Sex Modulates Intestinal Transformation by the Tumor-Suppressor GCC

Peng Li, M.D., Ph.D., Stephanie Schulz, Ph.D., Giovanni M. Pitari, M.D., Ph.D., and Scott A. Waldman, M.D., Ph.D.

Abstract

Background and Aims: Ovarian hormones oppose colorectal cancer, although mechanisms remain undefined. Similarly, the most commonly lost gene products in intestinal neoplasia include guanylin and uroguanylin, paracrine hormones for guanylyl cyclase C (GCC), which recently emerged as a tumor suppressor. However, the molecular intersection between intestinal paracrine and systemic sex hormones opposing intestinal neoplasia has not been explored.

Methods: Intestinal tumorigenesis was quantified in wild type ($Gcc^{+/+}$) and GCC-deficient ($Gcc^{-/-}$) mice carrying mutations in adenomatous polyposis coli (*Apc*) ($Apc^{Min/+}$) or exposed to the carcinogen azoxymethane (AOM). Proliferation of epithelial cells was examined employing cell cycle markers.

Results: Deletion of *Gcc* increased tumor multiplicity and growth in colons and small intestines, respectively, of $Apc^{Min/+}$ mice. While changes in multiplicity and growth increased tumor burden, females exhibited approximately 60% ($p = 0.040$) of the burden in males. Similarly, female $Gcc^{-/-}$ mice treated with AOM exhibited approximately 40% ($p = 0.048$) of the burden in males. Moreover, *Gcc* deletion promoted epithelial cell proliferation, quantified by increases in β-catenin, cMyc, cyclin D1, and phosphorylated retinoblastoma protein (pRb), in males but not females.

Conclusion: There is a previously unappreciated interaction between sex and GCC signaling restricting crypt cell proliferation. Thus, the invariable loss of guanylin and uroguanylin resulting in tumorigenesis is mitigated in females by hormonal components of the ovarian axis. In the context of the universal overexpression of GCC by tumors, these observations highlight the combination of GCC paracrine and ovarian hormones for targeted prevention and therapy of colorectal cancer.

Keywords: colon cancer, sex, guanylyl cyclase C, proliferation, hormone deficiency, prevention, therapy

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer and cancer-related mortality in the world.[1] Of all cases, approximately 20% reflect heritable genetic mutations, such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), and these patients characteristically develop CRC as young adults.[2–5] A majority (>80%) of cases are sporadic and the lifetime colon cancer risk in the general population is approximately 5%.[6] Carcinogenesis reflects accumulation of multiple mutations in oncogenes or tumor suppressors, which transform colonocytes producing invasive carcinoma.[7,8]

There is an established relationship between the ovarian axis and colorectal tumorigenesis. The annual age-adjusted incidence of colorectal cancer in the United States is higher in men than in women.[6,9] Also, there are sex differences regarding the anatomic location and clinicopathological characteristics of tumors.[6,9] Women have a higher incidence of right-sided colon cancers compared to men.[10–12] Interestingly, the clinical, histopathological, and molecular characteristics of colon tumors reflect the anatomic locations in which they arise.[11–13] Left-sided tumors more frequently exhibit chromosomal instability (CIN) reflected by inactivation of tumor-suppressor genes, including adenomatous polyposis coli (APC) and p53, and mutations in the oncogene K-ras.[8,14] In contrast, right-sided tumors are characterized by mutations in mismatch repair genes that underlie HNPCC.[3,15] Unlike left-sided colon cancer, right-sided tumors exhibit microsatellite instability (MSI).[3,15,16] Moreover, mechanisms underlying inactivation or mutation of tumor suppressors differ: DNA methylation and epigenetic changes, rather than loss of heterozygosity (LOH) in Apc and p53, are more frequent in right-sided colon cancers.[17]

Similarly, hormone replacement therapy (HRT) in postmenopausal women reduces the risk of colorectal cancer by approximately 30–40% and protects against the initiation and growth of adenomas, the precursor lesions of human colorectal cancers.[18,19] In that context, activation of estrogen receptor β-suppressed proliferation of human colon cancer cell by controlling key cell cycle modulators, producing G1-S phase arrest.[20] Moreover, overexpression of estrogen receptor α downregulated β-catenin and its downstream genes, including cyclin D1 and phosphorylated retinoblastoma protein (pRb), suppressing cell proliferation, and activated tumor necrosis factor, inducing apoptosis in human colon cancer cells.[21,22] Furthermore, estrogen receptor α and β inhibited tumorigenesis in proximal colons of $Apc^{Min/+}$ mice.[23] These considerations highlight the potential of HRT to prevent and treat colorectal cancer.

Recently, colorectal cancer has been defined as a disease of localized hormonal deficiency.[24] Indeed, the most commonly lost gene products in colorectal carcinogenesis include guanylin and uroguanylin, the endogenous ligands for guanylyl cyclase C (GCC).[25–27] GCC and its ligands regulate intestinal epithelial cell dynamics and crypt-villus homeostasis by restricting cell proliferation[28,29] through a transient arrest of the cell cycle.[28] Moreover, elimination of GCC suppresses intestinal tumorigenesis in mice carrying mutations in Apc or exposed to the carcinogen azoxymethane (AOM) by restricting proliferation and maintaining genomic integrity.[24] Further, oral administration of uroguanylin suppresses intestinal polyp formation and growth in $Apc^{Min/+}$ mice.[30]

Here, the intersection of sex and GCC signaling opposing tumorigenesis, converging at restriction of crypt cell proliferation, was defined in mice carrying mutations in Apc or exposed to AOM. This previously unrecognized differential effect on tumorigenesis in males and females supports the hypothesis that GCC signaling plays multiple roles in opposing intestinal carcinogenesis beyond the restriction of cell proliferation. The

Department of Pharmacology and Experimental Therapeutics, Thomas Jefferson University, 1100 Walnut Street, MOB 810, Philadelphia, Pennsylvania, USA.
Correspondence: P Li (Peng.Li@jefferson.edu)

DOI: 10.1111/j.1752-8062.2008.00029.x

synergistic protection afforded by GCC signaling and female sex opposing colorectal cancer underscores the potential utility of combining GCC paracrine and ovarian hormones for targeted prevention and therapy of colorectal cancer.

Material and Methods

Animal models

Apc$^{+/+}$*Gcc*$^{-/-}$ mice originally generated using I129/Svj embryonic stem cells[31] were backcrossed to the C57BL/6 strain. $N_{11\text{-}12}$ (11th to 12th generation) mice were crossed to *Apc*$^{Min/+}$ mice and $N_{10\text{-}11}$ mice were treated with AOM (Sigma, cat. A2853, St. Louis, MO, USA). All animal protocols were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. *Apc*$^{Min/+}$*Gcc*$^{+/-}$ (male) and *Apc*$^{+/+}$*Gcc*$^{+/-}$ mice were established from *Apc*$^{Min/+}$ (male) breeding founders to generate F2 *Apc*$^{Min/+}$*Gcc*$^{+/+}$ (23 males and 9 females) and *Apc*$^{Min/+}$*Gcc*$^{-/-}$ (15 males and 10 females) mice. The integrity of the Gcc gene was defined by genotype analysis and confirmed by analysis of ^{125}I-ST binding in membranes harvested from small or large intestine.[28,32] *Apc*$^{Min/+}$ mice were sacrificed at 8 weeks to examine intestinal tumors. Mice (6 weeks old) received intraperitoneal injections (12 mg/kg body weight) of AOM weekly for 6 weeks and 12 weeks after the first injection, and they (10 males and 15 females for *Apc*$^{+/+}$*Gcc*$^{+/+}$ and 13 males and 12 females for *Apc*$^{+/+}$*Gcc*$^{-/-}$) were sacrificed and intestines examined for tumors.

Tumor examination

Tumors were enumerated and their size quantified under a dissecting microscope (approximately 20–30 ×) in a blinded fashion. Tumor burden per animal was calculated as the sum of the sizes (diam^2) of individual tumors in each segment from each animal. All tumors from AOM-treated mice and selected tumors from *Apc*$^{Min/+}$ mice were histologically confirmed by a pathologist blinded to information for each case.

Immunoblot analysis

Epithelia were dissected from normal intestinal mucosas from five male and three female *Gcc*$^{+/+}$ or *Gcc*$^{-/-}$ mice, protein extracted in Laemmli buffer containing protease and phosphatase inhibitor cocktails (Pierce, Rockford, IL, USA), and extracts stored at –20°C. Extracts were subjected to immunoblot analyses employing antibodies to: β-catenin (cat. sc-7199), cMyc (cat. sc-764), cyclin D1 (cat. sc-718), and pRb (cat. sc-16671) from Santa Cruz (1:200 dilution, Santa Cruz, CA, USA) and GAPDH from Cell Signaling (cat. 2118, 1:1,000 dilution, Danvers, MA, USA). Bovine anti-goat and anti-rabbit secondary antibodies were from Santa Cruz (1:5,000 dilution, Santa Cruz, CA, USA). Staining intensity of specific bands quantified by densitometry was normalized to that for GAPDH. Average relative intensity reflects the mean of five male and three female mice.

Statistical analysis

Tumor multiplicity (number of tumors per animal) was analyzed by Poisson regression. Tumor burden and tumor size (mm^2) in the continuous scale were analyzed by linear mixed models, with random effect of animal to control for multiple measures per animal. Intensity of immunoblot staining was analyzed by *t*-test (2 tails) in both male and female groups.

Results

GCC differentially suppresses intestinal tumorigenesis in male and female *Apc*$^{Min/+}$ mice

The impact of *Gcc* deletion on intestinal tumorigenesis was examined in male and female mice heterozygous for wild-type

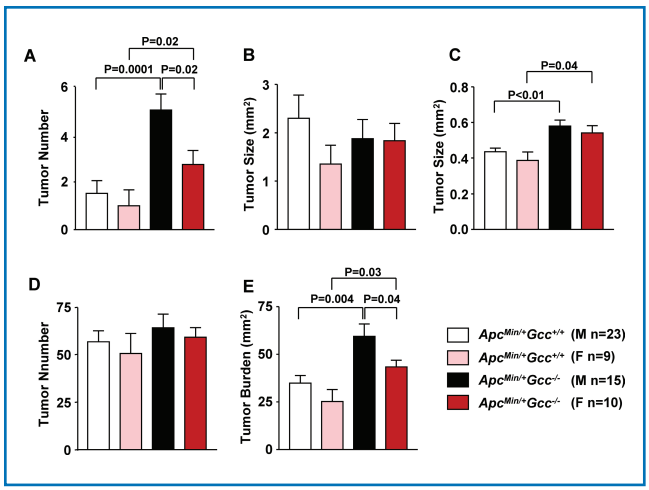

Figure 1. Impact of sex on tumorigenesis in *Apc*$^{Min/+}$*Gcc*$^{+/+}$ and *Apc*$^{Min/+}$*Gcc*$^{-/-}$. (**A**) Tumors were enumerated in the colon of *Apc*$^{Min/+}$*Gcc*$^{+/+}$ and *Apc*$^{Min/+}$*Gcc*$^{-/-}$ mice. Tumor multiplicity was significantly increased in male and female *Apc*$^{Min/+}$*Gcc*$^{-/-}$ mice, with a lower magnitude in females. (**B**) Tumor size (diam^2) was quantified in male and female *Apc*$^{Min/+}$*Gcc*$^{-/-}$ and *Apc*$^{Min/+}$*Gcc*$^{+/+}$ mice. (**C**) Similarly, tumor number and size were quantified in small intestines of *Apc*$^{Min/+}$*Gcc*$^{+/+}$ and *Apc*$^{Min/+}$*Gcc*$^{-/-}$ mice. The size of tumors (diam^2) in small intestines was increased in male and female *Apc*$^{Min/+}$*Gcc*$^{-/-}$, compared to *Apc*$^{Min/+}$*Gcc*$^{+/+}$ mice. (**D**) Tumor multiplicity was modestly increased in both sexes in *Apc*$^{Min/+}$*Gcc*$^{-/-}$ mice. (**E**) Tumor burden in each animal was calculated as the sum of the sizes of all tumors. Although tumor burden was significantly increased in male and female *Apc*$^{Min/+}$*Gcc*$^{-/-}$ mice, it was significantly higher in males, compared to females. Bars represent mean ± SEM.

Apc. *APC* is mutated in >80% of sporadic colorectal tumors and germline mutations in *APC* underlie the inherited intestinal neoplastic syndrome FAP. Elimination of *Gcc* selectively increased tumor multiplicity in colon (*Figure 1A*) without altering tumor size (*Figure 1B*). Loss of *Gcc* significantly increased multiplicity in male and female *Apc*$^{Min/+}$ mice, but with a lower magnitude in females (5.5-fold in males vs. 2-fold in females, $p = 0.02$). Thus, *Apc*$^{Min/+}$*Gcc*$^{-/-}$ male mice ($n = 15$) developed 5.5 tumors (median) while *Apc*$^{Min/+}$*Gcc*$^{+/+}$ male mice ($n = 32$) developed 1 tumor (median) in distal colon ($p = 0.0001$). In contrast, *Apc*$^{Min/+}$*Gcc*$^{-/-}$ female mice ($n = 10$) developed two tumors (median) while *Apc*$^{Min/+}$*Gcc*$^{+/+}$ female mice ($n = 9$) developed 1 tumor (median) in distal colon ($p = 0.02$). Conversely, in small intestine elimination of *Gcc* increased tumor size in males and females (*Figure 1C*) without affecting tumor multiplicity (*Figure 1D*). Increased tumor multiplicity in colon and tumor size in small intestine contributed to increased tumor burden in both sexes of *Apc*$^{Min/+}$*Gcc*$^{-/-}$ mice (*Figure 1E*). However, tumor burden was significantly lower in female *Apc*$^{Min/+}$*Gcc*$^{-/-}$, compared to male *Apc*$^{Min/+}$*Gcc*$^{-/-}$ mice (59 ± 7 mm^2 in male *Apc*$^{Min/+}$*Gcc*$^{-/-}$ mice vs. 43 ± 4 mm^2 in female *Apc*$^{Min/+}$*Gcc*$^{-/-}$ mice, $p = 0.04$).

GCC differentially inhibits AOM-induced colorectal tumorigenesis in male and female mice

In mice, AOM mimics the induction of sporadic colon cancer in humans by inducing DNA alkylation-dependent base pair mutations, resulting in random single base alterations and DNA strand breaks.[33] AOM-induced tumors were primarily restricted to the distal half of the colon. Similar to *Apc*$^{Min/+}$ mice, deletion of *Gcc* significantly increased tumor multiplicity (*Figure 2A*) and growth (*Figure 2B*) in male and female mice. Tumor burden induced by AOM, reflecting increased tumor multiplicity and tumor size, was enhanced 3.6-fold in males ($p = 0.01$) and 2.3-fold in females ($p = 0.06$) by elimination of *Gcc* (*Figure 2C*). Tumor burden was significantly lower in female *Gcc*$^{-/-}$, compared to male

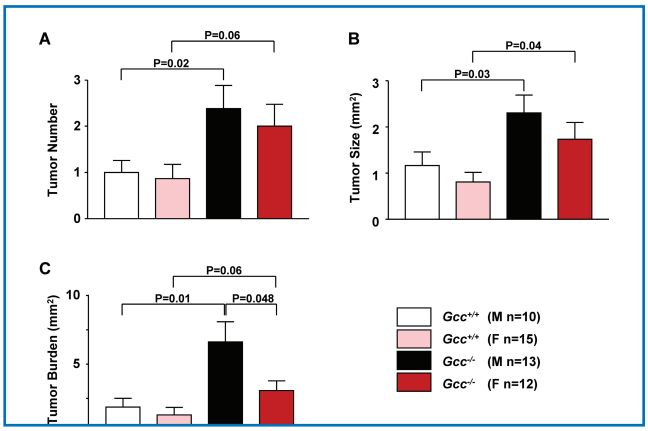

Figure 2. Impact of sex on tumorigenesis in *Gcc*$^{+/+}$ and *Gcc*$^{-/-}$ mice treated with AOM. (**A**) Tumors were enumerated in colons of mice treated with AOM. Tumor multiplicity was significantly increased in male and female *Gcc*$^{-/-}$ mice, with a lower magnitude in females. (**B**) Tumor size (diam^2) was significantly increased in male and female *Gcc*$^{-/-}$, compared to *Gcc*$^{+/+}$, mice. (**C**) Although tumor burden was significantly increased in male and female *Gcc*$^{-/-}$ mice, it was significantly higher in males, compared to females. Bars represent mean ± SEM.

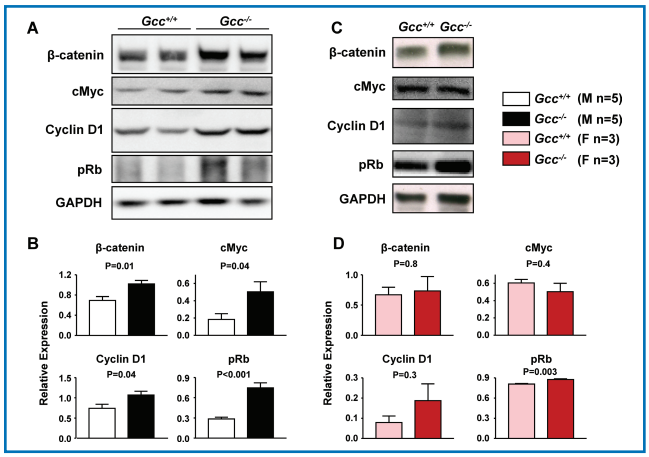

Figure 3. GCC suppresses cell proliferation in intestine of male, but not female, mice. (**A, C**) Cell proliferation was quantified by immunoblot analysis of mediators of the cell cycle, including β-catenin, cMyc, cyclin D1, and pRb in intestinal mucosa from male and female *Gcc*$^{+/+}$ and *Gcc*$^{-/-}$ mice. (**B, D**) Immunoblot intensity of specific bands quantified by densitometry was normalized to that for GAPDH. Average relative intensity reflects the mean of five animals in male and three animals in female. Bars represent mean ± SEM.

Gcc$^{-/-}$ mice (6.6 ± 1.5 mm^2 in male *Gcc*$^{-/-}$ mice vs. 3.0 ± 0.7 mm^2 in female *Gcc*$^{-/-}$ mice, $p = 0.048$).

GCC inhibits enterocyte proliferation in male, but not female mice

Elimination of *Gcc* in male mice increased proliferation of intestinal epithelia,[24,28] reflected by overexpression of β-catenin (*Figure 3A and 3B;* $p = 0.01$), a key regulator of cell proliferation that maintains intestinal epithelial cell renewal.[34,35] Hyperproliferation by upregulating β-catenin in male *Gcc*$^{-/-}$ mice reflects the established role of cGMP in targeting that protein for proteosomal degradation.[36] Acceleration of the cell cycle in *Gcc*$^{-/-}$ mice was associated with increase in the expression of β-catenin downstream targets,[34,35] including cMyc and cyclin D1 ($p = 0.04$; *Figure 3A and 3B*). Furthermore, upregulation of cyclin D1 resulted in phosphorylation of Rb ($p = 0.0003$), accelerating the cell cycle. These data suggest that GCC suppressed tumorigenesis by restricting proliferation and antagonizing the Wnt/β-catenin signaling cascade.[34,35,37,38] In contrast, elimination of *Gcc* did not affect cell proliferation in intestines of female mice (*Figure 3C and 3D*).

Discussion

Human colorectal cancer is a heterogeneous disease in which genetic mutations and environmental factors contribute to tumor initiation, promotion, growth, and malignant transformation.[7,8] Sex is a major factor in human intestinal neoplasia, and in the United States, women have a lower age-adjusted incidence of colon cancer compared to men. Moreover, this phenomenon is recapitulated in *Apc*$^{Min/+}$ mice. Indeed, although *Apc*$^{Min/+}$ mice, unlike humans, develop tumors predominantly in small intestine, there is a lower incidence and multiplicity of colorectal adenomas in females compared to males.[39] This study extended those previous observations beyond genetic models of intestinal neoplasia, demonstrating the impact of sex on intestinal tumorigenesis in a model of chemical carcinogenesis, in mice exposed to AOM. Abundant prospective and retrospective analyses have revealed the inverse relationship between HRT in postmenopausal women and risk of colon cancer.[40,41] Further, women experiencing surgical menopause exhibit an increased incidence of colorectal adenoma compared to those undergoing menopause naturally.[40] Taken together, these observations suggest the utility of HRT for colon cancer prevention.[40,41]

GCC has recently emerged as a novel tumor suppressor central to the initiation and promotion of colorectal cancer.[24,30,42] The endogenous paracrine hormones, guanylin and uroguanylin, are gene products most frequently lost during tumorigenesis, which occurs early along the adenoma-carcinoma sequence.[25–27] Conversely, oral administration of GCC ligand suppresses tumor multiplicity and size in intestine of *Apc*$^{Min/+}$ mice.[30] Further, activation of GCC signaling suppresses human colon cancer cell growth.[42–44] Moreover, GCC regulates homeostasis along the intestinal crypt-villus axis by restricting proliferation, the cell cycle and crypt hyperplasia, mechanisms that are corrupted by dysregulation of GCC signaling, which directly underlies intestinal tumorigenesis.[28,29] Observations here suggest that GCC signaling restricts proliferation by modulating the Wnt/β-catenin pathway, promoting PKG1β-dependent degradation of β-catenin and inhibiting its nuclear translocation,[36] reducing expression of downstream targets, including key regulators of the cell cycle such as cMyc, cyclin D1, and phosphorylated Rb. These observations suggest that colorectal cancer may represent, in part, a condition of paracrine hormone insufficiency. In that context, they implicate a role for paracrine hormone supplementation to prevent and treat intestinal tumorigenesis.[24,45]

The present observations reveal for the first time an intersection between sex and the GCC-based paracrine hormone axis regulating crypt homeostasis. They suggest that female sex and GCC signaling converge at restriction of crypt cell proliferation, a key mechanism underlying tumorigenesis promoted by dysregulated GCC signaling.[24,28] Indeed, elimination of GCC in males decreased expression of β-catenin and its downstream targets regulating the cell cycle, including cMyc, cyclin D1, and pRb, key mediators of proliferation,[34,35] and these changes were associated with tumor initiation and promotion. In striking contrast, elimination of GCC was without affect on β-catenin and its downstream effectors in females, reflected by diminished tumorigenesis in models of genetic or chemical carcinogenesis. These observations suggest that the invariable loss of guanylin and uroguanylin and the resultant downstream effects

on tumorigenesis through unrestricted proliferation and the associated acceleration of the cell cycle and crypt hypertrophy[24,28] are mitigated by select, but unknown, mechanisms in females. Moreover, they underscore the multiplicity of mechanisms by which dysregulated GCC signaling influences tumorigenesis.[24,45] Beyond proliferation, GCC regulates metabolic remodeling, epithelial-mesenchymal interactions, and genomic integrity,[24,45] which likely contribute to tumorigenesis in female mice in which GCC signaling was eliminated. In the context of the established association between HRT,[18,19] menopause,[40,41] and the risk of colon cancer, and the defined effects of estrogen on proliferative regulators,[21–23] it is likely that hormonal components of the ovarian axis mediate these protective effects, although a contribution of androgens to the promotion of tumorigenesis in males cannot be ruled out.[46,47]

The previously unappreciated synergy between sex and the GCC signaling axis suggests a unique opportunity for cancer prevention and treatment. Expression of guanylin and uroguanylin is invariably lost early along the continuum of neoplastic transformation, and the role of GCC in restraining proliferation suggests that receptor dysregulation reflecting ligand insufficiency is a central mechanism contributing to colorectal tumorigenesis. However, ligand insufficiency is associated with receptor overexpression, and GCC mRNA and protein are universally increased in human colorectal tumor, compared to normal epithelial, cells rendering them highly specific targets for therapy.[48–50] Taken together, in the context of the standard of care in which hormone deficiencies are treated by replacement, synergy between systemic female and intestinal paracrine hormones in antagonizing tumorigenesis underscores the potential for combination HRT and GCC ligand supplementation for targeted prevention and therapy in colorectal cancer.

Acknowledgments

These studies were supported by grants from NIH (CA75123, CA95026) and Targeted Diagnostic and Therapeutics Inc. to SAW, and the Pennsylvania Department of Health to GMP. The Pennsylvania Department of Health specifically disclaims responsibility for any analyses, interpretations, or conclusions. SAW is the Samuel M.V. Hamilton Endowed Professor.

References

1. Stec-Michalska K, Wichan P, Wojtun S. Early diagnosis of colorectal cancer. *Pol Merkur Lekarski.* 2004; 17(Suppl 1): 59–62.

2. Aaltonen LA, Peltomaki P, Leach FS, Sistonen p, Pylkkanen L, Mecklin JP, Jarvinen H, Powell SM, Jen J, Hamilton SR, et al. Clues to the pathogenesis of familial colorectal cancer. *Science.* 1993; 260(5109): 812–816.

3. Annie Yu HJ, Lin KM, Ota DM, Lynch HT. Hereditary nonpolyposis colorectal cancer: preventive management. *Cancer Treat Rev.* 2003; 29(6): 461–470.

4. Lynch HT, de la Chapelle A. Hereditary colorectal cancer. *N Engl J Med.* 2003; 348(10): 919–932.

5. Lynch HT, Cristofaro G, Rozen P, Vasen H, Lynch P, Mecklin JP, St John J. History of the international collaborative group on hereditary non polyposis colorectal cancer. *Fam Cancer.* 2003; 2(Suppl 1): 3–5.

6. Edwards BK, Brown ML, Wingo PA, Howe HL, Ward E, Ries LA, Schrag D, Jamison PM, Jemal A, Wu XC, Friedman C, Harlan L, Warren J, Anderson RN, Pickle LW. Annual report to the nation on the status of cancer, 1975–2002, featuring population-based trends in cancer treatment. *J Natl Cancer Inst.* 2005; 97(19): 1407–1427.

7. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med.* 2004; 10(8): 789–799.

8. Vogelstein B, Fearson ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. Genetic alterations during colorectal-tumor development. *N Engl J Med.* 1988; 319(9): 525–532.

9. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics, 2006. *CA Cancer J Clin.* 2006; 56(2): 106–130.

10. Konishi K, Fujii T, Boku N, Kato S, Kobal I, Ohtsu A, Tajiri H, Ochiai A, Yoshida S. Clinicopathological differences between colonic and rectal carcinomas: are they based on the same mechanism of carcinogenesis? *Gut.* 1999; 45(6): 818–821.

11. Liu LU, Holt PR, Krivosheyev V, Moss SF. Human right and left colon differ in epithelial cell apoptosis and in expression of Bak, a pro-apoptotic Bcl-2 homologue. *Gut.* 1999; 45(1): 45–50.

12. Nawa T, Kato J, Kawamoto H, Okada H, Yamamoto H, Kohno H, Endo H, Shiratori Y. Differences between right- and left-sided colon cancer in patient characteristics, cancer morphology and histology. *J Gastroenterol Hepatol.* 2007.

13. Park SY, Lee HS, Choe G, Chung JH, Kim WH. Clinicopathological characteristics, microsatellite instability, and expression of mucin core proteins and p53 in colorectal mucinous adenocarcinomas in relation to location. *Virchows Arch.* 2006; 449(1): 40–47.

14. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell.* 1990; 61(5): 759–767.

15. Iino H, Simms L, Young J, Arnold J, Winship IM, Webb SI, Furlong KL, Leggett B, Jass JR. DNA microsatellite instability and mismatch repair protein loss in adenomas presenting in hereditary non-polyposis colorectal cancer. *Gut.* 2000; 47(1): 37–42.

16. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science.* 1993; 260(5109): 816–819.

17. Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet.* 2006; 38(7): 787–793.

18. Chlebowski RT, Wactawski-Wende J, Ritenbaugh C, Hubbell FA, Ascensao J, Rodabough RJ, Rosenberg RJ, Rosenberg CA, Taylor VM, Harris R, Chen C, Adams-Campbell LL, White E. Estrogen plus progestin and colorectal cancer in postmenopausal women. *N Engl J Med.* 2004; 350(10): 991–1004.

19. Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, Bonds D, Brunner R, Brzyski R, Caan B, Chlebowski R, Curb D, Gass M, Hays J, Heiss G, Hendrix S, Howard BV, Hasia J, Hubbell A, Jackson R, Johnson KC, Judd H, Kotchen JM, Kuller L, LaCroix AZ, Lane D, Langer RD, Lasser N, Lewis CE, Manson J, Margolis K, Ockene J, O'Sullivan MJ, Phillips L, Prentice RL, Ritenbaugh C, Robbins J, Rossouw JE, Sarto G, Stefanick ML, Van Horn L, Wactawski-Wende J, Wallace R, Wassertheil-Smoller S. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the women's health initiative randomized controlled trial. *JAMA.* 2004; 291(14): 1701–1712.

20. Martineti V, Picariello L, Tognarini I, Carbonell Sala S, Gozzini A, Azzari C, Mavilia C, Tanini A, Falchetti A, Fiorelli G, Tonelli F, Brandi ML. ERbeta is a potent inhibitor of cell proliferation in the HCT8 human colon cancer cell line through regulation of cell cycle components. *Endocr Relat Cancer.* 2005; 12(2): 455–469.

21. Hsu HH, Cheng SF, Chen LM, Liu JY, Chu CH, Weng YJ, Li ZY, Lin CS, Lee SD, Kuo WW, Huang CY. Over-expressed estrogen receptor-alpha up-regulates hTNF-alpha gene expression and down-regulates beta-catenin signaling activity to induce the apoptosis and inhibit proliferation of LoVo colon cancer cells. *Mol Cell Biochem.* 2006; 289(1–2): 101–109.

22. Galluzzo P, Caiazza F, Moreno S, Marino M. Role of ERbeta palmitoylation in the inhibition of human colon cancer cell proliferation. *Endocr Relat Cancer.* 2007; 14(1): 153–167.

23. Cho NL, Javid SH, Carothers AM, Redstin M, Bertagnolli MM. Estrogen receptors alpha and beta are inhibitory modifiers of Apc-dependent tumorigenesis in the proximal colon of Min/+ mice. *Cancer Res.* 2007; 67(5): 2366–2372.

24. Li P, Schulz S, Bombonati A, Palazzo JP, Hyslop TM, Xu Y, Baran AA, Siracusa LD, Pitari GM, Waldman SA. Guanylyl cyclase C suppresses intestinal tumorigenesis by restricting proliferation and maintaining genomic integrity. *Gastroenterology.* 2007; 133(2): 599–607.

25. Notterman DA, Alon U, Sierk AJ, Levine AJ. Transcriptional gene expression profiles of colorectal adenoma, adenocarcinoma, and normal tissue examined by oligonucleotide arrays. *Cancer Res.* 2001; 61(7): 3124–3130.

26. Steinbrecher KA, Tuohy TM, Goss K, Scott MC, Witte DP, Groden J, Cohen MB. Expression of guanylin is downregulated in mouse and human intestinal adenomas. *Biochem Biophys Res Commun.* 2000; 273(1): 225–230.

27. Zhang L, Zhou W, Velculescu VE, Kern SE, Hruban RH, Hamilton SR, Vogelstein B, Kinzler KW. Gene expression profiles in normal and cancer cells. *Science.* 1997; 276(5316): 1268–1272.

28. Li P, Lin JE, Chervoneva I, Schulz S, Waldman SA, Pittari GM. Homeostatic control of the crypt-villus axis by the bacterial enterotoxin receptor guanylyl cyclase C restricts the proliferating compartment in intestine. *Am J Pathol.* 2007; 171(6): 1847–1858.

29. Steinbrecher KA, Wowk SA, Rudolp JA, Witte DP, Cohen MB. Targeted inactivation of the mouse guanylin gene results in altered dynamics of colonic epithelial proliferation. *Am J Pathol.* 2002; 161(6): 2169–2178.

30. Shailubhai K, Yu HH, Karunanandaa K, Wang JY, Eber SL, Wang Y, Joo NS, Kim HD, Miedema BW, Abbas SZ, Boddupalli SS, Currie MG, Forte LR. Uroguanylin treatment suppresses polyp formation in the Apc(Min/+) mouse and induces apoptosis in human colon adenocarcinoma cells via cyclic GMP. *Cancer Res.* 2000; 60(18): 5151–5157.

31. Schulz S, Lopez MJ, Kuhn M, Garberrs DL. Disruption of the guanylyl cyclase-C gene leads to a paradoxical phenotype of viable but heat-stable enterotoxin-resistant mice. *J Clin Invest.* 1997; 100(6): 1590–1595.

32. Carrithers SL, Parkinson SJ, Goldstein S, Park P, Robertson DC, Waldman SA. Escherichia coli heat-stable toxin receptors in human colonic tumors. *Gastroenterology.* 1994; 107(6): 1653–1661.

33. Sedgwick B. Repairing DNA-methylation damage. *Nat Rev Mol Cell Biol.* 2004; 5(2): 148–57.

34. Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC$^{-/-}$ colon carcinoma. *Science.* 1997; 275(5307): 1784–1787.

35. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW. Activation of beta-catenin-TCF signaling in colon cancer by mutations in beta-catenin or APC. *Science.* 1997; 275(5307): 1787–17890.

36. Thompson WJ, Piazza GA, Li H, Liu L, Fetter J, Zhu B, Sperl G, Ahnen D, Pamukcu R. Exisulind induction of apoptosis involves guanosine 3′,5′-cyclic monophosphate phosphodiesterase inhibition, protein kinase G activation, and attenuated beta-catenin. *Cancer Res.* 2000; 60(13): 3338–3342.

37. Takahashi M, Nakatsugi S, Sugimura T, Wakabayashi K. Frequent mutations of the beta-catenin gene in mouse colon tumors induced by azoxymethane. *Carcinogenesis.* 2000; 21(6): 1117–1120.

38. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Battle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R, Clevers H. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell.* 2002; 111(2): 241–250.

39. Cooper HS, Chang WC, Coudry R, Gery MA, Everley L, Spittle CS, Wang H, Litwin S, Clapper ML. Generation of a unique strain of multiple intestinal neoplasia (Apc(+/Min-FCCC)) mice with significantly increased numbers of colorectal adenomas. *Mol Carcinog.* 2005; 44(1): 31–41.

40. Woodson K, Lanza E, Tangrea JA, Albert PS, Slattery M, Pinsky J, Caan B, Paskett E, Iber F, Kikendall JW, Lance P, Shike M, Weissfed J, Schatzkin A. Hormone replacement therapy and colorectal adenoma recurrence among women in the polyp prevention trial. *J Natl Cancer Inst.* 2001; 93(23): 1799–1805.

41. Martinez ME. Hormone replacement therapy and adenoma recurrence: implications for its role in colorectal cancer risk. *J Natl Cancer Inst.* 2001; 93(23): 1764–1765.

42. Pitari GM, Di Guglielmo MD, Park J, Schulz S, Waldman SA. Guanylyl cyclase C agonists regulate progression through the cell cycle of human colon carcinoma cells. *Proc Natl Acad Sci USA.* 2001; 98(14): 7846–7851.

43. Pitari GM, Zingman LV, Hodgson DM, Alekseev AE, Kazerounian S, Bienengraeber M, Hajnoczky G, Terzic A, Waldman SA. Bacterial enterotoxins are associated with resistance to colon cancer. *Proc Natl Acad Sci USA.* 2003; 100(5): 2695–2699.

44. Pitari GM, Baksh RI, Harris DM, Li P, Kazerounian S, Waldman SA. Interruption of homologous desensitization in cyclic guanosine 3′,5′-monophosphate signaling restores colon cancer cytostasis by bacterial enterotoxins. *Cancer Res.* 2005; 65(23): 11129–11135.

45. Pitari GM, Li P, Lin JE, Zuzga D, Gibbons AV, Snook AE, Schulz S, Waldman SA. The paracrine hormone hypothesis of colorectal cancer. *Clin Pharmacol Ther.* 2007; 82(4): 441–447.

46. Slattery ML, Swweeney C, Murtaugh M, Ma KN, Wolff RK, Potter JD, Caan BJ, Samowitz W. Associations between ERalpha, ERbeta, and AR genotypes and colon and rectal cancer. *Cancer Epidemiol Biomarkers Prev.* 2005; 14(12): 2936–2942.

47. Kikuchi H, Uchida C, Hattori T, Isobe T, Hiramatsu Y, Kitagawa K, Oda T, Konno H, Kitagawa M. ARA54 is involved in transcriptional regulation of the cyclin D1 gene in human cancer cells. *Carcinogenesis* 2007; 28(8): 1752–1758.

48. Schulz S, Hyslop T, Haaf J, Bonaccorso C, Nielsen K, Witek ME, Birbe R, Palazzo J, Weinberg D, Waldman SA. A validated quantitative assay to detect occult micrometastases by reverse transcriptase-polymerase chain reaction of guanylyl cyclase C in patients with colorectal cancer. *Clin Cancer Res.* 2006; 12(15): 4545–4552.

49. Witek ME, Nielsen K, Walters R, Hyslop T, Palazzo J, Schulz S, Waldman SA. The putative tumor suppressor Cdx2 is overexpressed by human colorectal adenocarcinomas. *Clin Cancer Res.* 2005; 11(24 Pt 1): 8549–8556.

50. Birbe R, Palazzo JP, Walters R, Weinberg D, Schulz S, Waldman SA. Guanylyl cyclase C is a marker of intestinal metaplasia, dysplasia, and adenocarcinoma of the gastrointestinal tract. *Human Pathol.* 2005; 36(2): 170–179.

Synopsis

Sex Modulates Intestinal Transformation by the Tumor-Suppressor GCC

There is an established relationship between sex and colorectal tumorigenesis, and the annual age-adjusted incidence of colorectal cancer in the United States is higher in men than in women. In addition, there are sex differences regarding the anatomic location and clinicopathological characteristics of tumors. Moreover, hormone replacement therapy (HRT) in postmenopausal women reduces the risk of colorectal cancer by 30–40%. However, while ovarian hormones mitigate colorectal cancer risk by modifying genetic and environmental determinants, the precise mechanisms remain undefined. In that regard, the most commonly lost gene products in intestinal neoplasia include guanylin and uroguanylin, paracrine hormones for guanylyl cyclase C (GCC), an emergent tumor-suppressor regulating crypt hyperplasia, proliferation, and the cell cycle. Of note, the molecular intersection between intestinal paracrine and systemic sex hormones opposing intestinal neoplasia has not been explored. Here, the impact of GCC on tumorigenesis was examined in mice carrying mutations in *Apc* (*Apc*$^{Min/+}$) or exposed to azoxymethane (AOM), models of genetic and chemical intestinal carcinogenesis. Proliferation of intestinal epithelial cells was quantified employing molecular markers of the cell cycle. While elimination of GCC expression increased tumor burden in *Apc*$^{Min/+}$ and AOM-treated mice, females exhibited only approximately 50% of the burden observed in males. Of note, elimination of GCC expression promoted epithelial cell proliferation in males but not in females. These observations reveal an intersection between sex and the GCC-based paracrine hormone axis, converging at restriction of crypt cell proliferation. They suggest that the invariable loss of guanylin and uroguanylin and the resultant downstream effects on tumorigenesis are mitigated by sex. In the context of the established association between HRT, menopause, and the risk of colon cancer, and the inhibition by estrogen of cell cycle regulators, it is presumed that hormonal components of the ovarian axis mediate these protective effects. This previously unappreciated synergy between sex and the GCC signaling axis suggests a unique opportunity for cancer prevention and treatment. Indeed, while expression of guanylin and uroguanylin is invariably lost early along the continuum of neoplastic transformation, GCC is universally overexpressed by human colorectal tumors. In the context of the standard of care in which hormone deficiencies are treated by replacement, the present observations underscore the potential for combining systemic sex and GCC paracrine hormone supplementation for targeted prevention and therapy in colorectal cancer.

Peng Li, M.D., Ph.D., Stephanie Schulz, Ph.D.,
Giovanni M. Pitari, M.D., Ph.D,
and Scott A. Waldman, M.D., Ph.D.
Department of Pharmacology and Experimental Therapeutics, Thomas Jefferson University,
1100 Walnut Street, MOB 810,
Philadelphia, Pennsylvania, USA
Correspondence: P Li (Peng.Li@jefferson.edu)