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Regulation of cyclin A by FoxO3a drives proliferation in Anaplastic Thyroid Carcinoma

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Laura A. Marlow[1], Christina von Roemeling[1], Yilin Zhang[1], Simon J. Cooper[1], Stephen D. Rohl[1], Shilpi Arora[4], Irma M. Gonzales[4], David Azorsa[4] ,Han W. Tun[2], Peter Storz[1], Robert C. Smallridge[3] , and John A. Copland[1]

[1]Department of Cancer Biology, Mayo Clinic Comprehensive Cancer Center, and [2]Division of Hematology & Oncology, [3]Division of Endocrinology, Internal Medicine Department, Mayo Clinic, Jacksonville, Florida, [4]Pharmaceutical Genomics Division, The Translational Genomics Research Institute (TGen), Scottsdale, Arizona.

ABSTRACT

The Forkhead transcription factor, FoxO3a, is a known suppressor of primary tumor growth via transcriptional regulation of key genes regulating cell cycle arrest and apoptosis; it is negatively regulated by growth factors via phosphorylation and mislocalization from the nucleus to the cytoplasm. Here we show for the first time that FoxO3a remains nuclear in anaplastic thyroid carcinoma (ATC). FoxO3a transcriptionally upregulates cyclin A1 thereby promoting cell cycle progression and tumor growth in human ATC cells. Silencing FoxO3a leads to down-regulation of cyclin A1 mRNA and protein while over-expression of FoxO3a leads to upregulation of cyclin A1 protein. Transient co-transfection of cyclin A1 promoter linked to luciferase with FoxO3a leads to enhanced luciferase activity, which can be ablated when mutating the FoxO3a response element on the cyclin A1 promoter. This combined data implicates an entirely novel function for FoxO3a in modulating cancer progression by promoting cell cycle progression and tumor growth. ATC is an un-differentiated carcinoma with average survival upon diagnosis of 3 -4 months with 99% lethality, notably the most aggressive and deadliest cancers known. Our data indicate inactivation of FoxO3a may be effective at blocking tumor expansion in ATC. These new findings suggest caution related to current dogma focused upon reactivation of FoxO3a as a therapeutic strategy against cancers harboring signaling pathways such as Akt that inactivate FoxO3a.

INTRODUCTION

Anaplastic thyroid carcinoma (ATC) represents only 1.7 % of all malignant thyroid diseases, but it accounts for approximately 40% of thyroid carcinoma related deaths in the United States. Thus, the diagnosis of ATC is essentially a death sentence, since it is nearly always fatal and patients survive, on average, less than 5 months after diagnosis. Such a bleak prognosis is due to ATC aggressive growth, invasiveness, metastatic behavior of the cancer and lack of effective therapies. Due to high mortality rates, further preclinical studies of ATC pathogenesis and molecular signaling are critically needed for developing therapeutic interventions.

Now, our lab has recently identified the transcription factor, FoxO3a (FKHRL1), as a regulator of proliferation in ATC. FoxO3a is a member of the Fox superfamily that all share a conserved 110 amino acid DNA binding domain. FoxO3a is tightly controlled post-translationally by acetylation (p300/CBP, SIRT1), ubiquitination (Skp2), and phosphorylation (AKT, SGK1, AMPK, DYRK1a, IkKβ JNK, and Mst1) in order to regulate the metabolism, homeostasis, cell cycle, DNA repair, oxidative stress resistance and apoptosis. Regulation of FoxO3a phosphorylation is important for nuclear-cytoplasmic shuttling. Kinase phosphorylation of FoxO3a by p-AKT, DYRK1a, IKKβ or SGK1 leads to cytoplasmic export and inactivation of FoxO3a while JNK, AMPK or MSt1 phosphorylation leads to activation and nuclear localization.

While published data shows both nuclear and cytoplasmic FoxO3a in papillary and follicular thyroid cancers, we found that FoxO3a is exclusively nuclear in ATC. This is surprising since p-AKT negatively regulates FoxO3a and is elevated in most ATCs. This indicates that FoxO3a in ATC, but not in other thyroid cancers, may be uncoupled from its canonical regulation through AKT.

RESULTS

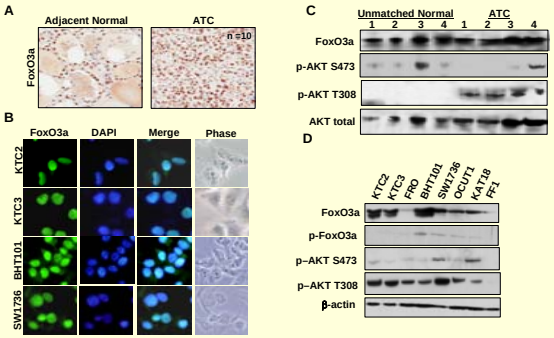

Figure 1. FoxO3a is predominantly nuclear in ATC. **A**. IHC staining in adjacent normal and ATC patient tissue shows FoxO3a is nuclear. **B**. ICC confirms nuclear localization of FoxO3a in ATC cells. DAPI staining is used as a nuclear control. **C**. Western blot panel of 4 different normal and ATC patient tissues shows equal FoxO3a expression levels, overall low p-AKT S473 and increased p-AKT T308. **D**. Western blot panel of 8 ATC cell lines show variable FoxO3a expression levels, generally little to no p-FoxO3a and p-AKT S473 and overall increased p-AKT T308 expression.

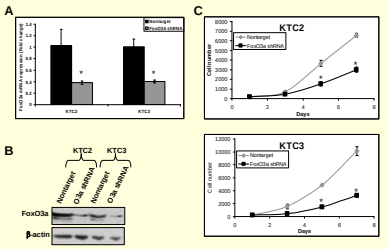

Figure 2. Silencing FoxO3a reduces proliferation.
A. QPCR verifies that FoxO3a has been silenced in KTC2 and KTC3 cells that have been infected with lentiviral FoxO3a 1566 shRNA verses nontarget control. Data is plotted as fold change ± S.D. as compared to nontarget control, *p < 0.05. **B**. Western blot of the same lentiviral infected cells confirms that FoxO3a is reduced in the FoxO3a shRNA cells when compared to its nontarget control. β- actin is the loading control. **C**. Proliferation curve over 7 days of the lentiviral infected cells show that when FoxO3a is silenced by FoxO3a shRNA, there is decreased proliferation. This data was verified independently by a high-throughput siRNA screen.

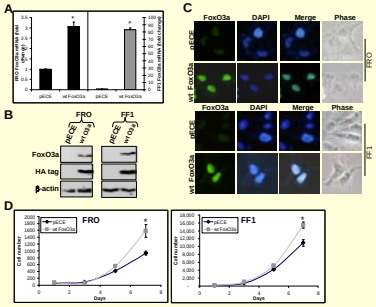

Figure 3. Over-expression of FoxO3a increases proliferation.
A. QPCR verifies that wild-type (wt) FoxO3a has been over-expressed in transiently transfected FRO and FF1 cells versus pECE control (24 hours post-transfection). **B**. Western blot panel of the same transiently transfected cells confirm that wt FoxO3a is over-expressed when compared to its empty control. **C**. ICC shows nuclear localization of transiently transfected wt FoxO3a in both ATC cells. **D**. Proliferation curve over 7 days of the transiently transfected cells show that over-expressed wt FoxO3a increases proliferation.

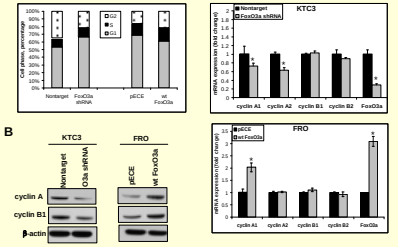

Figure 4. FoxO3a alters cyclin A expression.
A. Flow cytometry of lentiviral infected KTC3 cells for silencing FoxO3a and transiently transfected FRO cells for over-expressing wt FoxO3a. Prior to analysis, cells were synchronized for 24 hours and then released for 72 hours. Cell cycle was analyzed by FCS Express and phase percentages are as shown. **B**. Western blot of the same cells indicate that when FoxO3a is silenced in KTC3 cells, cyclin A and B expression decrease while over-expressing wt FoxO3a in FRO cells increases cyclin A and B expression. **C**. QPCR confirms that only cyclin A1 and A2 mRNA levels are decreased in FoxO3a silenced KTC3 cells and only cyclin A1 mRNA levels are increased in wt FoxO3a over-expressing FRO cells. There is no change in cyclin B1 and B2 mRNA levels.

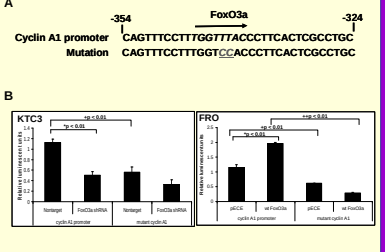

Figure 5. FoxO3a regulates the cyclin A promoter.
A. Cyclin A1 promoter region contains a putative FoxO3a binding site. The response element was mutated for a mutant cyclin A1 promoter lacking a functional FoxO3a consensus sequence. **B.** Luciferase activity comparison of the wild-type cyclin A1 promoter with the mutant cyclin A1 promoter. Cells were transiently transfected with the luciferase contructs along with renilla. The results are graphed as average relative luminescent units (firefly activity / renilla luciferase vector activity) ± S.D. with p values as shown, *comparison between control and FoxO3a shRNA or wt FoxO3a within a promoter, +comparison of activities across wild-type and mutant cyclin A1 promoters.

SUMMARY

- FoxO3a remains nuclear in anaplastic thyroid carcinoma (ATC)
- Cyclin A1 promoter activity is dependent upon FoxO3a
- FoxO3a modulates cancer progression by promoting cell cycle progression
- Our data goes against the current dogma focused upon reactivation of FoxO3a as a therapeutic strategy against cancers
- We suggest that FoxO3a is a potential molecular target for therapy in ATC

FUTURE DIRECTIONS

- Determine the mechanism of FoxO3a oncogenic activity
- Determine the role of FoxO3a in other thyroid cancers histotypes