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MYC activity mitigates response to rapamycin in prostate cancer through 4EBP1-mediated inhibition of autophagy

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Cancer cells have evolved exquisitely to ignore both intrinsic and extrinsic cell death signals, and resistance to cell death is a critical challenge facing clinical oncology. Autophagy, the catabolic recycling process that involves the fusion of autophagosomes containing sequestered cargo with lysosomes, has an enigmatic role in tumorigenesis. In times of metabolic stress due to deprived nutrition or hypoxia, tumor cells use autophagy as a scavenging mechanism for maintenance of critical processes and survival. However, modulation of the extent of autophagy plays a critical role, as excessive autophagy can result in a nonapoptotic and non-necrotic cell death (sometimes referred to as Type II programmed cell death). It is likely that the genetic context of specific cancers will have an impact upon whether autophagy is primarily a mechanism for survival or cell death.

Key words: MYC, prostate cancer, 4EBP1, rapamycin, mTOR, autophagy

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The tumor suppressor gene *PTEN* is frequently lost in the tumors of patients with advanced prostate cancer, resulting in constitutive activation of the PtdIns3K/AKT pathway. This pathway activates mammalian target of rapamycin, mTOR, which promotes anabolic cellular processes and inhibits autophagy. mTOR promotes growth through the action of two complexes referred to as TORC1 and TORC2. TORC1 activity results in the phosphorylation of S6 Kinase and 4EBP1 and activation of a cell's protein translational machinery; TORC2 activity phosphorylates AKT and accentuates the prosurvival, anti-apoptotic environment within the cell. Rapamycin, a naturally occurring macrolide antibiotic, specifically and preferentially inhibits TORC1 activity and can trigger autophagy. Rapamycin and its derivatives have been extensively explored for use in cancer therapies. In vitro and in vivo studies suggest that cancer cells dependent on the PtdIns3K/AKT pathway may be particularly sensitive to rapamycin, but clinical trials testing the clinical efficacy of the single agent rapamycin and rapamycin analogues in prostate cancer have shown little promise.

In our recently published work, we posited that specific genetic events are likely to have an impact upon a cancer cell's response to rapamycin. Advanced prostate cancer cells, and indeed most available prostate cancer cell lines, have accumulated multiple genetic alterations and demonstrate marked aneuploidy. This results in a poorly defined genetic context that challenges the interpretation of additional genetic perturbations even within isogenic cell lines. In order to control the genetic context of a cell and determine the impact of specific genetic events on a prostate cancer cell's response to rapamycin, we employed a series of genetically engineered prostate epithelial cell lines.

We used genetically engineered prostate epithelial cell lines expressing activated H-RAS, MYC, androgen receptor (AR) and different combinations thereof, to determine if any specific genetic event in these cells altered sensitivity to rapamycin. Whereas most introduced genes have little impact on rapamycin sensitivity, the cell line overexpressing MYC (referred to as LHMB-AR) requires 10-fold higher concentrations of rapamycin to achieve the same reduction in cell growth than

the other genetically engineered cell lines tested (including BH10i-AR which has the identical genetic constitution as LHMB-AR but does not overexpress MYC). MYC-induced changes in rapamycin sensitivity are also demonstrated in established, patient-derived prostate cancer cell lines by showing increased resistance when MYC is overexpressed (DU145 and LNCaP) and increased sensitivity when MYC expression is suppressed using an shRNA construct (DU145, LNCaP, and PC3).

While there is no rapamycin-induced apoptosis in any of the cell lines regardless of the expression of MYC, 1 nM of rapamycin induces cytostasis in BH10i-AR cells but has limited impact on the MYC-expressing LHMB-AR cells. In addition to a G1 cell cycle arrest, BH10i-AR cells have increased rapamycin-induced autophagy compared to the LHMB-AR cells based upon different amounts of lipidated LC3B and accumulation of acridine orange in acidic vesicles (presumably autolysosomes) as determined by microscopy and flow cytometry. Interestingly, MYC expression also reduces basal levels of autophagy seen even in the absence of rapamycin. In aggregate, these observations suggested that MYC confers resistance to rapamycin by maintaining cell cycle progression and reducing autophagy.

To determine how MYC affects the response to rapamycin, we investigated if MYC regulated downstream targets of mTOR. Using microarray analysis of the genetically engineered cell lines and a collection of human prostate cancers with known MYC-copy number gain, we find MYC expression increases 4EBP1 expression, and we subsequently demonstrate that MYC strongly binds to two E-box sequences near the transcription start site of the *4EBP1* gene. Importantly, suppression of 4EBP1 using a specific shRNA in LHMB-AR cells results in significant resensitization to rapamycin. This resensitization of LHMB-AR cells is associated with increased basal levels of autophagy as well as increased rapamycin-induced autophagy.

We tested several autophagy-inducing agents to dissect whether the differential autophagy observed in MYC overexpressing LHMB-AR cells is generalized or specific to rapamycin. Whereas the autophagy inducers N-acetyl sphingosine, Earle's balanced salt solution, and lithium chloride do not demonstrate a difference in the induction of autophagy between the MYC overexpressing LHMB-AR or the non-overexpressing BH10i-AR cells, autophagy induction by tunicamycin demonstrates a similar pattern as observed with rapamycin. Tunicamycin is an inhibitor of GlcNAc phosphotransferase, inhibits the synthesis of glycoproteins, and induces autophagy through a PtdIns3K/AKT/mTOR-independent mechanism. Furthermore, suppression of 4EBP1 in LHMB-AR cells increases tunicamycin-induced autophagy along with rapamycin-induced autophagy.

In aggregate, our recent work suggests that MYC significantly reduces rapamycin-induced cytostasis and autophagy and that MYC's impact is at least in part through direct transcriptional regulation of 4EBP1. This may help explain why rapamycin and rapamycin analogues are meeting with disappointing clinical results as MYC copy number gain is frequent in advanced prostate cancer. The observation that MYC regulation of 4EBP1 affects both rapamycin- and tunicamycin-induced autophagy suggests a generalized impact on autophagy by 4EBP1, although the potential mechanism for such an effect is not yet known.