AKT-MEDIATED REGULATION OF FOXK2 TRANSCRIPTION FACTOR CONTRIBUTES TO DOXORUBICIN RESISTANCE IN BREAST CANCER

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INTRODUCTION AND OBJECTIVES

Breast cancer has the highest incidence and mortality rates among women in the world. Recently, our group has demonstrated that FOXK2 transcription factor modulates drug sensitivity in breast cancer cells through transcriptional activation of *FOXO3a*. On the other hand, drug-resistant cells and poor outcome patients show constitutively high protein levels of FOXK2, suggesting that post-translational modifications might be inactivating FOXK2 functions, contributing to drug resistance. We then performed an *in silico* analyses and found the oncogenic kinase AKT (isoform 1) as the main possible regulator of FOXK2 **(Figure 1)**. Thus, our objective is to investigate the role of AKT phosphorylation in the regulation of FOXK2 expression and assess the impact of AKT-FOXK2 regulatory axis on drug resistance in breast cancer.

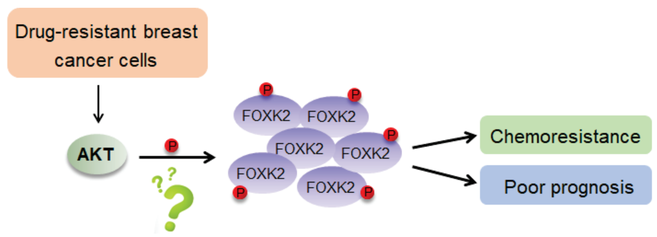

Figure 1 – Study hypothesis. The present study is based on the hypothesis that AKT promotes the phosphorylation of FOXK2, inactivating its tumor suppression function and contributing to a drug resistance phenotype and poor prognosis in breast cancer.

MATERIALS AND METHODS

The MTT and clonogenic assays were performed to assess drug-induced cytotoxicity. For AKT1 knockdown, cells were transfected with non-silencing control (NSC) and AKT1 siRNA using Lipofectamine RNAimax. The LY294002 PI3K inhibitor was used for pharmacological inhibition of AKT phosphorylation. For FOXK2 overexpression, cells were transfected with the empty vector (pCMV5) and the pCMV5-FOXK2 vector using Lipofectamine2000. For constitutively active AKT overexpression, cells were transfected with pBABE-(myr)Akt:ER vector and treated with 200 nM of 4-hydroxytamoxifen. The NE-PER kit was used for cytoplasmic and nuclear extractions. Protein expression in whole and fractioned lysates were examined by Western Blotting.

RESULTS AND CONCLUSIONS

Our results indicate that increased protein levels of FOXK2 are associated with higher levels of AKT phosphorylation **(Figure 2a)** and with drug resistance phenotype in breast cancer cells **(Figure 2b)**. Also, FOXK2 and phosphorylated AKT expression was found localized predominantly in the nucleus of drug-resistant MDA-MB-231 cells **(Figure 2c)**. Furthermore, AKT1-inhibited cells **(Figures 3a and 3b)** had impaired viability and clonogenicity **(Figures 3c, 3d, 3e and 3f)** and showed decreased FOXK2 levels, particularly of slower mobility bands **(Figure 4a)**. Additionally, we observed that FOXK2 expression pattern was slightly altered following inhibition of AKT phosphorylation **(Figure 4b)**, while there was no change in FOXK2 subcellular localization **(Figure 4c)**, suggesting that AKT phosphorylation does not regulate FOXK2 nuclear-cytoplasmic shuttling in our model. Consistently, co-transfection experiments **(Figure 5a)** revealed that AKT1 inhibition not only modified endogenous, but also exogenous expression of FOXK2 **(Figure 5b)**. Corroborating these data, the induction of AKT constitutive overexpression **(Figure 6a)** increased FOXK2 protein levels either in MDA-MB-231 and MCF-7 cells **(Figure 6b, 6c, 6d and 6e)**. In conclusion, our data suggest that AKT1 regulates FOXK2 expression in drug-resistant breast cancer cells. Experiments involving the allosteric inhibition of AKT by MK-2206 and treatment with lambda phosphatase are in progress and will allow a better understanding of AKT-FOXK2 regulatory axis.

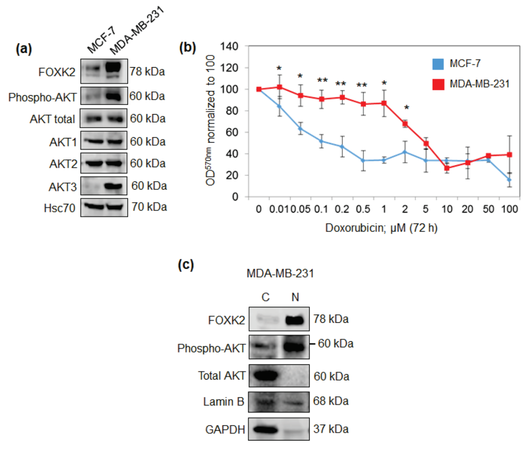

Figure 2 – Expression profile and subcellular localization of FOXK2 and AKT in breast cancer cell lines exhibiting distinct drug resistance phenotypes. (a) MCF-7 and MDA-MB-231 cell lines were harvested and subjected to Western blot analysis, where FOXK2, phospho-AKT, total AKT, AKT1, AKT2, AKT3 and Hsc70 levels were determined. **(b)** MCF-7 and MDA-MB-231 were seeded in 96-well plates and treated with increasing concentrations of doxorubicin for 72 h. Cell viability was assessed through the MTT assay. Bars represent average ± s.d. of three experiments. Statistical significance was determined by Student's t-test. (two-sided; (* $p \leq 0,05$; ** $p \leq 0,01$; significant). **(c)** MDA-MB-231 cells had their cytoplasmic and nuclear fractions separated by the NE-PER kit (Thermoscientific). Then, FOXK2, Phospho-AKT, total AKT, Lamin B and GAPDH expression was evaluated through Western blotting. C: Cytoplasm; N: nucleus.

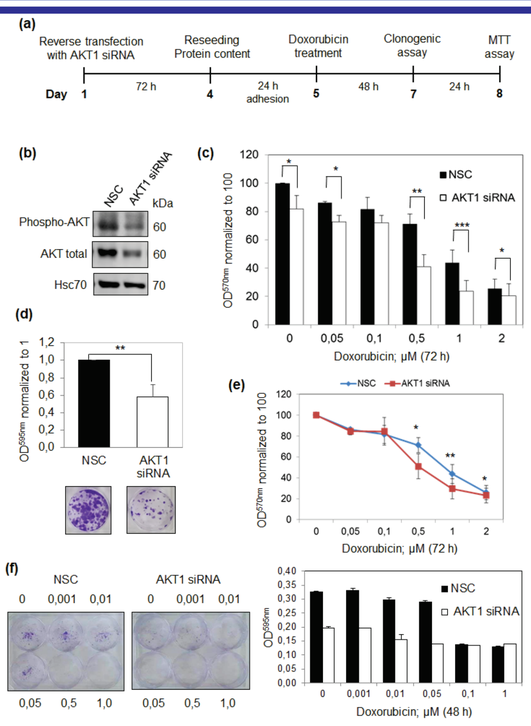

Figure 3 – AKT1 is required for survival and promotes doxorubicin resistance in MDA-MB-231 cells. (a) Experimental design. **(b)** MDA-MB-231 cells were transfected with non-silencing control (NSC) and AKT1 siRNA. After 72 h of incubation, cells were harvested for Western blot analysis, where phospho-AKT, total AKT and Hsc70 levels were determined. **(c)** Transfected cells were seeded in 96-well plates, treated with increasing concentrations of doxorubicin for 72 h and had viability assayed through the MTT assay. **(d)** Transfected cells were seeded in six-well plates, grown for 14 days and then stained with crystal violet. **(e)** Cells transfected with both NSC and AKT1 siRNA had optical densities read at 570 nm, normalized to 100 and compared for doxorubicin cytotoxicity by the MTT assay. Bars represent average ± s.d. of five experiments. Statistical significance was determined by Student's t-test. (two-sided; (* $p \leq 0,05$; ** $p \leq 0,01$;*** $p \leq 0,001$, significant). **(f)** Cells transfected with both NSC and AKT1 siRNA had optical densities read at 595 nm, normalized to 1 and compared for cytotoxicity by clonogenic assay. The graph is representative of three experiments.

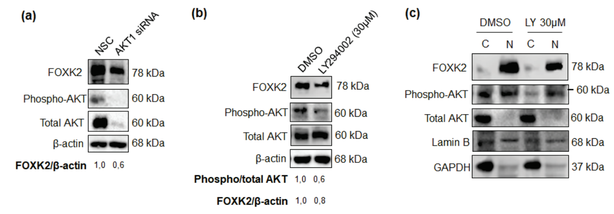

Figure 4 – FOXK2 expression and subcellular localization in AKT1-inhibited MDA-MB-231 cells. (a) MDA-MB-231 cells were transfected with non-silencing control (NSC) and AKT1 siRNA. After 8 days of incubation, cells were harvested for Western blot analysis, where phospho-AKT, total AKT, FOXK2 and β-actin levels were determined. MDA-MB-231 cells were treated with 30 µm LY294002 or DMSO vehicle control for 1 h. Cells were then harvested for Western blot analysis of FOXK2, phospho-AKT and total AKT levels in whole **(b)** and fractioned **(c)** protein extracts. Cytoplasmic and nuclear fractions were separated by the NE-PER kit (Thermoscientific). C: Cytoplasm; N: Nucleus.

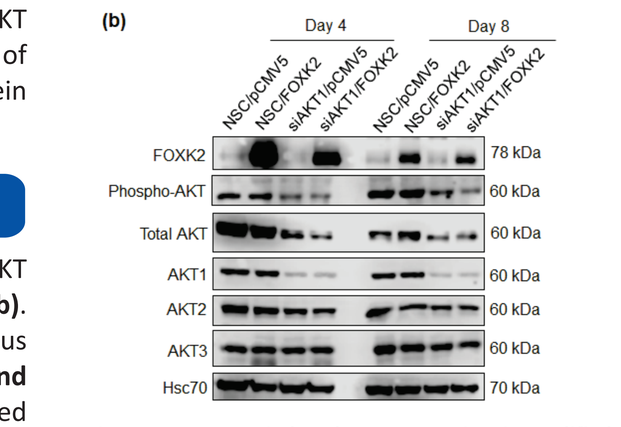

Figure 5 – FOXK2-induced overexpression is modified following AKT1 knockdown. (a) Co-transfection experimental design. **(b)** MDA-MB-231 cells were transfected with non-silencing control (NSC) and AKT1 siRNA. After 48 h of incubation, cells were transfected with the empty vector (pCMV5) and the wild-type FOXK2 vector. Following 24 h (Day 4) and 120 h (Day 8), cells were then harvested for Western blot analysis, where FOXK2, phospho-AKT, total AKT, AKT1, AKT2, AKT3 and Hsc70 levels were determined.

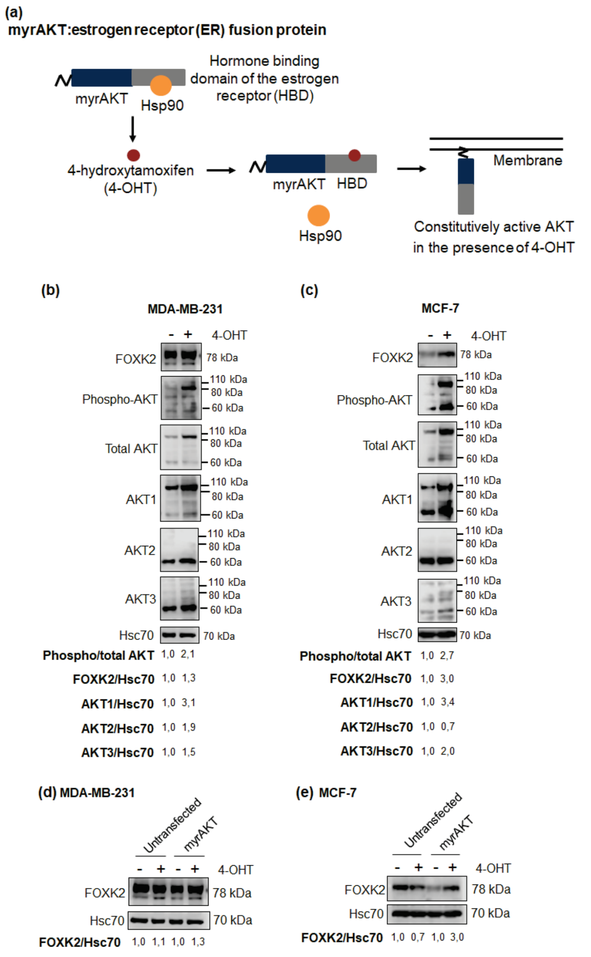

Figure 6 – FOXK2 expression is induced following myrAKT overexpression. (a) pBABE-(myr)Akt:ER vector operating scheme. **(b)** MDA-MB-231 and **(c)** MCF-7 were transfected with pBABE-(myr)Akt:ER vector. After 24 h, cells were treated with 200 nM 4-hydroxytamoxifen (4-OHT) or ethanol vehicle control for 24 h. Cells were then harvested for Western blot analysis, where FOXK2, phospho-AKT, total AKT, AKT1, AKT2, AKT3 and Hsc70 levels were determined. As experimental controls, untransfected MDA-MB-231 **(d)** and MCF-7 cells **(e)** were treated with 4-OHT and had FOXK2 levels seemingly assessed. 4-OHT: 4-hydroxytamoxifen.

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