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Guanylyl cyclase C in colorectal cancer: susceptibility gene and potential therapeutic target

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SUMMARY

Colorectal cancer is one of the leading causes of tumor-related morbidity and mortality worldwide. While mechanisms underlying this disease have been elucidated over the past two decades, these molecular insights have failed to translate into efficacious therapy. The oncogenomic view of cancer suggests that terminal transformation reflects the sequential corruption of signal transduction circuits regulating key homeostatic mechanisms, whose multiplicity underlies the therapeutic resistance of most tumors to interventions targeting individual pathways. Conversely, the paucity of mechanistic insights into proximal pathophysiological processes that initiate and amplify oncogenic circuits preceding accumulation of mutations and transformation impedes development of effective prevention and therapy. In that context, guanylyl cyclase C (GCC), the intestinal receptor for the paracrine hormones guanylin and uroguanylin, whose early loss characterizes colorectal transformation, has emerged as a component of lineage-specific homeostatic programs organizing spatiotemporal patterning along the crypt-surface axis. Dysregulation of GCC signaling, reflecting hormone loss, promotes tumorigenesis through reprogramming of replicative and bioenergetic circuits and genomic instability. Compensatory up-regulation of GCC in response to hormone loss provides a unique translational opportunity for prevention and treatment of colorectal tumors by hormone replacement therapy.

Keywords

Colorectal cancer; guanylyl cyclase C; guanylin; uroguanylin; hormone insufficiency; hormone replacement therapy; AKT; proliferation; cell cycle; metabolism; genomic instability; lineage-specific tumorigenesis

COLORECTAL CANCER is the 4th leading cause of cancer and the 2nd leading cause of cancer-related mortality in the world [1,2]. Mortality mainly reflects metastatic disease progression [2,3]. Current regimens for adjuvant chemotherapy combining thymidylate synthase inhibitors with topoisomerase inhibitors or platinum derivatives (FOLFIRI and FLOPOX) only marginally improve survival (overall survival less than 2 years.) [4-6]. The paucity of efficacious therapy associated with high rates of recurrence in advanced colorectal cancer (>60% in stage III disease and >90% in stage IV) underscores the unmet clinical need for therapeutic approaches targeting early-stage disease, before tumor cells acquire the

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malignant phenotype supporting accelerated growth, survival, and invasion [2,3,7-10]. Indeed, mechanistic insights into the pathophysiology of early tumorigenesis will facilitate evolution of therapeutic interventions to prevent, interrupt, or reverse disease progression by inhibiting precursor lesion formation and the adenoma-to-carcinoma transition.

COLORECTAL TUMORIGENESIS is currently envisioned as a process of progressive transformation of normal epithelium to invasive carcinoma reflecting accumulation of sequential mutations shared by sporadic and inherited colorectal tumors (Fig. 1) [11,12]. These genetic alterations corrupt normal homeostatic circuits, including cell cycle and proliferative regulation, DNA damage sensing and repair, and metabolic programming which, in the absence of appropriate apoptotic responses, recursively amplify the accumulation of genetic mutations [13-15]. Among genetic alterations occurring along the continuum of tumorigenesis, *APC* mutations represent the earliest event, followed by *KRAS*, *SMAD2*, *SMAD4* and *TP53* [11, 12,15]. *APC* is a master switch between proliferation and differentiation of intestinal epithelial cells [16] and its loss disturbs homeostasis, producing expansion of the proliferative compartment and loss of expression of epithelial differentiation markers [17]. *KRAS* is the downstream effector of tyrosine kinases, activating both MAPK and PI3K-AKT signaling critical for tumor progression [18]. *SMAD2* and *SMAD4* are components of TGF- β signaling, whose silencing promotes tumor progression in the *Apc^{min/+}* mouse model by recruiting immature myeloid cells, through increased expression of the chemokine CCL9, which support the evolution of neoplasia [19,20]. The role of *TP53* as a tumor suppressor is well-established, regulating critical processes in malignancy including replicative and metabolic circuits as well as DNA damage repair and apoptosis [21-24].

There is an evolving paradigm underlying tumorigenesis which expands the genetic basis of cancer in which progression of the malignant phenotype reflects accumulation of sequential mutations directing the evolution of autonomous growth and survival through corruption and co-option of basic homeostatic mechanisms. In this expanded model of lineage-dependent tumorigenesis, developmental programs imprint tissue-specific mechanisms entraining normal cellular homologues of oncogenes and tumor suppressors in survival circuits which coordinate fundamental homeostatic pathways (Fig. 2) [13]. Corruption of lineage-restricted programs by oncogenic insults, in turn, disrupt the subordinate homeostatic survival circuits, including the cell cycle, metabolism, DNA damage repair, and apoptosis, that universally contribute to transformation in all tissues [14,18]. In that context, progression of programs directing the invasive malignant phenotype reflects aberrant silencing (suppressor) or persistence (oncogenic) of normal lineage-dependent circuits [14,25-28]. This concept of lineage-dependence offers the possibility of restricted critical alterations in a small number of hierarchical pathways directing the earliest stages of tumorigenesis that precede induction of oncogenes, silencing of tumor suppressor genes, or accumulation of mutations and genetic instability that characterize the terminal stages of transformation that are refractory to therapeutic interventions.

DYNAMICS OF INTESTINAL EPITHELIAL CELLS along the vertical crypt-surface axis are controlled by coordinated homeostatic programs comprising a developmental continuum integrating proliferation, differentiation, metabolism and apoptosis. Crypts harbor stem cells at the base which continuously regenerate progenitor cells destined to differentiate along secretory (goblet, Paneth, and enteroendocrine cells) or absorptive (enterocytes) lineages (Fig. 3). This transition from proliferation to lineage commitment is associated with metabolic reprogramming from glycolysis to oxidative phosphorylation as they migrate toward the surface. Following this transition, goblet, enteroendocrine cells and enterocytes continue to migrate to the surface and ultimately undergo apoptosis, and are shed into the fecal stream [29-31]. In contRASt, Paneth cells migrate down to the base of the crypt where they reside.

The distinctive regenerative characteristic of the intestinal epithelium establishes a vertical axis representing a life cycle continuum, from cell birth to death, providing a unique model to explore the intersection between developmental programs and lineage-dependent tumorigenesis. Signaling mediators regulating homeostatic processes directing the spatiotemporal coordination of this continuum include WNT signaling and APC. [26,29-31]. In this pathway, APC plays a central role in the transduction of extrinsic WNT signals to the intestinal epithelial cells in coordinating the transition from proliferation in progenitor cells to differentiated enterocytes. Canonical signaling is mediated by the secreted WNT proteins which bind to the Frizzled receptor and the LRP5/LRP6 co-receptors in cell membranes[32, 33], promoting phosphorylation of LRP5/LRP6 by casein kinase 1 (CK1), GSK3, and other kinases[34,35]. Phosphorylation of LRP drives recruitment of dishevelled molecules and axin, leading to inactivation of β -catenin degradation[34,36-41]. Accumulated β -catenin translocates to the nucleus and activates TCF/LEF transcriptional activity resulting in proliferation and maintenance of stemness[42-44], in part through expression of cyclin D1 and Myc[45-47]. Signaling in this pathway is inhibited by antagonizing WNT-Frizzled (FZD) interactions or internalization of LRPs, releasing axin which permits the formation of destruction complexes with adenomatous polyposis coli (APC)-directed N-terminal phosphorylation of β -catenin by CK1 and GSK3[48-51]. Once phosphorylated, β -catenin is targeted for proteosomal degradation, preventing nuclear translocation. Failure to regulate WNT signaling by APC promotes tumorigenesis, in part, by permitting nuclear accumulation of β -catenin, driving gene expression supporting proliferation and antagonizing apoptosis, both of which disrupt survival homeostasis [11,26]. Indeed, APC is one of the most frequently mutated targets in sporadic and inherited colorectal carcinogenesis [52-56]. Disruption of WNT signaling in intestine through an intestinal-specific mechanism exemplifies the concept of lineage-dependent tumorigenesis, encompassing a key developmental mechanism essential for normal tissue-specific spatiotemporal patterning whose corruption, here reflecting inappropriate activation, engages subordinate downstream homeostatic mechanisms underlying the evolution of the tumorigenic phenotype (Fig. 2).

GUANYLYL CYCLASE C (GCC) is an intestine-specific receptor that catalyzes the conversion of GTP to cGMP upon activation by the endogenous paracrine hormones guanylin and uroguanylin or the exogenous diarrheagenic bacterial heat-stable enterotoxins (STs) [57, 58]. The latter are a principle cause of secretory diarrheal disease worldwide, and ST binding to GCC induces a signaling cascade regulating fluid and electrolyte efflux across the intestinal epithelium [58]. Beyond secretion, binding of the endogenous paracrine hormones regulates intestinal epithelial homeostasis and tumor susceptibility [59-61]. Guanylin and uroguanylin exhibit a pattern of expression along the crypt-villus axis that is associated with the transition from proliferation to differentiation, absent in the crypt but present in the differentiated compartment (Fig. 3) [57,58]. Additionally, guanylin and uroguanylin are the most commonly lost gene products in colorectal cancer, and their loss occurs early along the continuum of transformation [61-64]. Further, mice deficient in GCC signaling demonstrate a defect in differentiation, with preferential commitment along the enterocytic, compared to the secretory, lineage associated with disruption of regenerative and metabolic circuits revealing an accelerated cell cycle, disruption of genomic stability and a metabolic phenotype recapitulating tumorigenesis[59,60,65-70]. Further, elimination of GCC signaling increases the susceptibility of mice to intestinal tumorigenesis induced by carcinogens or inherited germline mutations [59]. Moreover, supplementation with uroguanylin decreases tumorigenesis in mouse models of intestinal carcinogenesis [71]. These observations suggest that GCC is a lineage-specific tumor susceptibility gene product normally involved in the spatiotemporal patterning of the intestinal vertical axis whose silencing, reflecting loss of expression of paracrine hormones, corrupts downstream process universally underlying neoplastic transformation[59,60,65, 67-70]Accordingly, colorectal tumorigenesis regulated by GCC signaling suggests a novel

pathophysiological paradigm in which colorectal cancer initiates, in part, as a disease of paracrine hormone insufficiency.

Hypo-differentiation and the selective impairment in maturation of the secretory lineage reflects a previously unrecognized role for GCC in regulating intestinal cell differentiation by discrete molecular mechanisms, including interaction with transcription factors specifying secretory lineage commitment including Hes-1 and Math [59,72]. Moreover, hyperproliferation and acceleration of the epithelial cell cycle in GCC deficient mice is associated with an increase in mediators promoting the G1/S transition (cyclin D1, pRb) and a decrease in cell cycle suppressors (p27), accompanied by hyperplasia of the crypt compartment, reflecting an increase in the number of progenitor cells (unpublished data).

Biological processes are absolutely dependent on the availability of ATP produced by mitochondrial respiration and glycolysis [73,74]. In mammals, cells adapt to changes in energy supplies, substrate availability, and metabolic demands by balancing the relative contributions of these ATP-generating systems [75]. Rapidly dividing cells exhibit a canonical anabolic profile, supporting increased proliferative energy demands by exploiting accelerated ATP generation characterizing glycolysis [76-78]. Conversely, differentiated cells characteristically exhibit a catabolic profile, leveraging the increased metabolic efficiency of oxidative phosphorylation to support energy requirements [79-82]. In that context, there is an established metabolic gradient along the intestinal crypt-surface axis, in which ATP is principally generated by glycolysis in the proliferating crypt compartment but by oxidative phosphorylation in the differentiated compartment (Fig. 3) [83,84]. The glycolytic phenotype of the crypt is identical to that universally characterizing metabolic reprogramming associated with tumorigenesis, presumably important in conferring a survival advantage to cancer cells undergoing rapid proliferation in a hypoxic environment [78,85-87]. Of significance here, elimination of GCC signaling in intestine disrupts the metabolic gradient along the crypt-surface axis, imposing a tumorigenic glycolytic phenotype along the entire vertical axis (unpublished data). This glycolytic phenotype reflects a decrease in mitochondrial genome, proteins, and organelles associated with reduced mitochondrial oxygen consumption, dehydrogenase activity and ATP production [88]. Conversely, it is accompanied by elevated glucose transport and lactate production associated with increased expression of rate-limiting glycolytic proteins up-regulated in tumors, including the glucose transporter I (GLUT1), hexokinase II (HKII), and pyruvate kinase (PK), recapitulating the tumor metabolic phenotype [85,87,89-95].

Elimination of GCC expression induces genomic instability in intestinal epithelial cells, increasing DNA double strand breaks, loss of heterozygosity, and point mutations in genes central to tumorigenesis, including APC and β -catenin [96]. Loss of GCC ligands early in tumorigenesis in the context of the established role of accumulated genetic alterations reinforcing genomic instability in carcinogenesis underscores the mechanistic contribution of dysregulated GCC signaling in colorectal cancer. Mechanisms by which GCC contributes to genomic integrity, including damage production, damage detection and assessment, mutation repair, and the associated coordination of replicative decision-making are currently being explored. However, proliferative restriction and genomic quality control reflect reinforcing mechanisms by which GCC opposes tumorigenesis [60]. Indeed, accelerated progression through G1 and premature entry into S are necessary for heritability and amplification of genetic instability [97,98]. Beyond corruption of DNA damage sensing and repair mechanisms and acceleration of the cell cycle, metabolic reprogramming directing ATP generation through aerobic glycolysis and the associated reduction in mitochondria increases the generation of reactive oxygen species which directly damage DNA [79,99,100].

Studies of murine and human colon cells provide further evidence for the role of GCC in regulating the cell cycle, proliferation, and metabolic programming in normal intestinal epithelium and tumors (unpublished data). GCC signaling inhibits proliferation of colonocytes and colon carcinoma cells *in vitro* and *ex vivo* [59,60,65,67-70]. GCC ligands induce a G1-S delay that restricts progression through the cell cycle without apoptosis or necrosis. Cytostasis induced by ligands was mediated by GCC, associated with accumulation of cGMP, mimicked by the cell-permeant analog 8-Br-cGMP, and reproduced and potentiated by the cGMP-specific phosphodiesterase inhibitor zaprinast, but not an inactive GCC receptor ligand analog [59, 60,67-70]. Cytostasis induced by GCC signaling was associated with altered expression of cell cycle mediators including cyclin D1, pRb, and p27 regulating the transition through G1-S [59,60,65,67-70]. Further, GCC reversed the tumorigenic metabolic phenotype in colon cancer cells. Ligand activation of GCC, or cGMP, inhibited glycolysis by reducing rate-limiting enzymes including GLUT1, HKII, and PK, associated with a decrease in glucose uptake and lactate production, limiting bioenergetic support for rapid proliferation. Moreover, activation of GCC promoted mitochondrial biogenesis, increasing mitochondrial expression and function, and reconstituting oxidative metabolism in normal colonocytes and cancer cells [59,60,65, 66,70].

SIGNALING PATHWAYS with a principle role in intestinal tumorigenesis include WNT/ β -catenin, receptor tyrosine kinases (RTK)-RAS, bone morphogenetic protein (BMP) and p53 [11,20,29,101-103]. These systems encompass primary molecular mechanisms mediating developmental programs beyond cell and tissue boundaries, coordinating proliferation, survival and differentiation [11,12,15,104]. Interestingly, recent experimental evidences suggest that these tumorigenic pathways converge into a signaling network through AKT (Fig. 4). As discussed above, canonical activation of WNT signaling stabilizes β -catenin by inactivating the destruction complex, leading to its cytoplasmic accumulation, nuclear translocation and activation of TCF transcriptional activity resulting in expression of genes essential for tumorigenesis. Similarly, activation of AKT can mimic WNT activation and promotes β -catenin nuclear translocation by inactivating the destruction complex by phosphorylating GSK3 β and by direct phosphorylation of β -catenin [105-107]. Further, Activation of AKT, in part, reflects the balance between phosphoinositide 3-kinase (PI3K) and the tumor suppressor phosphatase and tensin homologue (PTEN), which together dynamically regulate phosphatidylinositol bisphosphate (PIP₂) available for directing membrane translocation and PDK-1 activation mediating AKT phosphorylation. In that context, PI3K is activated by RTK-RAS, producing downstream AKT signaling [18,108]. Notably, prolonged activation of AKT signaling, in turn, can stabilize and constitutively activate wild-type RAS through eNOS, establishing a mutually reinforcing activation circuit involving RAS-PI3K-AKT potentiating tumorigenesis [109]. Moreover, BMP activation of receptor BMPRI1A in epithelial cells prevents inhibition of PTEN through phosphorylation and facilitates PTEN function. Thereby, disruption of BMP signaling by mutations silencing receptor function inactivates PTEN which, in turn, activates AKT mediating an accumulation of β -catenin [110]. Further, activated AKT phosphorylates and stabilizes MDM2, mediating its nuclear translocation, increasing MDM2-dependent p53 degradation [111,112]. Together, these observations highlight the convergence through AKT of the principle signaling pathways integrally involved in colorectal tumorigenesis [113,114].

In turn, AKT regulates downstream events that are essential for neoplastic transformation [18,97,107,108,111,113,114]. AKT controls proliferation and cell cycle progression through multiple downstream substrates, including GSK3 and mTOR (Fig. 4). GSK3 is a ubiquitously expressed serine/threonine protein kinase, whose activity can be inhibited by AKT-mediated phosphorylation at Ser21 of GSK3 α and Ser9 of GSK3 β [115]. The resulting inhibition of GSK3 phosphorylation of regulators driving the G1/S transition (β -catenin, c-myc, cyclin D1) mediates the AKT-GSK3 acceleration of the cell cycle [107,116-119]. AKT-GSK3 further

regulates cell fate and lineage commitment through phosphorylation of c-Jun, β -catenin, GLI, Notch, Snail, sterol-regulatory-element-binding transcription factor1, and c-Myc [18,116]. Additionally, AKT phosphorylates and activates the mTOR protein kinase by circumventing the inhibitory GTPase activity of the TSC2 tumor suppressor, tuberin, for the small G protein, Rheb, which binds directly to the kinase domain and activates mTOR in a GTP-dependent manner [120-122]. AKT-mTOR regulates cell growth and proliferation through initiation of translation and ribosome biogenesis [108,123,124]. Thus, AKT signaling activates eIF4E through inhibition of 4E-BP1 by mTOR, which promotes the cap-dependent translation of many target mRNAs essential for proliferation, including cyclin D1 and c-Myc [108,125].

Also, AKT contributes to the regulation of genomic instability. In the setting of radiation-induced damage, AKT regulates cell cycle progression, in part, by direct phosphorylation of the Chk1 protein kinase, a key component of the DNA damage sensing and repair mechanism, or through phosphorylation of MDM2 modulating p53 degradation [111,126]. Chk1 phosphorylation blocks cell cycle checkpoint function through nuclear exclusion, which sequesters it from key downstream targets, including the DNA damage-sensing kinases ATM and ATR [108,127,128].

Moreover, AKT coordinates metabolic programming with proliferation through multiple downstream substrates. AKT directly phosphorylates and activates phosphofructokinase 2 (PFK2) and ATP citrate lyase (ACL), stimulating glycolysis and fatty acid synthesis, respectively, which both support anabolic metabolism required for proliferation [129-131]. AKT phosphorylation of mTOR activates the hypoxia-inducible factor-1 α (HIF-1 α) transcription factor, which, in turn, increases expression of GLUT1 and other rate-limiting enzymes mediating glycolysis [108,124,132-134]. Further, AKT inhibits mitochondrial biogenesis and fatty acid oxidation through direct phosphorylation and inhibition of peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α). Additionally, AKT phosphorylates FOXO1, a transcriptional regulator supporting PGC-1 α function, resulting in its nuclear exclusion and targeting for ubiquitin-mediated proteosomal degradation (Fig. 4) [96,135]. Moreover, inhibition of FOXO1 reduces expression of superoxide dismutase (SOD2), a key antioxidant [135]. Decreased SOD2 impairs mitochondrial function by producing mutations in the mitochondrial genome, leading to a further increase in oxidative damage to organelle and somatic genomes [136-138]. Collectively, this discussion highlights the importance of AKT in coupling key signaling pathways central to colorectal tumorigenesis (WNT/ β -catenin, RTK, RAS, BMP, and p53) with homeostatic cellular mechanisms universally corrupted in all tumors (proliferation, metabolism, genomic instability) [108,114,126]. It suggests that targeting AKT could prevent or treat colorectal cancer through bypassing upstream oncogenic gene alterations, including mutations or gene amplification of RTK, KRAS, and PI3K, which induce neoplasia through disruption of homeostatic processes. In that context, AKT signaling also is critical for regulating normal cellular homeostatic processes, and off-target adverse effects resulting from their disturbance in non-neoplastic tissues hampers the utilization of AKT inhibitor in cancer prevention and therapy.

GCC IMPOSES LINEAGE-DEPENDENT COORDINATION of normal spatiotemporal patterning of the crypt-surface axis which opposes colorectal tumorigenesis through AKT modulation. Microarray expression profiling revealed that AKT pathways are the most significant differentially activated in intestine from mice deficient in GCC signaling, accompanied by an increase in downstream targets, including β -catenin and c-Myc (unpublished data). Elimination of GCC signaling in mice decreased expression of PTEN, associated with an increase in expression and activation of AKT1. In turn, elevated AKT activity increased phosphorylation and degradation of the downstream target TSC2, activating mTOR signaling, a key integrator coordinating proliferative and metabolic signals required for cell growth and survival (Fig. 4). Further, mTOR increased phosphorylation of S6K, activating

the translational machinery required for proliferation, while phosphorylation of FOXO1, a key transcription factor promoting expression of PGC1 α and mitochondrial biogenesis, resulted in nuclear exclusion and degradation, coordinately rebalancing metabolism favoring glycolysis (unpublished data). Conversely, transient restoration of GCC signaling by oral administration of the proximal downstream effector cGMP reduced AKT phosphorylation and downstream signaling through mTOR (unpublished data). Moreover, activation of GCC signaling in both human and murine colon cancer cells decreased AKT activation and downstream signaling through mTOR (unpublished data). In that context, GCC signaling increases PTEN expression inhibiting AKT activity (unpublished data). Also, pharmacologic and genetic inhibition of AKT signaling mimics, while constitutive activation blocks, GCC effects in regulating replicative and metabolic circuits (unpublished data). In close agreement with these observations, activation of GCC reverses the tumorigenic phenotype by limiting growth of colorectal cancer cells by restricting progression through the G1/S transition and reprogramming metabolic circuits from glycolysis to oxidative phosphorylation, limiting bioenergetic support for rapid proliferation by modulating AKT signaling. Therefore, regulation of AKT signaling in a lineage-specific fashion by GCC might represent a unique therapeutic opportunity for the prevention and treatment of colorectal cancer.

CONCLUSION

These observations suggest a novel pathophysiological paradigm in which GCC is a lineage-dependent tumor suppressing receptor which coordinates proliferative, metabolic and genomic homeostasis along the crypt-surface axis, in part, by restricting AKT signaling whose dysregulation reflecting hormone loss contributes to tumorigenesis (Figure 4). In the context of the loss of guanylin and uroguanylin at the earliest stages of colorectal carcinogenesis, this paradigm offers the provocative suggestion that colorectal cancer initiates as a disease of hormone insufficiency. Of significance, while paracrine hormones are lost in tumorigenesis, there is compensatory over-expression of the receptor GCC in almost all primary and metastatic tumors examined [139,140]. Persistence of receptors in the face of hormone loss suggests that colorectal cancer is a disease of hormone insufficiency that can be prevented or treated by oral hormone replacement therapy [60,65,66,70].

FUTURE PERSPECTIVE

Appreciation for pathophysiological mechanisms underlying the earliest stages of tumorigenesis will promote the evolution of current paradigms to prevent, interrupt, or reverse disease progression by inhibiting precursor lesion formation or the adenoma-to-carcinoma transition. The paradigm in which GCC is a lineage-specific tumor susceptibility gene product whose dysregulation reflecting hormone loss contributes to the initiation of colorectal tumorigenesis provides opportunities for novel cancer prevention and treatment. In that context, it is anticipated that cells undergoing early transformation reflecting paracrine hormone insufficiency exhibit a compensatory over-expression of GCC receptors in the absence of the profusion of genetic mutations corrupting subordinate survival pathways that produce therapeutic resistance. This distinctive window of opportunity in pathophysiology could be exploited, in conjunction with hormone replacement therapy, to prevent and treat primary colorectal cancer. Indeed, in this model, the earliest stages of colorectal cancer might be treated like classical endocrine insufficiency syndromes, for example like diabetes is treated with insulin. In that context, future therapeutic efforts in patients at risk for colorectal cancer could involve a component of GCC ligand replacement therapy [65,70,71]. Ligand replacement may take the form of oral GCC hormone supplementation with guanylin or uroguanylin [65, 70,71]. Alternatively, the preferred ligand may be the bacterial heat-stable enterotoxins, which are specific GCC ligands with 10- to 100-fold greater affinity for GCC than their endogenous hormone analogues [57,58]. Moreover, these approaches could include novel peptide delivery systems, for example chronic colonization of the colorectum with enteric bacteria secreting

ST. This approach exploits the ability of these bacteria to synthesize and process bioactive ST and their natural facility to specifically occupy the microbial niche in the colon and rectum and, consequently, deliver peptide directly to the target organ (colorectum) without exposing off-target tissues (small intestine), reducing the potential for adverse effects (hypersecretion). Beyond these specific considerations, elucidating the GCC signalome coordinating multiple pathways opposing transformation (Fig. 5) will expand the current paradigm of disease initiation and the armamentarium of therapeutic strategies to prevent and treat colorectal cancer.

Executive Summary

COLORECTAL CANCER

- Colorectal cancer is the 4th leading cause of cancer and the 2nd leading cause of cancer-related mortality in the world.

COLORECTAL TUMORIGENESIS AND THE DYNAMICS OF INTESTINAL EPITHELIAL CELLS

- While terminal transformation and the evolution of invasive and metastatic colorectal cancer reflects accumulation of sequential mutations in pathways regulating key homeostatic pathways, there is a paucity of mechanistic insights into proximal pathophysiological processes that initiate and amplify oncogenic circuits preceding global mutations.

GUANYLYL CYCLASE C

- Guanylyl cyclase C (GCC), the intestinal receptor for the paracrine hormones guanylin and uroguanylin, has emerged as a component of lineage-specific homeostatic programs organizing spatiotemporal patterning of proliferation, DNA damage and repair mechanisms, and the balance of glycolysis and oxidative phosphorylation along the crypt-surface axis.
- Guanylin and uroguanylin are the most frequently lost gene products in colorectal carcinogenesis and their loss occurs early along the continuum of transformation, preceding the development of the invasive metastatic phenotype.

SIGNALING PATHWAYS AND LINEAGE-DEPENDENT COORDINATION BY GCC

- Loss of GCC signaling reprograms the cell cycle, mechanisms maintaining genomic integrity, and metabolic circuits that recapitulate the tumorigenic phenotype. Moreover, loss of GCC signaling promotes tumorigenesis in mouse models of colorectal cancer.
- Conversely, re-engaging GCC by ligand replacement rescues control of the cell cycle, genomic integrity and metabolism and opposes tumorigenesis.

CONCLUSIONS

- These observations suggest a novel pathophysiological paradigm in which dysregulation of GCC signaling, reflecting early hormone loss, promotes tumorigenesis through reprogramming of replicative and bioenergetic circuits and genomic instability. This model suggests that initiation of colorectal cancer is, at least in part, a disease of hormone insufficiency.

FUTURE PERSPECTIVE

- In the context of colorectal cancer as a disease of hormone loss, and the universal compensatory over-expression of GCC by tumor cells, there is a unique translational

opportunity for prevention and treatment of colorectal tumors by hormone replacement therapy, analogous to the treatment of other diseases of endocrine insufficiency, for example the treatment of diabetes with insulin.

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Abbreviations

AKT, v-akt murine thymoma viral oncogene homolog
 APC, adenomatous polyposis coli
 BMP, bone morphogenetic protein
 CHK1, the checkpoint kinase 1
 cGMP, cyclic GMP
 CNG, cyclic nucleotide-gated channel
 DVL, dishevelled
 FOXO, the forkhead box O transcription factor
 FZD, frizzled
 GCC, guanylyl cyclase C
 GLUT1, glucose transporter I
 GSK3, glycogen synthase kinase 3
 HIF, hypoxia inducible factor
 HKII, hexokinase II
 hMSH2, hMLH1, human mismatch repair genes
 MDM2, mouse double-minute 2
 mTOR, mammalian target of rapamycin
 PDE, phosphodiesterase
 PGC1 α , peroxisome proliferator-activated receptor gamma, coactivator 1 α
 PK, pyruvate kinase
 PKG, cyclic GMP-dependent protein kinase
 PKA, cyclic AMP-dependent protein kinase
 pRb, phosphorylated retinoblastoma
 Rheb, RAS homolog enriched in brain
 ROS, reactive oxygen species
 RTK, receptor tyrosine kinase
 ST, diarrheagenic bacterial heat-stable enterotoxin
 TGF- β , transforming growth factor- β
 TSC, tuberous sclerosis complex

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Figure 1. The adenoma-carcinoma hypothesis

suggests that sequential accumulation of genetic alterations underlies the development of colorectal tumorigenesis. Mutations in the *APC* gene (5q21) are the earliest known event. Inactivation of *APC* stabilizes β -catenin and accelerates cell cycle progression. *hMSH2* and *hMLH1* are human mismatch repair genes (MMR) that encode key molecules that repair DNA replication errors. Deletions or mutations in MMR genes exists in 95% of tumors in patients with hereditary non-polyposis colorectal cancer (HNPCC), but only 10~15% of sporadic tumors. *KRAS* mutations are also found in nondysplastic lesions and hyperplastic polyps. *KRAS* activates both MAPK and PI3K-AKT signaling pathways. *SMAD4*, also known as deleted in pancreatic cancer (*DPC4*), and *SMAD2* are downstream mediators of TGF- β signaling. Inactivation of TGF- β signaling, identified in juvenile polyposis and late stage colorectal tumors, drives malignant progression of colorectal tumors. *TP53* mutations occur in ~75% of colorectal carcinomas, but infrequently in benign lesions. Adopted from [12]. Dashed arrow, loss of function, solid arrow, gain of function.

Figure 2. Lineage-specific tumorigenesis

There is an emerging paradigm that extends the oncogenomic view of tumorigenesis suggesting that there are developmental and tissue-specific programs that indelibly restrict signaling circuits which define homeostatic repertoires in specific tissues. These executive circuits entrain and drive subordinate signaling mechanisms that control key cellular processes universally contributing to carcinogenesis across different tissues. These subordinate processes include cancer cell autonomous mechanisms like genetic stability, the balance between glycolysis and oxidative phosphorylation, replication and the cell cycle, and apoptosis. Also, they include non-cancer cell autonomous mechanisms like mesenchymal remodeling of the stromal niche, angiogenesis, and inflammation. In this model of lineage-specific tumorigenesis, corruption (⚡) of tissue-specific executive circuits, for example by environmental insults, disrupts subordinate homeostatic programs, initiating the transformational continuum ultimately producing the invasive metastatic phenotype. Adopted from [141]

Figure 3. Organization of the vertical crypt-surface axis in intestinal epithelium

The intestinal epithelium undergoes continuous homeostatic cycles of proliferation, migration, differentiation, apoptosis and shedding. Rapidly proliferating progenitor cells originate from stem cells residing near the bottom of crypts. When migrating along the vertical axis, these cells reprogram nuclear and cytoplasmic circuits, coordinating proliferative restriction, lineage commitment, and metabolic programming. Progenitor cells differentiate into four cell types, including enterocytes, goblet cells, enteroendocrine cells and Paneth cells. Enterocytes, goblet cells and enteroendocrine cells reside in the villus (small intestine) or upper crypt (colon and rectum) compartments while Paneth cells reside at the bottom of crypts. Guanylin and uroguanylin are secreted in a gradient along the crypt-surface axis, lowest in the crypt and

greatest near the surface. Proliferative cells are indicated by red nucleus, and differentiated cells are by blue nucleus. The pericryptal myofibroblasts are the yellow cells. Adopted from [70]

Figure 4. AKT signaling and tumorigenesis

Activation of receptor tyrosine kinases (RTKs) recruits PI3K directly or indirectly via RAS to the plasma membrane, where it phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP₂) to generate the phosphatidylinositol-3,4,5-trisphosphate (PIP₃). PIP₃ recruits AKT and PDK1 to the plasma membrane, and activates AKT. AKT phosphorylates a variety of downstream substrates and regulates the cell cycle, cell growth and metabolism, and cell death. PTEN antagonizes the PI3K-AKT pathway by dephosphorylating PIP₃. BMP activation of receptor BMPR1A in epithelial cells prevents inhibition of PTEN through phosphorylation and facilitates PTEN function. AKT activates mTORC1 through phosphorylation of TSC2, and inactivates the GTPase activity of TSC2 for Rheb. Rheb-GTP activates mTORC1 and downstream S6 kinases. Phosphorylation of FOXO by AKT inhibits its regulation of cell metabolism. Phosphorylation of Chk1 results its nuclear exclusion and sequestration from key downstream targets, including the DNA damage-sensing kinases ATM and ATR. Phosphorylation of MDM2 by AKT activates p53 degradation. In the presence of WNT, Frizzled (FZD) interacts with Dishevelled (DVL) and inactivates the APC-destruction complex leading to the stabilization of β-catenin and its nuclear translocation. Phosphorylation of β-catenin or GSK3 mimics the effect of WNT signaling activation that stabilizes and mediates β-catenin nuclear translocation, which accelerates the cell cycle and potentiates tumorigenesis, in part through CyclinD1 and Myc transcription. GCC signaling suppresses AKT activation

through a multiple mechanisms, in part by increasing PTEN expression and function. Proteins activated during tumorigenesis are shown in red, and those inactivated are in blue. Proteins activated by phosphorylation are indicated by a red phosphate, while those inactivated by phosphorylation are indicated by a blue phosphate. Cellular processes regulated by AKT downstream substrates are illustrated by underline colores.

Figure 5. The GCC signalome

Reproduced with permission from [70]. Activation of GCC by its ligands coordinates a complex system of intracellular signaling composed of dynamically regulated proteins that control discreet biological processes maintaining intestinal epithelial cell and crypt-villus homeostasis, the corruption of which contributes to the initiation and promotion of cancer. GCC, through cGMP, activates cGMP (PKG)- and cAMP (PKA)-dependent protein kinases; phosphodiesterases (PDE), which control cGMP concentration, spatially and temporally shaping GCC signaling; and cyclic nucleotide-gated channels (CNGs), which regulate calcium signaling through calcium-sensing receptors (CaRs). PKG is the canonical intracellular effector for guanylyl cyclases, and in intestinal cells induces phosphorylation of (1) the cystic fibrosis transmembrane conductance regulator (CFTR), mediating fluid secretion; (2) vasodilator-stimulated phosphoprotein (VASP), constraining actin polymerization dynamics, migration, and adhesion; and (3) β -catenin, resulting in its proteosomal degradation, restricting proliferation and promoting terminal differentiation. In turn, CNGs and CaRs form the extracellular calcium-dependent limb of the GCC signalome, cooperatively promoting differentiation and restricting proliferation of intestinal epithelial cells. GCC also inhibits the secretion of matrix metalloproteinase-9 (MMP-9) and transforming growth factor- β (TGF- β), key regulators of epithelial and mesenchymal remodeling and enterocyte survival and maturation. Specifically, TGF- β promotes tumor cell survival and proliferation through an oncogenic switch, recruiting stress-activated protein kinase p38 and phosphorylated c-Jun-N-terminal kinase (pJNK). Further, GCC and cGMP control genomic integrity and cellular metabolism, in part, by activating peroxisome proliferator-activated receptor γ , coactivator 1 α (PGC1 α), either directly or by inhibiting the Akt pathway. Akt regulates cell survival, cell cycle progression, glycolysis, the number and function of mitochondria, and the DNA damage response pathway by targeting intracellular effector proteins, including the forkhead (FOXO) family of transcription factors, glycogen synthase kinase 3 (GSK3), tuberous sclerosis complex 1 (TSC1) and tuberous sclerosis complex 2 (TSC2), and the mammalian target of rapamycin (mTOR). Akt-dependent functional complexes regulating proliferation and cell survival include (1) Myc, cyclin D1, and phosphorylated retinoblastoma protein (pRB), which promote G1-S cell cycle transition; (2) p27kip1 and retinoblastoma-like 2 (RBL2), which induce cell cycle arrest; (3) Fas-ligand (FasL) and BCL2-like 11 (Bim), mediators of cell death; (4) BCL2-antagonist of cell death (BAD), which inhibits the B-cell lymphoma-like protein 1 (BCL-XL) and induces apoptosis; and (5) the mouse double-minute 2 (MDM2), which prevents cell arrest and apoptosis by promoting proteasome-dependent p53 degradation. The hypoxia-inducible factor (HIF) promotes glycolysis and inhibits respiration; reactive oxygen species (ROS) induce genomic instability and HIF stability and function; phosphoenolpyruvate carboxykinase

(PEPCK) and glucose-6-phosphatase (G6Pase) are key enzymes mediating gluconeogenesis; glycogen synthase promotes HIF degradation and glycogen synthesis; and Rheb (Ras homolog enriched in brain) is a small G protein inhibited by the TSC1/TSC2 complex that promotes mTOR-mediated functions, including proliferation and metabolism. Lines and arrows, indicating functional relations between molecules, are a schematic representation of key interactions mediating biological processes in intestinal epithelial cells, and they do not exhaustively portray the complexity of signaling interconnections mediated by each molecule of the GCC signalome.