Chapter 8
Krüppel-like Factors in Gastrointestinal Tract Development and Differentiation

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Abstract A number of KLF family members are known to play important roles in the regulation of proliferation, differentiation, and development of the gastrointestinal tract. Of these, KLF4 (previously known as GKLF or EZF) and KLF5 (previously known as IKLF or BTEB2) have been the most extensively studied. In this chapter, we review the expression patterns and established functions for KLF family members in the gastrointestinal tract and offer insight into possible future areas of investigation of the KLFs in gastrointestinal differentiation and development.

Development and Differentiation of the Gastrointestinal Tract

In the developing embryo, changes leading to the origins of the organs of the gastrointestinal tract are first detected during the period of gastrulation (Katz and Wu 2004; Lebenthal 1989; Moore and Persaud 1998). The gut tube develops from two invagination events (one at the anterior end and the other at the posterior end) that allow incorporation of the endoderm into the body cavity. These invaginations elongate in the endoderm and fuse in the midline of the embryo to form an elongated tube. Beginning at about the fourth week of human development (from embryonic day 7.5 to 9.5 in mice), the endoderm is internalized as the embryo folds to become the primitive gut (Hogan and Zaret 2002; Wells and Melton 1999). The primitive gut can be divided into three distinct parts during fetal life: foregut, midgut, and hindgut. Each segment of the primitive gut contributes to different components of the gastrointestinal tract. The foregut gives rise to the esophagus, stomach, proximal half of the duodenum, liver, and pancreas. The midgut forms the distal half of the duodenum, as well as the jejunum, ileum, cecum, appendix, ascending colon,

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and parts of the transverse colon. The hindgut develops into the transverse colon, descending colon, sigmoid colon, and rectum down to the anorectal line. Whereas the gut epithelium originates predominantly from the endoderm, the smooth muscle layers and connective tissue surrounding the epithelium are derived from the mesoderm. Complex interactions occurring between the gut endoderm and different mesenchymal tissues lead to the morphogenesis and differentiation of the esophagus, stomach, small intestine, and colon (Roberts 2000).

Esophageal Development and Differentiation

The esophagus has its origins in the foregut immediately caudal to the primordial pharynx, beginning at around E9.5 in mice and the fourth week of human embryonic development (Katz and Wu 2004; Kaufman 1995; Lebenthal 1989; Moore and Persaud 1998; Que et al. 2006). Initially, a laryngotracheal diverticulum develops from the ventral side of the foregut. As the diverticulum elongates, a tracheoesophageal septum is formed, dividing the trachea and the esophagus by 34–36 days of human gestation. During the seventh and eight weeks of human embryonic development, the esophageal epithelium proliferates and almost completely occludes the lumen. Beginning at 10 weeks, the esophagus recanalizes, eventually forming a columnar ciliated epithelium. By birth, the ciliated epithelium is replaced by a stratified squamous epithelium. This stratified squamous epithelium persists throughout adulthood under normal conditions and consists of three compartments: basal layer, suprabasal (prickle) layer, and superficial layer (Karam 1999). Proliferation occurs in the basal layer, and cells undergo differentiation as they migrate through the suprabasal layer to the superficial layer of the epithelium. The stem cells of the esophagus are located in the basal layer of the epithelium (Seery 2002). In some cases, metaplasia develops in cells of the esophageal epithelium with the formation of a columnar lining similar to that normally found in the intestine. This condition is called intestinal metaplasia or Barrett's esophagus (Fitzgerald 2006). Of note, the esophagus is keratinized in mice but not in humans (Hogan and Zaret 2002).

Gastric Development and Differentiation

Starting around 4 weeks of gestation in the human and approximately E10 in the mouse, the stomach begins to form as a dilatation of the distal foregut (Hogan and Zaret 2002; Katz and Wu 2004; Kaufman,1995; Moore and Persaud 1998). During development the stomach shifts position, and the dorsal wall of the stomach grows faster than the ventral wall, forming the greater and lesser curvatures of the stomach. The stomach continues to rotate until about 8 weeks' gestation and is eventually lined by a columnar epithelium composed of a pit-gland unit with four

compartments: foveolus, isthmus, neck, and base. All cells lining the pit-gland unit originate from progenitor cells located in the isthmus, which then migrate inward and/or outward to give rise to several cell types: surface mucus (pit) cells, mucus neck cells, zymogenic (chief) cells, parietal (oxyntic) cells, and enteroendocrine cells (Karam 1999). In the mouse, the proximal stomach (forestomach) is lined by a stratified, keratinized, squamous epithelium, and the distal stomach is lined by a glandular epithelium. Keratinization of the forestomach is first detected at E16.5 (Hogan and Zaret 2002).

Intestinal Development and Differentiation

Up until approximately E14 in the developing mouse and week 8 in humans, the intestine is lined by pseudostratified epithelium (Hogan and Zaret 2002; Traber and Wu 1995). Between E14 and E15 in mice and gestational weeks 9–10 in humans, the intestinal lining undergoes a transition into a single-layered simple columnar epithelium, with the development of finger-like projections called villi as a wave along the craniocaudal axis of the small intestine. This critical transition occurs after less than 25% of gestation in humans but after nearly 75% of gestation in mice (Traber and Wu 1995). Thus, the mouse intestinal epithelium is relatively immature at birth, and changes in gene expression and histogenesis continue over the first 3 weeks of postnatal life in mice (de Santa Barbara et al. 2003). In humans, the crypts begin to form between gestational weeks 10 and 12, and by about gestational week 16 the intestinal epithelium appears similar to that in the adult. In mice, crypts develop from the intervillous zone beginning around E19 and continuing into the second week of life, and the villi continue to lengthen during this time. At the suckling–weaning transition in mice, occurring between postnatal days 18 and 22, further functional differentiation occurs with dramatic changes in gene expression, such that by the middle of the fourth postnatal week the mouse small intestine has attained its adult form (Traber and Silberg 1996).

The adult small intestine contains four cell types: enterocytes, goblet cells, enteroendocrine cells, and Paneth cells (Karam 1999). These cells can be grouped into two functional classes—absorptive (enterocytes) and secretory (goblet cells, enteroendocrine cells, Paneth cells)—with Notch signaling being the key "molecular switch" between the two classes (Yang et al. 2001). The colon generally lacks Paneth cells but contains the other three cell types (enterocytes, goblet cells, enteroendocrine cells). In both the small and large intestine, as in the other luminal gastrointestinal organs, there is a spatial separation of proliferating and differentiating cells. In the small intestine, stem cells and transit-amplifying cells are located in the crypts, with differentiation occurring as cells migrate out of the crypts and along the villi. In the colon, which lacks villi, proliferation is restricted to the lower third of the crypts.

KLFs in Gastrointestinal Development and Differentiation

Among all the KLF family members characterized thus far, KLF4 (previously known as GKLF or EZF) and KLF5 (previously known as IKLF or BTEB2) have been the most extensively studied KLFs in the gastrointestinal tract (McConnell et al. 2007).

KLF4

KLF4 is highly expressed in the differentiated compartments of the gastrointestinal epithelia, including: the suprabasal and superficial layers of the esophagus (Fig.1A); the mid to upper portion of the gastric unit; small intestinal villi (Fig. 1B); and the upper portion of the colonic crypts (Garrett-Sinha et al. 1996; Goldstein et al. 2007;

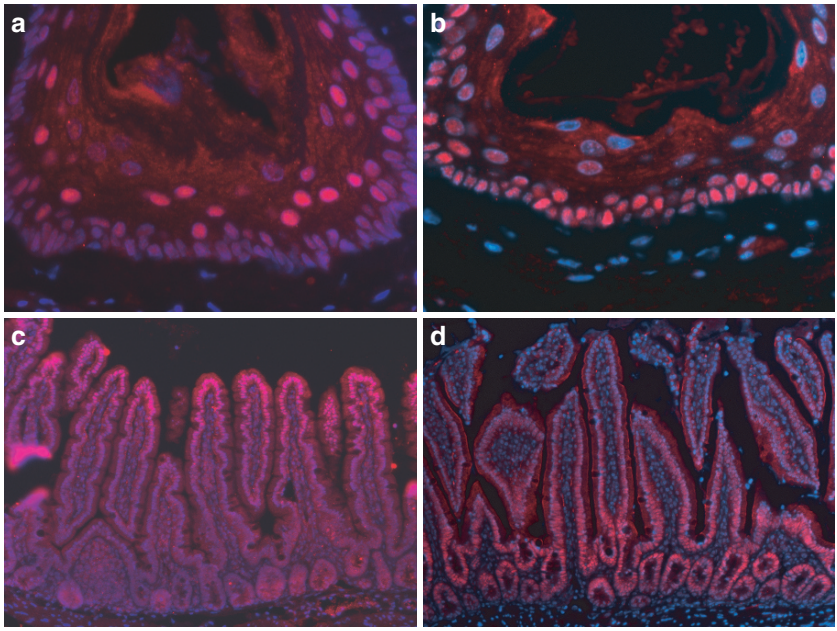

Fig. 1 Klf4 and Klf5 are expressed in the differentiating and proliferative compartments, respectively, in murine gastrointestinal epithelia. In the squamous esophagus, nuclear Klf4 staining (*red*) is seen in cells of the suprabasal layer (**A**), and Klf5 (*red*) is expressed in basal cell nuclei (**B**). In columnar epithelial cells, such as those of the small intestine, Klf4 (*red*) is expressed along the villi (**C**), and Klf5 (*red*) is localized to the crypts (**D**). Similar patterns of expression are seen in proliferative and differentiating regions of the stomach and colon (not shown). Hoechst 33258 (*blue*) is used as a counterstain. **A, B** ×400. **C, D** ×100

Katz et al. 2005; Shields et al. 1996). Expression of the gene encoding *Klf4* is regulated during development, with maximal expression occurring in the late stage of mouse fetal development (Garrett-Sinha et al. 1996; Ton-That et al. 1997). No expression of *Klf4* is detected at E9.5, and the level of expression is very low from E10 to E12. Beginning on E13, the level of the *Klf4* transcript starts to rise, peaking at E17, before decreasing moderately by E19. *Klf4* expression during embryonic development is therefore coincident with the transition in the intestine from squamous cells to columnar epithelium as well as with gut tube morphogenesis (Hogan and Zaret 2002; Traber and Wu 1995).

In 12.5-day embryos, *Klf4* mRNA expression is apparent in epithelial cells of the dorsal surface of the tongue, whereas epithelial cells of the esophagus and colon begin to express *Klf4* transcripts at E15.5 (Garrett-Sinha et al. 1996). In newborn mice, many gastrointestinal epithelia express high levels of *Klf4* mRNA, including the tongue, esophagus, stomach, and colon (Garrett-Sinha et al. 1996; Ton-That et al. 1997). Interestingly, levels of *Klf4* transcripts have been reported to be higher in the colon than in the small intestine in the embryo, newborn, and adult mice. Homozygous null mice for *Klf4* have been generated but die within 15 hours after birth owing to a defect in the barrier function of the skin (Segre et al. 1999). *Klf4* null mice show perturbations of the late-stage differentiation structures of the skin and tongue and abnormal differentiation of goblet cells in the colon (Katz et al. 2002; Segre et al. 1999). Tissue-specific gene ablation of *Klf4* in the glandular epithelium of the stomach results in increased proliferation and altered differentiation of parietal, zymogenic, pit, and mucus neck cells of the gastric epithelia (Katz et al. 2005). Gastric epithelia of these *Klf4* mutant mice are also hypertrophic and display precancerous changes.

Expression of KLF4 in terminally differentiated cells of the gastrointestinal tract suggests an important role for this transcription factor in the switch from cell proliferation to cell differentiation (Ghaleb et al. 2005). In support of this, overexpression of KLF4 in vitro in a human colonic adenocarcinoma cell line (HT-29) results in growth arrest, and DNA synthesis increases following suppression of KLF4 in these cells (Shie et al. 2000). KLF4 also regulates cell proliferation by controlling several genes critical for cell cycle checkpoint control. For example, in the human colon cancer cell line RKO, inducible expression of KLF4 blocks cell cycle progression at the G_1/S transition point (Chen et al. 2001). Furthermore, increased expression of cell cycle checkpoint protein $p21^{WAF1/CIP1}$ is observed in RKO cells following induction of KLF4, and transcriptional profiling reveals that KLF4 induction also leads to increased expression of $p27^{KIP2}$ and to reduced expression of cyclin D1 and CDC2. The protein $p21^{WAF1/CIP1}$ is also a target of Klf4 in the gastric epithelium in vivo (Katz et al. 2005). KLF4 controls p53-dependent G_1/S cell cycle arrest and inhibits the expression of cyclin B1, cyclin D1, and cyclin E following DNA damage in HCT-116 colon cancer cells (Shie et al. 2000; Yoon et al. 2003, 2005). It has also been proposed that KLF4 is a tumor suppressor in gastric and colon cancers (Wei et al. 2005; Zhao et al. 2004). In esophageal cancer cells, KLF4 promotes apoptosis and inhibits invasion (Yang et al. 2005). However, KLF4 is also suggested to be a context-dependent oncogene (Rowland et al. 2005),

and nuclear localization of KLF4 in skin and breast is associated with a more aggressive phenotype (Chen et al. 2008; Pandya et al. 2004). Nonetheless, these data generally support a role for KLF4 as a negative regulator of proliferation in gastrointestinal epithelial cells.

In addition to its role in the regulation of cell proliferation, Klf4 regulates a number of genes critical for epithelial differentiation. In esophageal epithelial cells, KLF4 transcriptionally activates keratin 4, a marker of keratinocyte differentiation (Jenkins et al. 1998; Okano et al. 2000). Klf4 also increases expression of another keratinocyte differentiation marker keratin 13 (Goldstein et al. 2007). In newborn colon, loss of *Klf4* results in decreased expression of the goblet cell-specific gene *muc2* and diminished numbers of goblet cells, suggesting that this factor may be critical for goblet cell differentiation (Katz et al. 2002). Klf4 also activates the enterocyte differentiation marker intestinal alkaline phosphatase (Hinnebusch et al. 2004). Thus, in sum, Klf4 appears to play an essential role in epithelial differentiation throughout the gastrointestinal tract.

A number of studies have suggested that KLF4 acts as a tumor suppressor in the gastrointestinal tract. Decreased expression of KLF4 has been observed in dysplastic epithelium of the colon (Shie et al. 2000). Similarly, colonic adenomas and carcinomas from patients with familial adenomatous polyposis have decreased levels of KLF4 expression compared to adjacent normal mucosa (Dang et al. 2000). Reduced levels of *Klf4* mRNA are also observed in the intestine of APC^{Min} mice; and $APC^{Min}/Klf4^{+/-}$ mice develop an increased number of adenomas compared to APC^{Min} mice (Ghaleb et al. 2007). Furthermore, dysregulation of KLF4 gene expression has been reported in a number of human colorectal cell lines (Zhao et al. 2004), gastric cancers (Katz et al. 2005; Wei et al. 2005), and human esophageal squamous cell carcinomas (Ghaleb et al. 2007; Luo et al. 2003; Wang et al. 2002; Wei et al. 2005).

KLF5

Klf5, in contrast to *Klf4*, is expressed predominantly in the proliferative compartments of the gastrointestinal epithelia in adults, including: the basal layers of the esophagus (Fig. 1C); the small intestinal crypts (Fig. 1D); and the lower third of the colonic crypts (Conkright et al. 1999; Goldstein et al. 2007; Ohnishi et al. 2000; Yang et al. 2008). In the mouse embryo, *Klf5* expression is abundant from E7 and is expressed throughout the primitive gut beginning at E10.5 (Ohnishi et al. 2000). Progressively, expression of *Klf5* becomes confined to the crypts of the small intestine by E17.5. Expression is also seen in the epithelium of the tongue at E16.5 and E17.5. Thus, *Klf5* expression remains consistently high throughout development of the gastrointestinal tract.

Klf5 appears to function as a positive regulator of proliferation in non-transformed epithelial cells (Sun et al. 2001). For example, Klf5 expression in IEC-6, IEC-18, and IMCE intestinal epithelial cells increases cell proliferation (Bateman et al. 2004; Chanchevalap et al. 2004), and transgenic expression

of *Klf5* in murine esophageal epithelia in vivo results in increased basal cell proliferation (Goldstein et al. 2007). Interestingly, in this model, expression of *Klf5* in the suprabasal layer did not appear to alter keratinocyte proliferation or differentiation, suggesting that context is important for Klf5 function. Other studies suggest that Klf5 may be important in maintaining the transit-amplifying cell state in esophageal epithelia by transcriptionally activating epidermal growth factor receptor (EGFR) and integrin-linked kinase (ILK) to regulate proliferation and migration, respectively (Yang et al. 2007, 2008). Some controversy, however, exists regarding the role of KLF5 in transformation. Although some studies suggest that KLF5 contributes to cellular transformation induced by K-Ras during intestinal tumorigenesis (Nandan et al. 2008), Klf5 appears to inhibit proliferation in some intestinal cancer cell lines; moreover, reduced expression of Klf5 is observed in intestinal adenomas from APC^{Min} mice and familial adenomatous polyposis (FAP) patients (Bateman et al. 2004). Klf5 also inhibits proliferation in esophageal squamous cancer cells (Yang et al. 2005). Mice homozygous for a null mutation in *Klf5* die by E8.5, and heterozygous loss of *Klf5* results in abnormally shaped small intestinal villi (Shindo et al. 2002). In sum, the function of Klf5 in gastrointestinal epithelia appears to be generally pro-proliferative but is also context-dependent.

KLF6

KLF6 (also known as Zf9 or CPBP) was initially cloned from a human placental expression library and is ubiquitously expressed in adult tissues (Koritschoner et al. 1997). During development, *Klf6* is abundantly expressed at E16.5 and E18.5, where it is restricted to the mucosa of the hindgut (Laub et al. 2001). In mice, loss of *Klf6* is lethal to embryos by E12.5 due to markedly reduced hematopoiesis in the yolk sac (Matsumoto et al. 2006). $Klf6^{-/-}$ mice also have a poorly defined liver. To date, the early lethality of the $Klf6^{-/-}$ mice has precluded analyses of the effects of *Klf6* loss on gastrointestinal development and homeostasis at later stages in the embryo and in the adult. A growing body of evidence implicates KLF6 as a negative regulator of proliferation and/or a potential inducer of differentiation. For example, KLF6 increases expression of the Cdk inhibitor $p21^{Waf1/CIP1}$, which promotes cell cycle arrest in a p53-independent manner (Narla et al. 2001). Moreover, interaction of KLF6 with cyclin D1 has been shown to inhibit proliferation via disruption of the cyclin D1–Cdk4 complex assembly and activity (Benzeno et al. 2004). In human esophageal cells, KLF6 interacts with KLF4 to co-activate the keratin 4 promoter, which is important for epithelial differentiation (Okano et al. 2000). KLF6 has been identified as a putative tumor suppressor in the stomach (Cho et al. 2005) and colon (Cho et al. 2006a, 2006b; Reeves et al. 2004). It is downregulated in Barrett's associated esophageal adenocarcinoma (Peng et al. 2008) and silenced by promoter hypermethylation in esophageal squamous cell cancer (Yamashita et al. 2002).

KLF9

Klf9 (previously known as BTEB) was originally isolated as a positive transcriptional regulator of the CYP1A1 gene promoter (Imataka et al. 1992). Northern blot analyses demonstrated that KLF9 is ubiquitously expressed. In the embryo, *Klf9* is expressed by E8 in the epithelia and smooth muscle layers of the developing gut, and this expression pattern is sustained to E16 (Imataka et al. 1992). To study the function of KLF9 in vivo, homozygous null mice for *Klf9* were generated. *Klf9* null mice are viable but have defects in brain development and in female reproductive capacity (Morita et al. 2003; Simmen et al. 2004). Further analysis of *Klf9*$^{-/-}$ mice revealed significantly reduced levels of proliferation and migration in intestinal epithelial cells of mutant mice (Simmen et al. 2007). These mice also show slight alterations of Paneth and goblet cell differentiation. Whereas these studies suggest that Klf9 is pro-proliferative in vivo, KLF9 expression is decreased in human colorectal cancers by a quantitative real-time polymerase chain reaction (PCR), Western blot analysis, and immunohistochemistry (Kang et al. 2008). More studies are necessary to confirm the function of KLF9 in gastrointestinal homeostasis and carcinogenesis.

KLF13

Klf13 (also known as BTEB3, RFLAT-1, or FKLF2) was identified by screening mouse databases for the presence of C_2H_2 DNA-binding domains (Martin et al. 2000; Scohy et al. 2000). Klf13 is expressed ubiquitously. *Klf13* mRNA is widely expressed at different stages of the developing embryo, and transcripts can be detected by E8 (Martin et al. 2001). Klf13 expression is detected in the muscle and epithelial layers of the developing gut at E11 and E13, but it becomes restricted to the epithelium by E16. The predominant phenotype of the *Klf13*$^{-/-}$ mice is a reduced number of erythrocytes, increased number of lymphoid cells, and a large spleen (Gordon et al. 2008; Zhou et al. 2007). Although homozygous null mutants for *Klf13* are viable, an increase in the number of deaths was observed among mutant mice at 3 weeks of age. No changes in the gastrointestinal tract of these animals have been reported to date.

KLF16

Klf16 (also called BTEB4 or DRRF) was initially described as a zinc finger transcription factor that modulates the activity of the dopamine receptor promoters (Hwang et al. 2001). Using in situ hybridization, *Klf16* can be detected in the intestine at E12, E14, and E16 (D'Souza et al. 2002). In the adult, Klf16 is

expressed in a number of tissues, where it is localized to cell nuclei and acts as a transcriptional repressor (Kaczynski et al. 2002).

Conclusion

Krüppel-like factors are known to play a critical role in the differentiation and development of numerous organs and tissues in the body. Among them, KLF4 and KLF5 have been the most extensively characterized in the gastrointestinal tract and are known to play important roles in development and gastrointestinal homeostasis. Although some tissue-restricted KLFs are likely to have little or no role in the gastrointestinal tract in the normal state, the expression and function of these factors during/after gastrointestinal tract injury and cancer have not been well studied. Moreover, numerous other KLFs are expressed ubiquitously or are known to be expressed in the luminal organs of the gastrointestinal tract, but their precise role in differentiation and development is still unclear. In addition, little is known about the interactions among these factors themselves. Finally, given their similar binding sites, some KLFs may be able to replace others when the expression of the latter factors is lost (e.g., through the cellular differentiation process or in cancer and other diseases). Thus, further identifying the roles of the KLFs, their specific interactions, and their ability to antagonize and/or compensate for each other will provide fertile ground for future investigations of KLF function in the gastrointestinal tract.

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