Mice Expressing Activated PI3K Rapidly Develop Advanced Colon Cancer

Alyssa A. Leystra, Dustin A. Deming, Christopher D. Zahm, Mohammed Farhoud, Terrah J. Paul Olson, Jamie N. Hadac, Laura A. Nettekoven, Dawn M. Albrecht, Linda Clipson, Ruth Sullivan, Mary Kay Washington, Jose R. Torrealba, Jamey P. Weichert, and Richard B. Halberg

Abstract
Aberrations in the phosphoinositide 3-kinase (PI3K) signaling pathway play a key role in the pathogenesis of numerous cancers by altering cellular growth, metabolism, proliferation, and apoptosis. Mutations in the catalytic domain of PI3K that generate a dominantly active kinase are commonly found in human colorectal cancers and have been thought to drive tumor progression but not initiation. However, the effects of constitutively activated PI3K upon the intestinal mucosa have not been previously studied in animal models. Here, we show that the expression of a dominantly active form of the PI3K protein in the mouse intestine results in hyperplasia and advanced neoplasia. Mice expressing constitutively active PI3K in the epithelial cells of the distal small bowel and colon rapidly developed invasive adenocarcinomas in the colon that spread into the mesentery and adjacent organs. The histologic characteristics of these tumors were strikingly similar to invasive mucinous colon cancers in humans. Interestingly, these tumors formed without a benign polypoid intermediary, consistent with the lack of aberrant WNT signaling observed. Together, our findings indicate a noncanonical mechanism of colon tumor initiation that is mediated through activation of PI3K. This unique model has the potential to further our understanding of human disease and facilitate the development of therapeutics through pharmacologic screening and biomarker identification.

Introduction
Colorectal cancer remains a leading cause of cancer-related death, despite significant advances in treatment options. Targeting oncogenic pathways has been and continues to be a significant interest of many investigators. The PI3K/AKT signaling cascade has been identified as a promising target for drug development. Many new inhibitors of phosphoinositide 3-kinase (PI3K) and the downstream signaling molecules are currently in clinical development; however, their role in the clinical setting has yet to be well defined.

The PI3K/AKT pathway transmits signals from various transmembrane growth factor receptors through a kinase cascade to nuclear transcription factors. PI3K initiates this signaling pathway through the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 then activates the serine/threonine kinase AKT, which phosphorylates multiple downstream targets responsible for a wide variety of vital cellular functions. One of the prominent targets is mTOR, a serine/threonine kinase that is an important regulator of cell growth and metabolism. This kinase then mediates activation of the eukaryotic translation initiation factor 4E-binding protein (4E-BP1) and the p70S6 ribosomal kinase (S6) that are involved in protein synthesis.

Mutations of the PIK3CA gene, encoding the p110 catalytic subunit of the PI3K kinase, are present in 20% to 30% of human colon cancers. Three mutations are common: E542K, E545K, and H1047R, and result in a dominantly active form of the PI3K protein. PIK3CA mutations have been investigated in numerous cancer cell lines; however, the effect of a dominant-active PI3K has not previously been investigated in the mammalian intestine. We describe a novel mouse model designed to provide insight into the biologic effects of a dominantly active form of PI3K in the colon and, once characterized, to further test new therapeutic agents and identify biomarkers.

Materials and Methods
Mouse husbandry
All animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee.
at the University of Wisconsin (Madison, WI) following the guidelines of the American Association for the Assessment and Accreditation of Laboratory Animal Care. FC mice [FVB/N-Tg(Fabp1-Cre))Jig: National Cancer Institute Mouse Repository; strain number - 01X38] were maintained by backcrossing to FVB mice (FVB/J: The Jackson Laboratory; stock number - 001800). PIK3ca+/+ mice [C57BL/6-Gr(ROSA)26Sortm7(Pik3ca⊥EGFP)JbK/J: The Jackson Laboratory; stock Number - 012343] were maintained by crossing siblings for fewer than 5 generations. FC + PIK3ca+/+ and F0 + PIK3ca+/+ littermates were generated by crossing FC females with PIK3ca+/+ males (herein, + denotes carrier and 0 denotes non-carrier for FC and PIK3ca). For comparison, F1 Apc(Ki) males (herein, Apc denotes carrier for FC and PIK3ca) were kept under anesthesia for 60 minutes and then prepared for dual hybrid microPET/CT colonography as described previously (9). A 10-minute positron emission tomography (PET) acquisition was carried out after injection of 18F-FDG (160 mg) from experimental mice and controls was loaded onto precast 10% PAGE gels. The gels were run at 200 volts for 30 minutes and then transferred to polyvinylidene difluoride (PVDF) Immobilon-P Membranes (Millipore) at 100 volts for 45 minutes. The membranes were blocked with 5% nonfat dry milk for 1 hour and then probed with primary antibodies against p110α (1:1,000; Cell Signaling Technology), rabbit anti-cytokeratin 20 (clone SP33, Ventana), rabbit anti-lysozyme (1:500, DAKO), rabbit anti-PCNA (1:1,000, Cell Signaling Technology), and rabbit anti-synaptophysin (1:500, Abcam). Immunohistochemistry was carried out using the Histo mouse Max Broad Spectrum (DAB) Kit as instructed by the manufacturer (Invitrogen) except for the following modification: antigen unmasking was carried out by boiling the samples for 20 minutes in citrate buffer (pH 6.0) or treating samples with proteinase K for 5 minutes. The primary antibodies included rabbit anti-pAKT (Ser473, 1:100, Cell Signaling Technology), mouse anti-β-catenin (1:400, BD Biosciences—Clone 14), rabbit anti-cytokeratin 7 (clone SP52, Ventana), rabbit anti-cytokeratin 20 (clone SP33, Ventana), rabbit anti-lysozyme (1:500, DAKO), rabbit anti-PCNA (1:1,000, Cell Signaling Technology), and rabbit anti-synaptophysin (1:500, Abcam). Western blot analysis Tissue samples were collected and flash frozen. After 24 hours, the samples were sonicated in T-PER tissue protein extraction reagent (Thermo Scientific), proteasome inhibitor cocktail (Sigma-Aldrich), and phenylmethylsulfonylfluoride (PMSF; Sigma-Aldrich). Extracted protein (30 μg) from experimental mice and controls was loaded onto precast 10% PAGE gels. The yield was quantified using the NanoDrop DU-800 (Thermo Scientific) and reverse 5′-ATCATGCAAATCCAGTGCAA-3′ and reverse 5′-
CAGCTGTCGTCACTCTTCA-3' and ribosomal protein L13A (RPL13A; NM_009438) forward 5'-TTCCGGTAGAGGCTCACCTGAAGAT-3' and reverse 5'-TCTCCGGATAGTCATTTGGA-3'. Real-time PCR was carried out with a CFX96 Real-Time PCR machine (Bio-Rad). Melt-curve analysis and serial dilution of cDNA confirmed amplification of a single product with high efficiency. Gene expression was normalized to RPL13A and reactions were conducted in triplicate.

Results and Discussion

We sought to determine whether activation of the PI3K/AKT cascade affects homeostasis in the mammalian intestine. Mice carrying a transgene in which the fatty acid–binding protein promoter is fused to Cre recombinase (FC) were crossed to mice carrying a transgene encoding a chimeric protein with the iSH2 domain of the p85 regulatory subunit fused to the N-terminus of p110 catalytic subunit (PIK3ca+; Supplementary Fig. S1A; refs 7,10). (FVB/J × C57Bl/6)F1 progeny carrying both transgenes (FC' PIK3ca+−) express this dominantly active form of p110 in epithelial cells of the distal small bowel and colon because the Cre recombinase excises a stop sequence upstream of PIK3ca+.

The majority of FC' PIK3ca+− mice became moribund between 40 and 60 days of age (Supplementary Fig. S1B). These mice were imaged with a Siemens Dual Hybrid PET/CT scanner, revealing high avidity for fluorodeoxyglucose (18F; FDG), and CLR1404 (124I; a phospholipid ether analogue; ref. 11) in the proximal colon and low avidity for FDG between 40 and 60 days of age (Supplementary Fig. S1B). These mice became moribund and had marked dilation of the small bowel and cecum (Fig. 2A). Obstructive enteropathy was always caused by the presence of a massive tumor in the proximal colon. On gross examination, the colon tumors penetrated through the serosa and had an impressive enlargement of blood vessels and mesenteric lymphatic tissue (Fig. 2A). In a group of 5 FC' PIK3ca+− mice, we sectioned en bloc the tumor and mesenteric lymphatic tissue. The lymphatic tissue was hyperplastic in all mice. Tumor deposits were identified within the mesenteric adipose tissue in 3 of 5 mice. In other FC' PIK3ca+− mice, the intestines were removed, split lengthwise, and splayed open, revealing large, flat, thickened rugal folds and plaque-like tumors in the proximal colon without a significant luminal exophytic component (Fig. 2A).

Histologic examination of the proximal colon confirmed the presence of moderately differentiated, diffusely invasive mucinous adenocarcinomas with extension through the muscularis propria and serosa into the pericolonic adipose tissue (Figs. 2B and 3A). These tumors exhibited mucinous differentiation with islands of malignant glands within mucin lakes (Fig. 2B) as well as budding at the leading edge of invasion fronts. The masses elicited a desmoplastic reaction as well as inflammatory infiltration (Fig. 2B). The neoplastic cells exhibited high-grade nuclear atypia, phosphorylation of AKT, and an increase in cellular proliferation, as compared with the normal colonic epithelium from FC' PIK3ca+− or FC' PIK3ca+− mice and adenomas from ApcMin+− mice (Fig. 3B; Supplementary Figs. S2 and S3). In addition, neoplastic cells were weakly positive for cytokeratin 20 and negative for cytokeratin 7 (Supplementary Fig. S4), indicating that the transformed cells originated from the intestinal epithelium (12). Areas of invasion were covered by hyperplastic crypts with focal crypt dilatation and branching or at times a denuded epithelium.

Some of the FC' PIK3ca+− mice (6 of 17) also developed cecal tumors. Direct extension was observed of one tumor into the lymphatic, ovarian, uterine, and pancreatic tissue (Fig. 2C), which would be consistent with stage T4 invasion of human cancers. No gross evidence of liver or lung metastasis was identified.

The development of advanced cancers in this model is mediated by the expression of the dominantly active form of PI3K and consequently activation of several of its targets (Figs. 3B and 4A; Supplementary Fig. S5). The presence of the PIK3ca+

Figure 1. FC' PIK3ca+− mice develop intestinal obstruction due to large proximal colon tumors. The tumors exhibit a high avidity for FDG (A) and CLR1404 (C). The mouse was injected with the imaging agent and scanned, and the data were reconstructed with standard algorithms. Following scan acquisition, each mouse was sacrificed and the abdominal wall was dissected revealing tumors in the proximal colon (B and D, arrows). Scale bars, 1 cm.
protein was confirmed in the mucosa of the proximal and distal colon (Fig. 4A). In addition, downstream activation of AKT and subsequent phosphorylation of S6 and 4E-BP1 were observed (Fig. 4A). These observations confirm that the transgene was transcribed and translated in the colon of \( FC^+ PIK3ca^{+/+} \) mice and that its expression resulted in increased downstream activation of the PI3K/AKT/mTOR pathway. In contrast, phosphorylation of extracellular signal-regulated kinase (ERK)1/2 was not noted above baseline, indicating that activation of the RAF/MEK/ERK cascade is not involved in tumorigenesis in this model (Fig. 4B).

Most human colorectal adenomas carry truncating mutations in \( APC \) and the loss of this gene is thought to be the tumor-initiating event (13). These mutations result in translocation of \( \beta \)-catenin to the nucleus (Supplementary Fig. S3) and consequently change the pattern of gene expression. \( \beta \)-Catenin was examined in the invasive adenocarcinomas of \( FC^+ PIK3ca^{+/+} \) mice and found to be localized to the cell membrane and cytoplasm (Fig. 3B). This pattern of localization indicates that tumor initiation in \( FC^+ PIK3ca^{+/+} \) mice is not mediated through aberrant WNT signaling. The lack of aberrant WNT signaling also correlates with the lack of an exophytic or polyp-like morphology in this model, as polyp formation would be expected in \( Apc \)-mutant tumors. Given the lack of other induced genetic abnormalities and the short time frame in which these tumors develop, the initiating event in \( FC^+ PIK3ca^{+/+} \) cancers appears to be PI3K-mediated.

With the goal of identifying precursor lesions or early tumors, \( FC^+ PIK3ca^{+/+} \) mice were sacrificed at fixed points in time. Cohorts of at least 4 mice were evaluated at 20, 30, 40, and 50 days of age. On histologic examination, the distal small bowel and colon were hyperplastic in all mice examined, and lesions similar to serrated sessile adenomas that are seen in humans were identified (Supplementary Fig. S6). Invasive mucinous adenocarcinomas were first observed in the 40-day cohort.

To examine whether intestinal cell fate is altered before tumorigenesis, we assessed the 4 major epithelial cell lineages...
in the colon (Supplementary Fig. S7). The relative number of absorptive cells, Paneth cells, and endoendocrine cells in samples from FC⁺ PIK3ca⁺ mice were similar to that observed in samples from controls. In contrast, goblet cells were slightly more abundant in the samples from experimental mice than in samples from controls.

Animal models have led to significant advances in our understanding of the biology of many cancer types, including colorectal cancer. This study is the first to describe highly invasive mucinous adenocarcinomas in the mammalian intestine resulting from the expression of a dominantly active form of PI3K. These tumors are quite comparable with cancers in humans, especially those on the right side of the colon. Shared histologic characteristics include high-grade nuclear atypia, budding at the leading edge of the invasion fronts, and mucin lakes (14).

Recently, a model lacking the expression of PTEN in mice was described (15). This model has a drastically different phenotype with only 19% of mice developing tumors in the small intestine by 12 months of age. Despite having activated AKT signaling, only one invasive adenocarcinoma was identified. We hypothesize that the primary difference between these two models relates to the roles of PTEN and PI3K in the AKT signaling pathway (i.e., tumor suppressor gene vs. an oncogene). These models also have different genetic backgrounds, and Cre expression was controlled by different promoters.

Interestingly, in FC⁺ PIK3ca⁺ mice, tumor initiation appears to be independent of WNT signaling and invasive cancers develop rapidly without a benign polypoid or exophytic precursor lesion. These tumors appear to develop through a novel noncanonical pathway to tumor initiation mediated by PI3K. Human tumor cell lines, including the commonly investigated RKO human colon cancer cell line, that possess activating mutations of PIK3CA but lack mutations of APC and CTNNB1 have been described (15). In addition, human colorectal tumors carrying activating mutations in PIK3CA often express normal levels of β-catenin (16). Thus, nonpolyloid tumors that arise quickly as a consequence of mutations in PIK3CA might explain the development of interval cancers that occur between screening colonoscopies. Further investigations need to examine this novel pathway to tumorigenesis described in this study.

This model will also aid in the development and testing of pharmacologic agents. Targeting oncogenic pathways has led to recent advances in the treatment of multiple cancers, including the use of vemurafenib to treat melanomas harboring BRAF mutations, erlotinib for lung cancers possessing EGFR mutations, and crizotinib for lung cancers harboring the EML4-ALK translocation. PIK3CA has been identified as an important oncogene in multiple cancers and thus modeling this mutation in the mammalian colon is important. This murine model with rapidly developing invasive colorectal cancers is an exciting model of human colon cancer that has the potential to be instrumental in the development of targeted therapeutics and biomarker identification.

Disclosure of Potential Conflicts of Interest
The authors declare competing financial interests. J.P. Weichert is the founder of Cellectar, Inc., which holds the licensing rights to the CLR1404 technology, and therefore has a financial interest in this agent. J.P. Weichert is also employed as Director, CSO and has ownership interest (including patents) in NovoLog. No potential conflicts of interests were disclosed by the other authors.

Authors’ Contributions
Conception and design: A.A. Leystra, D.A. Deming, C.D. Zahm, R.B. Halberg
Writing, review, and/or revision of the manuscript: A.A. Leystra, D.A. Deming, C.D. Zahm, J.N. Hadac, D.M. Albrecht, L. Clipson, R. Sullivan, M.K. Washington, J.R. Torrealla, R.B. Halberg
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.A. Leystra, D.A. Deming, M. Farhoud
Study supervision: R.B. Halberg
Director of small animal imaging laboratory where imaging studies were done, helped with imaging study design: J.P. Weichert
Acknowledgments
The authors thank Ella Ward and Jane Weeks in Experimental Pathology at the University of Wisconsin Carbone Cancer Center for technical assistance and Drs. Jeff Baker, William F. Dove, Norman Drinkwater, Greg Kennedy, Paul Lambert, Mark Reischelderfer, H. Ian Robbins, and William Schelman for critical review of the manuscript. The article is dedicated in the memory of Joseph E. Hoeger.

Grant Support
The project was supported by the Conquer Cancer Foundation of the American Society of Clinical Oncology through a Young Investigator Award (D.A. Deming), the National Cancer Institute of the U.S. NIH through P01 AI084853 (J.R. Torrealba), P30 CA014520 (Core Grant, University of Wisconsin Carbone Cancer Center), F50 CA095103 (Gastrointestinal Specialized Program of Research Excellence Grant, Vanderbilt-Ingram Cancer Center), R01 CA123438 (R.B. Halberg), T32 CA009614 (D.A. Deming), T32 CA09135 (J. N. Hadac and C.D. Zahn), and T32 CA09217 (T.J.P. Olson), and start-up funds (R.B. Halberg) from the Division of Gastroenterology and Hepatology, the Department of Medicine, and the School of Medicine and Public Health at the University of Wisconsin.

Received December 21, 2011; revised April 10, 2012; accepted April 11, 2012; published OnlineFirst April 23, 2012.

References

15. Wellcome Trust Sanger Institute Cancer Genome Project. Cambridge, UK: Wellcome Trust Genome. Available from: http://www.sanger.ac.uk/genetics/CGP.
Mice Expressing Activated PI3K Rapidly Develop Advanced Colon Cancer

Alyssa A. Leystra, Dustin A. Deming, Christopher D. Zahm, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-4097

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/04/23/0008-5472.CAN-11-4097.DC1

Cited articles
This article cites 15 articles, 4 of which you can access for free at:
http://cancerres.aacrjournals.org/content/72/12/2931.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/72/12/2931.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.