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The Role of Akt Isoforms in Colorectal Cancer

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14. ABSTRACT Akt activation is found in many cancers, including CRC, and is a marker for CRC initiation and progression. However, since Akt has a central role in cell signaling, targeting the three Akt isoforms concurrently may give rise to unacceptable toxicity. Therefore, selective inhibition of one or more Akt isoforms or their target phosphoproteins may be a more effective treatment strategy for CRC. Though a phosphoproteomics screen, we identified 20 Akt isoform-specific phosphorylation targets as likely to be involved in tumor growth and metastasis. We hypothesized that Akt isoforms and their downstream effectors play essential roles in CRC induction, growth and metastasis. In the first year of the award, we have found that knockout of Akt1 or Akt2 substantially reduces colorectal tumorigenesis in our genetically engineered mouse model. We also successfully ablated novel downstream targets of Akt in our novel murine colorectal cancer cell lines: IWS1, MTSS1 or MIM, FRMD6, SEMA4B, MYH9, and Liprin-β1. We found that each phosphorylation target promotes tumorigenesis in vitro. MTSS1 had the greatest effect on in vitro tumorigenesis.					
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Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality worldwide, with approximately 600,000 deaths each year. The five-year prognosis for patients with newly diagnosed metastatic CRC is less than 20%.¹ Understanding the key cellular signaling pathways that promote formation of CRC metastases is critical to the development of novel treatment strategies.

Akt activation is a common theme in many cancers, including CRC, and is a marker for CRC initiation and progression.² As a result, Akt has been identified as an important molecular target for cancer therapy. However, since Akt has a central role in cell signaling, targeting the three Akt isoforms (Akt1, Akt2, and Akt3) concurrently may give rise to unacceptable toxicity. Therefore, selective inhibition of one or more Akt isoforms or their target phosphorylated proteins may be a more effective treatment strategy for CRC.

Using triple Akt knockout cells from mice which were engineered to express one Akt isoform at a time but were otherwise identical, the Tschlis laboratory collaborated with Cell Signaling Technologies, Inc., to identify 20 Akt isoform-specific phosphorylation targets using an unbiased phosphoproteomics screen which are likely to be involved in tumor invasion and metastasis.³ We selected six of these targets which are strongly expressed in colonic tumors relative to normal colonic mucosa: IWS1, the metastasis suppressor MTSS1L or MIM, the FERM domain protein and Merlin interactor FRMD6, the type I transmembrane protein SEMA4B, the non-muscle myosin heavy chain NMHCIIA/MYH9 which interacts with the metastasis-associated protein S1004A and LIPRIN-β1, which interacts with the metastasis-promoting protein S1004A.

Body

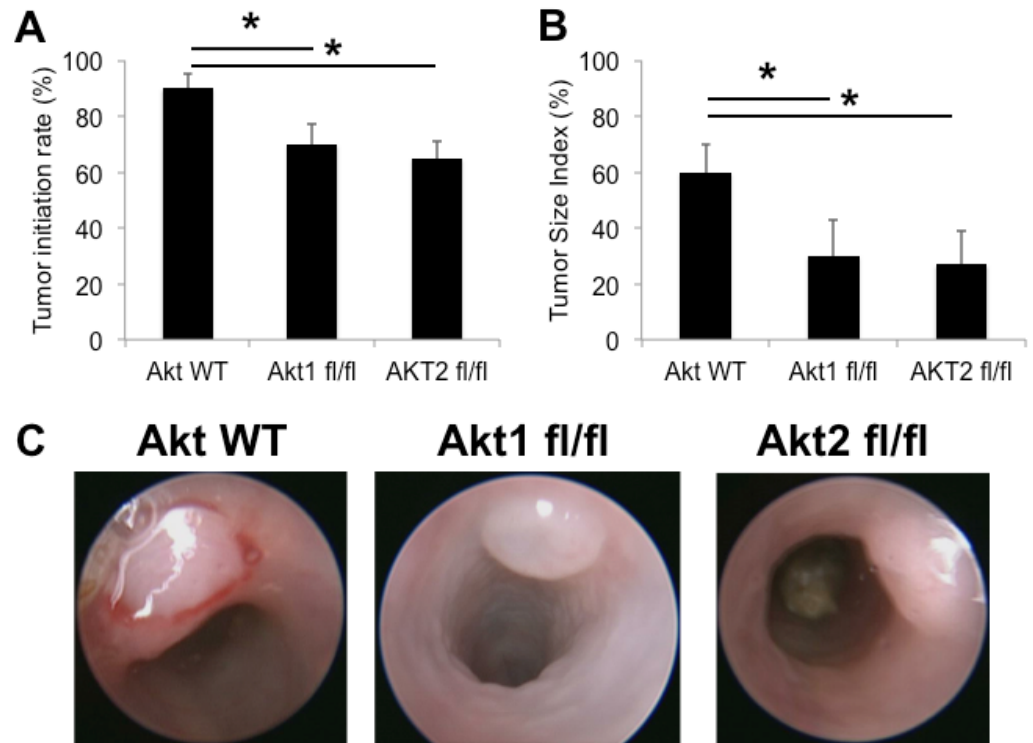
Aim 1: To determine the role of Akt1 and Akt2 in the induction, invasiveness and metastatic potential of colorectal tumors.

Sub-aim 1a: Evaluate the effect of colon-specific Akt1 and Akt2 ablation on tumorigenesis.

We have extensive experience with a novel genetically engineered mouse (GEM) model of CRC in which adenovirus expressing cre recombinase (adeno-cre) is delivered to the distal colon of mice carrying a floxed *Apc* exon 14 allele (*Apc* mice with deletion of the *Apc* gene) and an additional lox-stop-lox (LSL) *Kras* G12D allele (*Apc-Kras* mice). These mice are a more accurate model for sporadic and metastatic CRC: 1) animals develop one or two tumors in the distal colon; 2) the tumors derive from somatic modification of genes known to be involved in CRC; 3) the somatic mutations involve the colonic epithelium; 4) tumors recapitulate the entire adenoma-carcinoma-metastasis sequence, with liver metastases forming in approximately 50% of mice six months following tumor induction; 5) tumor size can be monitored *in vivo* using optical colonoscopy; and 6) sequential tumor biopsy can be performed for genetic and biochemical analysis.⁴⁻⁷

We crossed *Akt1^{fl/fl}* mice and *Akt2^{fl/fl}* mice with *Apc^{fl/fl}Kras^{G12D}* mice to derive *Apc^{fl/fl}Kras^{G12D}Akt1^{fl/fl}* *Apc^{fl/fl}Kras^{G12D}Akt2^{fl/fl}*, *Apc^{fl/fl}Kras^{G12D}Akt^{WT}* mice. We then performed surgical laparotomies on 20 mice from each group, isolated the distal colon, and delivered adeno-cre by rectal enema, as previously described. Tumor formation was assessed by optical colonoscopy four weeks after tumor induction using a custom endoscopy system. We found that ablation either *Akt1* or *Akt2* resulted in decreased tumor formation rate and tumor size index (a proxy for tumor size) (Figure 1).

Figure 1: Distal colonic tumors were induced in *Apc^{fl/fl}Kras^{G12D}Akt WT*, *Apc^{fl/fl}Kras^{G12D}Akt1^{fl/fl}*, and *Apc^{fl/fl}Kras^{G12D}Akt2^{fl/fl}* mice via surgical administration of adenocarcinoma. 4 weeks later, we assessed tumor initiation rate (A) and tumor size (B) by optical colonoscopy. Representative colonoscopy images are shown in (C). * P<0.01.



6 months after tumor induction, we performed MRI scans of mice with colonoscopic tumors following administration of gadolinium. Liver metastases were visible on MRI (Figure 2). The liver metastasis rate was significantly lower in Akt2 and Akt3 fl/fl groups compared to the control group. All MRI imaging was performed at the Small Animal Imaging/Tumor Biology Facility of Tufts University.

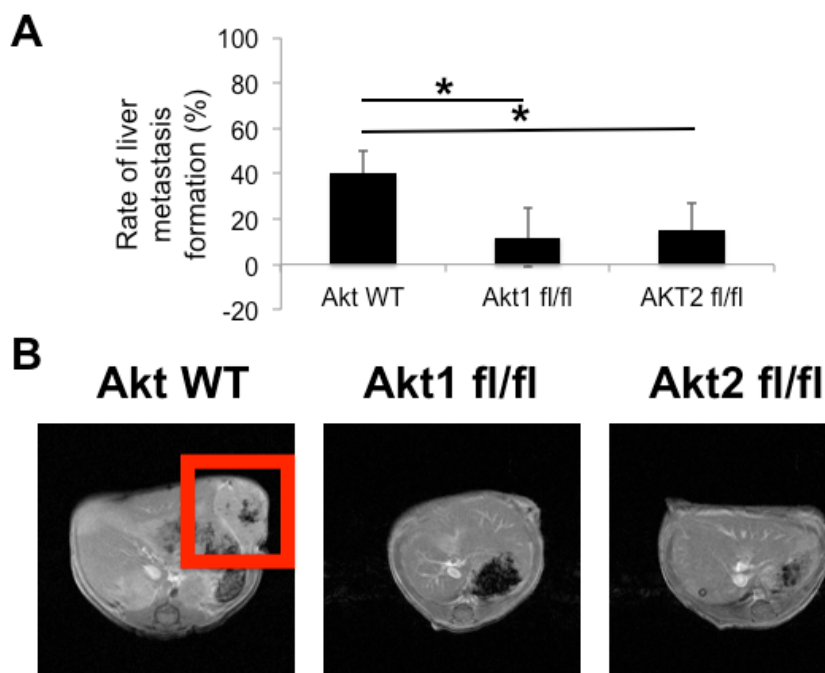


Figure 2: Liver metastasis formation was assessed in *Apc^{fl/fl}Kras^{G12D}Akt WT*, *Apc^{fl/fl}Kras^{G12D}Akt1^{fl/fl}*, and *Apc^{fl/fl}Kras^{G12D}Akt2^{fl/fl}* mice that had colonic tumors present on colonoscopy. We performed MRI scans post gadolinium administration. * P<0.01. (Square; tumor metastasis)

Following MRI scanning, mice were sacrificed and tumor (primary colon and liver) tissue was divided for subsequent histology and biochemical analysis. These studies are ongoing.

Aim 2: To determine the role of Akt isoform-dependent phosphorylation events in CRC growth and metastasis.

Sub-Aim 2a: To evaluate the role of Akt isoform-dependent phosphorylation targets in cellular growth and invasion.

We transduced the C57BL/6 mouse-derived colorectal tumor cell line F62 with lentiviral short hairpin (sh) RNA constructs or shControl to knock down the endogenous proteins IWS1, MTSS1 or MIM, FRMD6, SEMA4B, MYH9, and Liprin- β 1. We then replaced with the exogenous wild type or phosphorylation site mutant proteins that were created using insertional mutagenesis. Immunoprecipitation studies were performed for each phosphorylation target to demonstrate that the wild-type, but not mutant, protein is phosphorylated by Akt. A representative immunoprecipitation study for MTSS1 is shown in Figure 3.

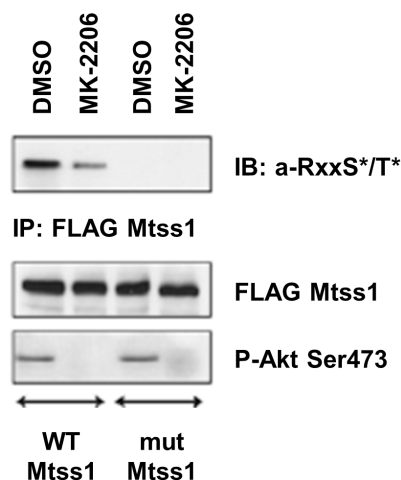


Figure 3. Mtss1 is phosphorylated by Akt at Ser594. Mtss1 was ablated in F62 colorectal cancer cells with short hairpin (sh) RNA to the 5' UTR, then rescued with wild-type (WT) FLAG-tagged Mtss1 or mutant (mut) FLAG-tagged Mtss1 expressing Ala594 instead of Ser594. WT or mut Mtss1 cells treated with DMSO or a pan-AKT inhibitor (MK-2206, 5 μ M) were immunoprecipitated with anti-FLAG antibody bound to sepharose beads, then probed with a universal phospho-Akt substrate antibody (RxxS*/T*).

Next, we assess cellular proliferation in wild-type and mutant IWS1, MTSS1, FRMD6, SEMA4B, MYH9, and Liprin- β 1. 1000 cells were seeded in 96 well plates, grown for 72 hours, then assessed cell viability by the Cell Titer Glo chemiluminescent assay. We then determined cell migration by seeding cells to confluence in 6 well plates, creating a wound with a pipette tip, then assessing percent wound closure after 24 hours (Figure 4).

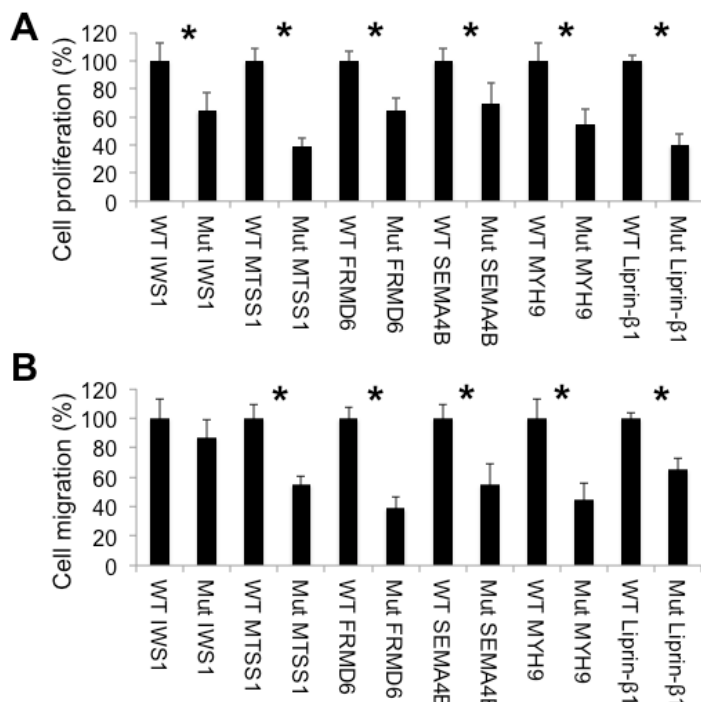


Figure 4. We determined cell proliferation (A) and cell migration (B) in F62 murine colorectal cancer cells expressing wild-type (WT) or mutant (mutant) FLAG-tagged protein with the endogenous protein knocked down by shRNA. Cell proliferation and migration in mutant cells were normalized to wild-type cells.

Key research accomplishments

1. Akt1 and Akt2 promote colorectal tumorigenesis.
2. Akt1 and Akt2 promote liver metastasis of colorectal primary tumors.
3. We successfully created lentiviral constructs expressing wild-type IWS1, MTSS1, FRMD6, SEMA4B, MYH9, and Liprin- β 1 or mutant protein that is not phosphorylated by Akt. Endogenous protein was knocked down by shRNA.
4. For all selected phosphorylation targets, Akt-specific phosphorylation promotes cellular proliferation and migration in vitro.

Reportable outcomes

I have received a V Scholar Award from the V Foundation based on work supported by the DOD Career Development Award.

Conclusions

Akt isoforms are independently important for colorectal carcinogenesis. The six selected novel Akt phosphorylation targets play an important role colorectal cell growth and migration in vitro.

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Appendices

None