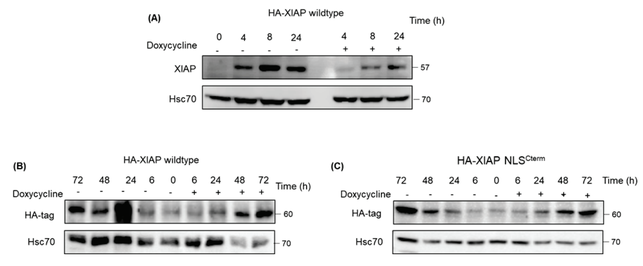

Figure 8: Generation of MCF-7 cells stably expressing XIAP forms by doxycycline-inducible Tet-On system. Clones stably expressing XIAP and the TET repressor were selected with puromycin and hygromycin, prior to doxycycline treatment. MCF-7 cells stably expressing the wildtype were exposed to doxycycline for 0, 4, 8 and 24 hours and harvested for Western blotting analysis of XIAP (A). After reselection to TET repressor with hygromycin, cells stably expressing wildtype (B) and NLS^{Cterm} (C) forms of XIAP were exposed to doxycycline for different times and harvested for Western blotting analysis of HA-tag labelling. Hsc70 was used as a constitutive control.

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