The Pathobiology of Krüppel-like Factors in Colorectal Cancer

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The Krüppel-like factor (KLF) proteins are zinc finger–containing transcription factors that exert important functions in regulating diverse biologic processes such as growth, proliferation, differentiation, development, inflammation, and apoptosis. Many KLFs have also been shown to play significant roles in tumorigenesis of various organs and tissues. Three in particular—KLF4, KLF5, and KLF6—are often dysregulated in tumors of the gastrointestinal tract, including colorectal cancer. This article reviews the functions of these three KLFs in normal gastrointestinal biology and their pathobiologic roles in colorectal cancer.

Introduction

Colorectal cancer (CRC) is the fourth most common cancer in men and women in the United States and is the second leading cause of cancer-related deaths, after lung cancer [1]. It was estimated that CRC would be diagnosed in approximately 153,000 Americans in 2007, and over 52,000 would die from it. These figures render CRC a significant health concern. Hereditary risks contribute to approximately 25% of all cases of CRC, with the others considered to be sporadic cases [2•]. Both hereditary and sporadic CRC develop through a series of distinct histopathologic stages from normal colonic epithelial cells to carcinoma through the intermediate stage of adenoma [3]. Although it has been proposed that the development of CRC is the result of multistep and sequential accumulations of genetic mutations in a number of key genes with important functions in the control of cell growth [4], recent studies have indicated that the genomic landscape of CRC is far more complex and heterogeneous than previously thought [5••]. It has also become clear that the pathways governed by the various mutated cancer-related genes are important in the molecular pathogenesis of CRC [6]. Future investigation is likely to require a focus on the biochemical functions of cancer-related genes and their locations in the various pathways that, when perturbed, eventually lead to CRC.

Krüppel-like Factors

Krüppel is a member of the "gap" class of segmentation genes of *Drosophila melanogaster*, the mutation of which causes contiguous groups of segments of the fruit fly embryo to fail to develop [7]. Krüppel encodes a protein that contains a DNA-binding motif composed of several "zinc fingers" of the C_2H_2 type—a structure that uses two cysteine and two histidine amino acid residues to tetrahedrally coordinate a single zinc atom in a fingerlike configuration [7]. In mammals, a group of proteins were found to exhibit close homology to the zinc finger portion of Krüppel. These proteins (currently 16 in number) are called Krüppel-like factors (abbreviated KLFs) [8–11]. It is now well established that KLFs are involved in many important cellular processes such as growth, development, differentiation, proliferation, and apoptosis.

Several members of the KLF family—including KLF4 [12•,13•], KLF5 [12•,13•], KLF6 [14], KLF8 [15], and KLF11 [16]—have been implicated in the pathobiology of various human cancers; they exert either a tumor-suppressing or tumor-promoting function. KLF4, KLF5, and KLF6 have been shown to be involved in tumorigenesis of the gastrointestinal tract, including CRC. This review will focus on the pathobiologic role in CRC played by these three KLFs.

Krüppel-like factor 4

KLF4 (also called gut-enriched Krüppel-like factor or GKLF) was initially identified during screening of an NIH3T3 cDNA library under reduced stringency of hybridization with a cDNA probe containing the zinc finger portion of the immediate early gene product, Zif268 or Egr1 [17]. KLF4 is a nuclear protein that contains two potent nuclear localization signals (NLSs), one within the three zinc fingers and the other in a cluster of basic amino acids immediately adjacent to the first zinc finger

[18]. These two NLSs define a subfamily of three closely related KLFs: KLF1, KLF2, and KLF4 [18]. KLF4 binds to DNA sequence elements that are GC-rich. A consensus DNA binding sequence was empirically determined and is present in the promoters of many genes, including the CACCC element and the basic transcription element (BTE) [19]. Subsequently, KLF4 was shown to inhibit the promoter of the cytochrome P-450IA1 (*CYP1A1*) gene in a BTE-dependent manner [20].

Expression of the *KLF4* gene is developmentally regulated, with a higher level of expression occurring toward the later stage of fetal development [21]. In adults, KLF4 is highly enriched in epithelial tissues, including the skin, lung, and intestine [17]. In the intestinal tract, KLF4 is predominantly present in the terminally differentiated, postmitotic epithelial cells lining the villus border of the small intestine and the upper crypt region of the large intestine [13•]. In cultured cells, the level of *KLF4* mRNA is associated with the growth-arrested state in a manner similar to that observed in the intestinal epithelium [17]. Forced expression of KLF4 in cultured cells results in the inhibition of DNA synthesis [17]. These results indicate that KLF4 inhibits cell proliferation and may be required in the maintenance of the quiescent state in vitro and in vivo.

The inhibitory effect of KLF4 on cell proliferation is further substantiated by its ability to inhibit the transition between the G1 and S phases of the cell cycle in an inducible expression cell culture system [22]. The induction of KLF4 is also correlated with an increase in the level of $p21^{WAF1/CIP1}$, a critical checkpoint protein that inhibits cell cycle progression [22]. Using a well-established DNA damage model induced by ionizing irradiation, which causes a p53-dependent cell cycle arrest, it was shown that *KLF4* is transcriptionally activated following induction of p53 and is essential in mediating the cell cycle arrest at both the G_1/S and G_2/M boundaries [23,24]. Importantly, KLF4 was shown to be essential in the induction of expression of the $p21^{WAF1/CIP1}$ gene in response to DNA damage and does so by binding to a specific *cis*-DNA element in the $p21^{WAF1/CIP1}$ proximal promoter to activate $p21^{WAF1/CIP1}$ expression [25]. Subsequent cDNA microarray analysis of the transcriptional profiles of KLF4 further demonstrates that KLF4 inhibits the cell cycle by coordinately regulating expression of numerous cell cycle regulatory genes [26].

The findings that KLF4 is an inhibitor of cell proliferation and that it mediates the cell cycle checkpoint function of the tumor suppressor p53 suggest that KLF4 may itself act as a tumor suppressor. Indeed, levels of *KLF4* mRNA are reduced in intestinal adenomas of $Apc^{Min/+}$ mice and colonic adenomas of patients with familial adenomatous polyposis (FAP) when compared with surrounding normal tissues [27]. Conversely, overexpression of KLF4 in the human colon cancer cell line RKO, which does not express endogenous KLF4, results in reduced tumorigenesis in vitro and in vivo [28]. In cultured human CRC cell lines, the tumor suppressor adenomatous polyposis coli (*APC*) gene has been shown to activate expression of the *KLF4* gene; this activation depends on an intestine-specific transcription factor, CDX2 [29]. Because *APC* is mutated in most sporadic CRCs [2•], these studies underscore the importance of KLF4 in mediating the tumor-suppressive function of APC. APC exerts its tumor-suppressive effect by modulating the subcellular distribution of β-catenin, a member of the Wnt signaling pathway of tumorigenesis [30••]. KLF4 can downregulate the level of β-catenin, suggesting a role for KLF4 in the Wnt/β-catenin pathway [31]. More recently, KLF4 was shown to bind directly to the transcriptional activation domain of β-catenin to inhibit β-catenin–mediated transcription [32], further indicating an important role for KLF4 in inhibiting Wnt-mediated signaling.

A recent study has further substantiated the in vivo function of KLF4 as a suppressor of intestinal tumorigenesis [33••]. Mice heterozygous for *Klf4* alleles ($Klf4^{+/-}$) were crossbred with $Apc^{Min/+}$ mice [34], which carry a germline mutation in the mouse *Apc* gene and develop adenomas in the intestine early in life, to determine the effect of haploinsufficiency of *Klf4* on tumor burdens. Between 10 and 20 weeks of age, $Klf4^{+/-}/Apc^{Min/+}$ mice developed significantly more intestinal adenomas than $Apc^{Min/+}$ mice. Immunohistochemical staining showed that Klf4 protein levels are lower in the normal-appearing intestinal tissues of $Klf4^{+/-}/Apc^{Min/+}$ mice than in wild-type, $Klf4^{+/-}$, or $Apc^{Min/+}$ mice. In contrast, the levels of β-catenin and cyclin D1 are higher in the normal-appearing intestinal tissues of $Klf4^{+/-}/Apc^{Min/+}$ mice than in the other three genotypes. Klf4 levels are further reduced in intestinal adenomas derived from both $Apc^{Min/+}$ and $Klf4^{+/-}/Apc^{Min/+}$ mice compared with their normal-appearing tissues. There is an inverse correlation between adenoma size and *Klf4* mRNA levels in both $Klf4^{+/-}/Apc^{Min/+}$ and $Apc^{Min/+}$ mice. Finally, there is a progressive loss of heterozygosity (LOH) of the wild-type *Apc* allele in adenomas of increasing size from $Klf4^{+/-}/Apc^{Min/+}$ and $Apc^{Min/+}$ mice. Results from this study demonstrate that KLF4 plays an important role in promoting the development of intestinal adenomas in the presence of the Apc^{Min} mutation.

The relevance of KLF4 in the pathogenesis of human CRC is demonstrated by a significant reduction of *KLF4* mRNA levels in colorectal adenoma and adenocarcinoma compared with matched normal colonic tissues [35,36]. There is also evidence for LOH in a subset of CRC and in a panel of CRC cell lines [36]. Moreover, the 5'-untranslated region of the *KLF4* gene is found to be hypermethylated in a subset of CRC. Lastly, several point mutations are identified in KLF4 that result in a diminished ability to activate the $p21^{WAF1/CIP1}$ promoter in some of the CRC cell lines [36]. These studies suggest that KLF4 is a tumor suppressor, at least in a fraction of patients with CRC. Recent studies demonstrating that KLF4 is involved in maintaining centrosome duplication and thus genomic stability further illustrate the mechanism by which KLF4 may be involved in tumor suppression [37].

Mice homozygous for a null mutation in the *Klf4* gene die shortly after birth, for unknown reasons [38]. Immediately following birth, $Klf4^{-/-}$ mice have a 90% reduction in the number of goblet cells in their colon, show abnormal expression of the goblet cell–specific marker Muc2, and have abnormal goblet cell morphology [38]. These studies indicate that one of the in vivo functions of KLF4 is to direct goblet cell differentiation in the intestine. Recent studies have also implicated the Notch pathway of signal transduction in both intestinal tumorigenesis and goblet cell differentiation. Specifically, inhibition of Notch by γ-secretase inhibitors turns proliferative intestinal crypt epithelial cells and intestinal adenomas into goblet cells in $Apc^{Min/+}$ mice [39•]. Preliminary studies from our group show that Notch inhibition in adenomas from $Apc^{Min/+}$ mice is accompanied by an increase in KLF4 expression and goblet cell formation, suggesting that KLF4 is subject to regulation by the Notch pathway (Ghaleb and Yang, unpublished data). Taken together, these studies place KLF4 in an interesting and important position between the Wnt and Notch signaling pathways, both of which are crucial for intestinal tumorigenesis [40••]. Additional studies are likely to further reveal the exact mechanism by which KLF4 mediates the crosstalk functions of these two key pathways in CRC.

Krüppel-like factor 5

KLF5 (also known as intestinal-enriched Krüppel-like factor or IKLF) was identified based on its homology with KLF2 [41] and later was found to be identical to the previously identified basic transcription element binding protein 2 (BTEB2) [42]. Expression of KLF5 is temporally regulated during embryonic development and is highest in the adult intestinal tract [41,43]. In the intestine, KLF5 is present primarily in the epithelial cells lining the bases of the crypts [13•,41,43], in contrast to the pattern of expression for KLF4, which is enriched in the epithelial cells toward the luminal surface [13•,17]. A similar contrast in the expression patterns for KLF4 and KLF5 has also been described in the epidermis of the skin [43].

Studies in knockout mice suggest that one physiologic function of KLF5 lies in the regulation of cardiovascular remodeling, in which KLF5 is shown to be markedly induced in activated smooth muscle cells and fibroblasts in injured blood vessels [44]. In response to external stress, transgenic mice heterozygous for the *Klf5* alleles ($Klf5^{+/-}$) show diminished levels of arterial-wall thickening, angiogenesis, cardiac hypertrophy, and interstitial fibrosis [44]. The fact that KLF5 is expressed in the proliferating crypt-cell component of the intestinal epithelium also begs the question whether KLF5 promotes cell proliferation. Indeed, forced expression of KLF5 in transfected mouse fibroblasts increases the rate of proliferation and leads to anchorage-independent growth and foci formation in soft agar [45]. Similarly, another study indicates that overexpression of KLF5 in transfected intestinal epithelial cells IEC6 increases the rate of proliferation [46]. This study also demonstrates that the growth-inhibitory effect of all-*trans* retinoic acid (ATRA) on IEC6 cells is mediated by the ability of ATRA to inhibit KLF5 expression. Conversely, lysophosphatidic acid has been shown to facilitate proliferation of colon cancer cells by inducing expression of KLF5 [47]. Moreover, *KLF5* was shown to be a target gene of the Wnt signaling pathway [48].

The relationship between KLF5 and tumorigenesis is still under debate. On one hand, KLF5 is thought to function as a tumor suppressor in breast and prostate cancers [49,50]. On the other hand, KLF5 has been shown to mediate the transforming effect of activated HRAS in transformed fibroblasts [51•,52]. Here, KLF5 is shown to be a transcriptional target for activated HRAS. The induction of KLF5 then increases proliferation by transcriptional upregulation of the expression of cyclin D1, cyclin B1, and Cdc2, all essential for timely cell cycle progression [51•,52]. The pro-proliferative activity of KLF5 is further increased by interaction with protein inhibitor of activated STAT1 (PIAS1), a small, ubiquitin-like modifier (SUMO) ligase that regulates transcription factors through SUMOylation or physical interaction, with resultant synergistic induction of the cyclin D1 and Cdc2 promoters [53].

Although a previous report shows that intestinal tumor progression is associated with downregulation of KLF5 [54], a recent study indicates otherwise, at least in intestinal tumors containing mutated (activated) KRAS [55••]. In this study, the effect of KLF5 on proliferation and transformation of intestinal epithelial cells was examined both in vitro and in vivo. Induction of mutated $KRAS^{V12}$ in IEC6 intestinal epithelial cells resulted in increased KLF5 levels accompanied by increased rates of proliferation and anchorage-independent growth. Inhibition of KLF5 expression by mitogen-activated protein kinase/extracellular signal–regulated kinase (MEK) inhibitors or KLF5-specific small interfering RNA reduced proliferation and anchorage-independent growth despite $KRAS^{V12G}$ induction. Human CRC cell lines with mutated KRAS contained high levels of KLF5; reduction of KLF5 by MEK inhibitors or KLF5 small interfering RNA also reduced proliferation and transformation. In vivo, both intestinal tumors derived from mice transgenic for villin-$KRAS^{V12G}$ and human primary CRCs with mutated KRAS contain high levels of KLF5 and increased staining of the proliferative marker Ki67. Thus, elevated KLF5 levels are strongly correlated with activating KRAS mutations in intestinal tumors in vitro and in vivo. Inhibition of KLF5 expression in these tumors reduces proliferation and transformation. Because activating KRAS mutations are found in more than 50% of cases of CRC [56], KLF5 is an important downstream mediator for activated KRAS during colorectal carcinogenesis and may serve as a novel therapeutic target for tumors with KRAS mutations.

Krüppel-like factor 6

KLF6, also called transcription factor ZF9 or core promoter–element binding protein (CPBP), was initially isolated by target site screening of a placental expression library [57]. As a transcription factor, KLF6 interacts with the core promoter element of TATA boxless genes [57]. The *KLF6* gene can be induced by a wide range of physiologic stimuli and exhibits characteristics resembling an immediate early gene [10]. As with many other members of the KLF family, KLF6 directly affects the activity of other proteins through protein-protein interactions. For example, KLF6 physically interacts with KLF4, and the two co-activate the human keratin 4 promoter, the expression of which is associated with a switch from proliferation to differentiation [58]. Similar to KLF4, KLF6 also upregulates expression of the $p21^{WAF1/CIP1}$ gene in a p53-dependent manner to suppress growth [59,60]. In addition, KLF6 interacts with cyclin D1 and disrupts complexes of cyclin D1 and cyclin-dependent kinase 4 (Cdk4), producing cell cycle arrest [60]. These studies therefore indicate that KLF6, like KLF4, is a checkpoint protein that inhibits cell cycle progression.

The first implication of KLF6 as a tumor suppressor came from studies in prostate cancer, in which the *KLF6* gene was found to be mutated in a subset [59]. One *KLF6* allele was deleted in 77% of the prostate cancers examined, and the remaining *KLF6* allele was mutated in most of the tumors from which the first allele was deleted. Functional studies show that although wild-type KLF6 upregulates $p21^{WAF1/CIP1}$ in a p53-independent manner and significantly reduces cell proliferation, tumor-derived KLF6 mutants do not. Therefore, the data support a role for KLF6 as a tumor suppressor involved in human prostate cancer [59]. KLF6 mutation subsequently has been identified in several other human cancers, including hepatocellular carcinoma [61] and gastric cancer [62].

Reeves et al. [63] were the first to make clear that KLF6 is frequently inactivated in both sporadic CRC and CRC associated with inflammatory bowel disease. They showed that the *KLF6* locus is deleted in 55% of tumors and mutations are identified in 44%. KLF6 mutations are present in tumors with either microsatellite or chromosomal instability and are more common in the presence of wild-type APC, particularly in cancers related to inflammatory bowel disease. Similar to the findings with prostate cancer, cancer-derived KLF6 mutants neither suppress growth nor induce $p21^{WAF1/CIP1}$ following transfection into cultured cells. The conclusion is that deregulation of KLF6 by a combination of allelic imbalance and mutation may pay a role in the development of CRC. Subsequent mutational studies of KLF6 in CRC support this conclusion [64,65]. These studies showed that loss of KLF6 expression is significantly associated with tumor size but not with Dukes' class, tumor location, or lymph node metastasis, suggesting that loss of KLF6 expression may be an early event in colorectal carcinogenesis [65].

KLF6 mutations, but not *KRAS* or *BRAF* mutations, have been implicated in the development of nonpolypoid CRC [66]. Another recent study compares the frequencies of LOH in the *KLF6* locus in patients with sporadic CRC and those with hereditary CRC including FAP and hereditary nonpolyposis colorectal cancer (HNPCC) [67•]. It was found that the frequencies of *KLF6* LOH in sporadic cases were 4% in adenomas, 0% in intramucosal carcinomas, 35% in invasive carcinomas, and 33% in liver metastases. Invasive carcinomas in patients with FAP showed only 6% LOH, and invasive carcinomas from patients with HNPCC exhibited no LOH. These data suggest that LOH of the *KLF6* locus contributes to the invasion step from an intramucosal carcinoma to an invasive carcinoma in the carcinogenesis of sporadic CRC but is rarely involved in the carcinogenesis of CRC in patients with FAP or HNPCC. Also, mutation analysis of the *KLF6* gene in 298 colorectal tumors examined in this study detected no somatic mutations. Another study with similar findings has also been reported [68]. The absence of somatic mutations raises some uncertainty about the *KLF6* gene as a classic tumor-suppressor gene in colorectal carcinogenesis.

Conclusions

KLF4, KLF5, and KLF6 are members of the Krüppel-like factor family and play important roles in the pathogenesis of CRC. The three factors converge on shared signaling pathways to either promote cellular proliferation (KLF5) or to induce cell cycle arrest (KLF4 and KLF6). They also often work to antagonize each other's function (KLF4 and KLF5) or they cooperate to enhance each other's activity (KLF4 and KLF6). Although both KLF4 and KLF6 exert a tumor-suppressive effect in CRC, KLF5 promotes tumorigenesis, at least in the context of oncogenic KRAS activation. The intricacy of the networks and pathways in which the three KLFs are involved in CRC are proving to be interestingly complex and challenging. Further investigation is warranted to determine the mechanisms by which the three KLFs are regulated in normal colonic epithelial cells and in CRCs with regards to the upstream and downstream mediators as well as the interaction among the three factors themselves.

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Disclosures

No potential conflicts of interest relevant to this article were reported.

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