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RUNX3 in Oncogenic and Anti-Oncogenic Signaling in Gastrointestinal Cancers

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ABSTRACT
The reputation of RUNX3 as a strong candidate for a tumor suppressor originated from studies of gastric carcinogenesis and now extends to a variety of other human cancers. The RUNX3 transcription factor is a downstream effector of the TGF-β superfamily signaling pathway and has a critical role in the regulation of cell proliferation, cell death by apoptosis, and cell adhesion. Recently, RUNX3 was proposed as a gatekeeper linking oncogenic Wnt and anti-oncogenic TGF-β/BMPs signaling pathways in intestinal tumorigenesis in mouse and human. Also, loss of RUNX3 leading to elevated oncogenic Wnt activity was found to be a key event in inducing a precancerous state of the stomach. Chronic *Helicobacter pylori* infection is reported to inactivate RUNX3 in gastric carcinogenesis by multiple mechanisms. This "Prospect" focuses on our current understanding of the tumor suppressive functions of RUNX3 in the context of gastrointestinal cancer initiation and progression. J. Cell. Biochem. 112: 1243–1249, 2011.

KEY WORDS: RUNX3; GASTROINTESTINAL CANCERS; WNT; TGF-BETA; BMP

As a strong candidate for a tumor suppressor, *RUNX3*, a member of Runt-related transcription factors (*RUNXs*; *RUNX1*, *RUNX2*, and *RUNX3*), has been widely studied in a variety of human cancers [Blyth et al., 2005; Chuang and Ito, 2010]. The studies of RUNX3 originate from phenotypical observations of *Runx3*-deficient mice and characterization of $Runx3^{-/-}$ mouse gastric epithelial cell lines, and both models have provided substantial evidence for RUNX3 functions in gastric epithelial cells. Disruption of *Runx3* in mouse causes epithelial hyperplasia and reduced epithelial apoptosis in the stomach. Unfortunately, most $Runx3^{-/-}$ mice with a C57BL/6 background die soon after birth [Li et al., 2002]. Mouse *G*astric epithelial cell lines generated by Kosei *I*to, Yoshiaki *I*to, and *F*ukamachi (GIF), which were established from gastric epithelium of 16.5 dpc $Runx3^{-/-}$ and $Runx3^{+/+}$ C57BL/6 mouse fetuses with the $p53^{-/-}$ genetic background ($Runx3^{-/-}$ GIF and $Runx3^{+/+}$ GIF), provide a substitute for the short-lived mouse model. $Runx3^{-/-}$ GIF cells grow faster than $Runx3^{+/+}$GIF and, most importantly, $Runx3^{-/-}$ GIF cells form gastric adenocarcinomas in nude mice [Li et al., 2002; Fukamachi et al., 2004]. This observation is a clear indication of the epithelial-autonomous tumor suppressor function of Runx3, although inflammatory lesions in the $Runx3^{-/-}$ gastrointestinal tract were also reported [Brenner et al., 2004].

Currently, functional inactivation of RUNX3 is known to be caused by mutation, epigenetic gene silencing (promoter hypermethylation) or cytoplasmic protein mislocalization in more than 80% of gastric and 40% of colorectal cancers [Ito et al., 2005, 2008]. The CpG island methylator phenotype (CIMP) has been proposed as a mechanism for human sporadic colorectal cancer [Toyota et al., 1999] and around one-third of colorectal cancer cases have been classified as CIMP [Goel et al., 2007]. *RUNX3* is one of the established markers used to classify CIMP+ tumors [Weisenberger et al., 2006].

RUNX3 REGULATES CELL-GROWTH, APOPTOSIS, AND CELL–CELL ADHESION DOWNSTREAM OF ANTI-ONCOGENIC TGF-β SIGNALING IN GASTRIC EPITHELIUM

RUNX3 binds directly to SMADs, major mediators in the TGF-β/BMP signaling pathway, which leads to synergistic activation of the Immunoglobulin Cα promoter [Shi and Stavnezer, 1998; Hanai et al., 1999]. RUNX3 has a function in TGF-β-induced growth inhibition and apoptosis; upon stimulation by TGF-β, RUNX3 cooperates with SMADs to directly upregulate targets related to cell-growth inhibition and apoptosis. *RUNX3* is encoded in chromosomal region 1p36, which is frequently deleted in a variety of human cancers. Deficiency of *RUNX3* corresponds with impaired tumor suppressive TGF-β signaling [Ito and Miyazono, 2003]. In vitro and ex vivo studies using $Runx3^{-/-}$ and $Runx3^{+/+}$ GIF cell lines and

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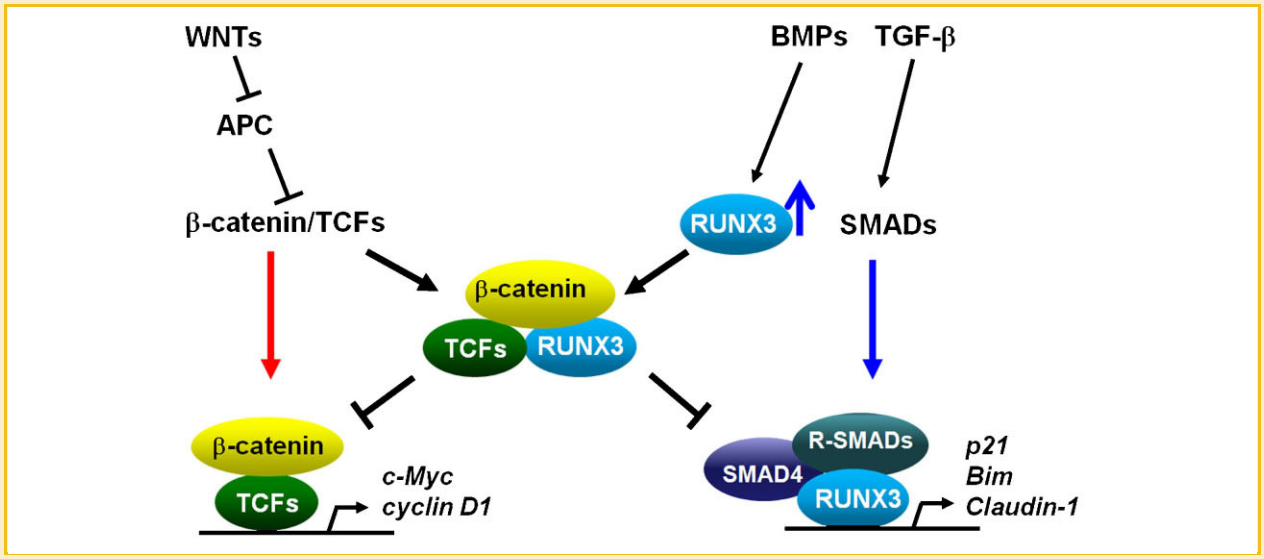

Fig. 1. Ternary complex formation by β-catenin/TCFs and RUNX3. The ternary complex is a novel node of the oncogenic Wnt-β-catenin/TCFs and anti-oncogenic TGF-β/BMPs-RUNX3, and is inhibitory on both sides. RUNX3 is upregulated by the stimulation of BMPs in colorectal cancer cells.

SNU16 cell line, a *RUNX3*-positive and TGF-β-sensitive human gastric cancer cell line, have revealed transcriptional target genes of RUNX3; it upregulates transcription of a cyclin-dependent kinase inhibitor, $p21^{WAF1/Cip1}$ and a proapoptotic gene, *Bim*, downstream of TGF-β signaling in gastric epithelial cells [Chi et al., 2005; Yano et al., 2006] (Fig. 1).

Tumorigenic $Runx3^{-/-}$ GIF cells attach weakly to each other and do not form any glandular structures when cultured on collagen gel, while non-tumorigenic $Runx3^{+/+}$ GIF cells form simple columnar epithelia with occasional glandular structures, suggesting abnormal epithelial sheet formation and cell polarity in $Runx3^{-/-}$ cells [Fukamachi et al., 2004]. A major tight junction protein, claudin-1, is transcriptionally regulated by RUNX3 [Chang et al., 2010] (Fig. 1). Exogenous expression of claudin-1 suppresses tumor growth of $Runx3^{-/-}$ GIF cells and knockdown of *claudin-1* enhances the tumor growth of SNU16 cells [Chang et al., 2010]. The tight junction provides a link to the actin cytoskeleton for the transduction of regulatory signals to and from the junction [Furuse and Tsukita, 2006]. Because of this ability, tight junction proteins are believed to be involved in the regulation of proliferation and differentiation, and most likely in the regulation of epithelial tumorigenesis. In fact, the expression of claudin-1 and RUNX3 is highly correlated in normal human gastric epithelia and human gastric cancers [Chang et al., 2010], and claudin-1 is, therefore, assumed to function as a gastric tumor suppressor.

RUNX3 ATTENUATES ONCOGENIC WNT SIGNALING IN INTESTINAL EPITHELIUM

Mutations of the *APC* gene, a key regulator of the canonical Wnt pathway, initiate intestinal carcinogenesis in human and mouse. The $Runx3^{-/-}$ intestinal epithelium shows increased proliferation and β-catenin/Tcfs activity, as indicated by the upregulation of β-catenin/Tcfs target genes (CD44, cyclin D1, c-Myc, Axin2/conductin, and EphB2). β-catenin, TCFs, and RUNX3 form a ternary complex. RUNX3 inhibits the DNA binding of β-catenin/TCF4 in vivo and in vitro, and prevents the transactivation of β-catenin/TCFs [Ito et al., 2008] (Fig. 1).

The inactivation of either *RUNX3* or *APC* can induce intestinal adenomas in human and mouse. $Runx3^{+/-}$ and $Apc^{Min/+}$ BALB/c mice develop the same number and size of small intestinal adenomas. Biallelic inactivation of either *Runx3* or *Apc* is sufficient for adenoma induction. Tumors isolated from those mouse lines display similar phenotypes and both bear upregulated cyclin D1 and c-Myc, which indicate increased transcriptional activity of β-catenin/TCFs. This feature is consistent with human adenomatous polyp cases that show either downregulated *Runx3* expression or nuclear β-catenin accumulation, but never both. In contrast, $Runx3^{+/-}Apc^{Min/+}$ double compound mutant mice develop adenocarcinomas. Human colorectal adenocarcinoma tissues display nuclear/cytoplasmic β-catenin accumulation and reduced RUNX3 expression simultaneously, suggesting that the alteration of both causes strong Wnt activation [Ito et al., 2008; Subramaniam et al., 2009] (Fig. 2). Thus, both the inactivation of RUNX3 that acts as a "brake" and the activation of β-catenin that acts as an "accelerator" in Wnt signaling appear to be necessary for the progression from adenoma to adenocarcinoma.

Recently, *RUNX3* was found to be transcriptionally upregulated by the stimulation of BMP-2 and -4 in BMP-sensitive human colorectal cancer cells [Lee et al., 2010]. Tumor suppressive roles for BMPs in colon cancer were defined by the discovery of germline mutations in the BMPRIa gene of patients with the rare inherited gastrointestinal cancer predisposition syndrome, familial juvenile polyposis (JP) [Howe et al., 2001]. Inhibition of BMP signaling in epithelial cells by transgenic overexpression of noggin, a BMP antagonist, results in the formation of ectopic crypts and polyps in the mouse intestine, which mimicks the intestinal histopathology of

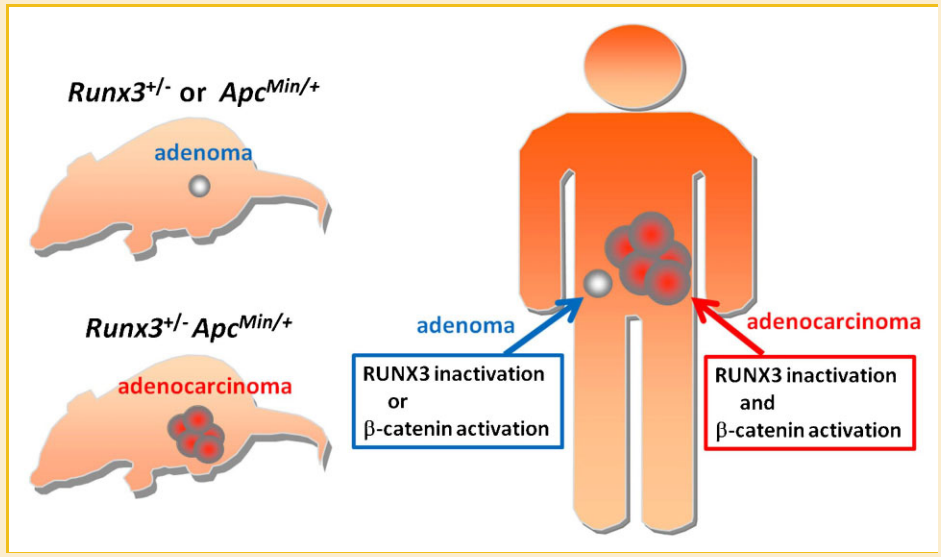

Fig. 2. Inactivation of either *RUNX3* or *APC* (activation of β-catenin) induces adenomas, and the inactivation of both *RUNX3* and *APC* (activation of β-catenin) causes the development of adenocarcinomas in mouse and human intestine.

JP [Haramis et al., 2004]. Similarly, conditional inactivation of BMPRIa and BMPRII results in hyperplasia and the development of hamartomatous polyps in the colon, which also occurs in the human JP syndrome [He et al., 2004; Beppu et al., 2008]. These findings further reinforce the case for a BMP signaling role in colonic malignancy. Upregulation of RUNX3 by BMPs, but not by TGF-β, reduces c-Myc expression; the inference, therefore, is that RUNX3 down-regulates c-Myc expression through attenuation of β-catenin/TCFs, downstream of BMPs in colorectal carcinogenesis (Fig. 1). This synthesis provides new insight into the mechanisms by which BMP suppresses cell growth and c-Myc expression in colorectal cancer; however, the precise molecular mechanism remains to be fully explained.

RUNX3 ATTENUATES ONCOGENIC WNT SIGNALING IN GASTRIC EPITHELIUM

Activation of the Wnt pathway is one of the major causes of gastric cancer development. Around 30% of gastric cancers have nuclear localization of β-catenin, a hallmark of Wnt activation [Clements et al., 2002]. Acceleration of *Wnt1* and deficiency of *Apc* are reported to be the initiators of gastric tumorigenesis in mouse models [Oshima et al., 2006; Tomita et al., 2007].

RUNX3 is expressed in gastric epithelial cells and particularly in chief cells [Ito et al., 2005, 2009] that differentiate in the lower compartment of gastric fundic glands (Fig. 3A). *Runx3*$^{-/-}$ adult mice with BALB/c rather than C57BL/6 background can survive to

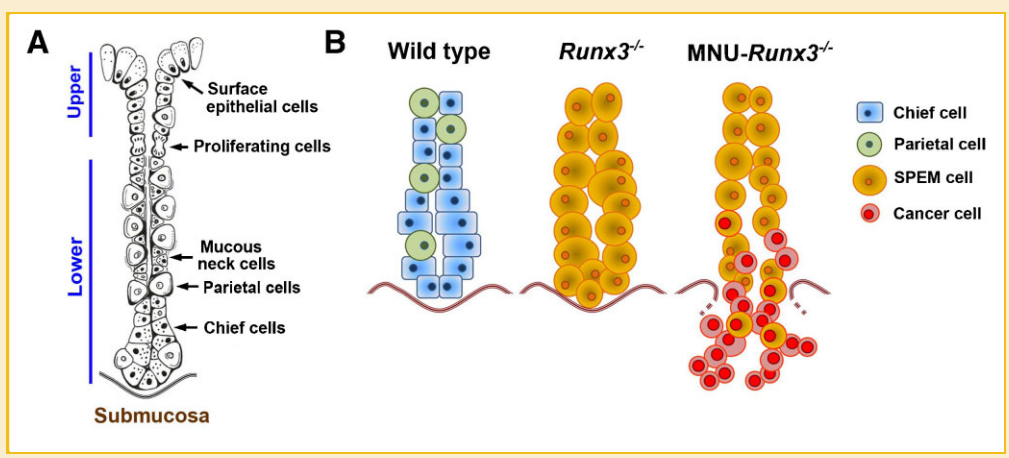

Fig. 3. Oncogenesis in *Runx3*$^{-/-}$ gastric glands. A: A diagram of a gastric gland. In the fundic area, a proliferating cell zone exists at the neck of the gland. The fundic gland can be separated into an upper and a lower compartment by the zone. B: RUNX3 is particularly expressed in chief cells in the lower compartment of fundic glands. Gastric epithelia of *Runx3*$^{-/-}$ adult mice exhibited hyperplasia, accompanied by the loss of chief cells and development of SPEM. The stomachs of *N*-methyl-*N*-nitrosourea-treated *Runx3*$^{-/-}$ (MNU-*Runx3*$^{-/-}$) adult mice frequently develop adenocarcinomas, which invade submucosa and further muscular layers.

around 1 year of age and the gastric epithelia exhibit hyperplasia accompanied by the loss of chief cells and the development of spasmolytic polypeptide/trefoil factor family 2 (TFF2)-expressing metaplasia (SPEM). SPEM is currently thought to progress to gastric cancer (see below). Following carcinogenic insult with *N*-methyl-*N*-nitrosourea (MNU), *Runx3*$^{-/-}$ mice, unlike the wild-type counterpart, consistently develop adenocarcinoma, which invades the submucosa and further muscular layers of the stomach. The *Runx3* deficiency gives rise to a precancerous state in the gastric epithelial glands [Ito et al., 2011] (Fig. 3B).

Furthermore, *Runx3*$^{-/-}$ gastric epithelium displays an intestinal phenotype marked by expression of a key inducer of intestinal metaplasia (IM), Cdx2 [Yuasa, 2003], and elevates Wnt activity with increased expression of Wnt target genes, c-Myc and CD44 (Fig. 4). In gastric cancer development, Cdx2 is also found as a positive target of oncogenic Wnt signaling and its expression is indirectly inhibited by Runx3, which attenuates β-catenin/TCF4 activity. In the absence of Runx3, Cdx2 expression is aberrantly upregulated in the gastric epithelium, which results in the induction of an intestinal phenotype in the lower area of the *Runx3*$^{-/-}$ SPEM glands (Fig. 4). Interestingly, some of the cells located at the bottom of the glands in the stomach were found to accumulate β-catenin in nuclei [Supplementary Information in Ito et al., 2011] in the same way that some cells at the bottom of large and small intestinal glands are known to accumulate nuclear β-catenin physiologically [Batlle et al., 2002; van de Wetering et al., 2002]. Although the significance of Wnt activation in the lower compartment of gastric glands is not yet clear, most of the cancerous cells in the MNU-treated *Runx3*$^{-/-}$ stomach invade the submucosa while showing nuclear accumulation of β-catenin [Supplementary Information in Ito et al., 2011]. These results suggest that highly active Wnt signaling at the bottom of gastric glands, coupled with the extreme sensitivity of *Runx3*$^{-/-}$ cells to the Wnt and MNU-enhanced carcinogenic process, eventually stimulate Wnt signaling (activation of β-catenin/Tcfs) to accelerate carcinogenesis and invasion of *Runx3*$^{-/-}$ cells (Fig. 4).

By itself, ectopic expression of Cdx2 caused by oncogenic Wnt does not seem to be sufficient to support the tumorigenic growth of GIF cells in a nude mouse assay. Knockdown of Cdx2 in tumorigenic *Runx3*$^{-/-}$ GIF cells or the addition of exogenous Cdx2 to non-tumorigenic *Runx3*$^{+/+}$ GIF cells does not alter their tumorigenicity in nude mice. On the other hand, the expression of constitutively active β-catenin in *Runx3*$^{+/+}$ GIF cells induces tumorigenic activity, while the expression of a dominant negative form of TCF4 in *Runx3*$^{-/-}$ GIF cells abolishes it, demonstrating the indispensability of Wnt signaling activity for tumorigenicity [Ito et al., 2011]. Thus, although Cdx2 is a target for Wnt signaling, Cdx2 induction alone does not appear to exert strong oncogenic activity in the stomach. The observation suggests that the induction of IM by Cdx2 in the absence of other factors is not sufficient for Wnt-driven gastric oncogenesis. This notion has important implications and warrants further studies to clarify whether IM induced by aberrant Cdx2 expression is pathologically a pre-neoplastic or para-neoplastic change of gastric epithelial cells in gastric carcinogenesis [Tatematsu et al., 2003].

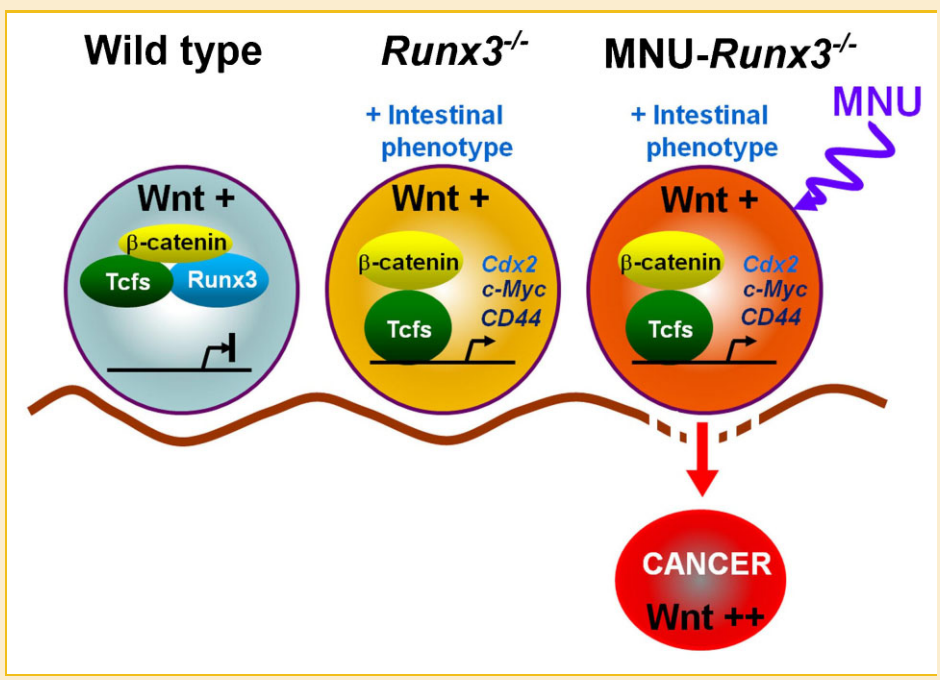

Fig. 4. Runx3 attenuates β-catenin/TCFs in gastric epithelial cells in the lower compartment of the glands. *Runx3*$^{-/-}$ gastric epithelial cells elevate Wnt activity with the increased expression of Wnt target genes, c-Myc, CD44, and Cdx2 which induces an intestinal phenotype. Some cells located at the bottom of gastric glands physiologically accumulate β-catenin in nuclei, and most cancerous cells in the MNU-treated *Runx3*$^{-/-}$ (MNU-*Runx3*$^{-/-}$) stomach invade the submucosa while showing nuclear accumulation of β-catenin. The active Wnt signaling, the extreme sensitivity of *Runx3*$^{-/-}$ cells to the Wnt, and the MNU-enhanced carcinogenic process are thought to accelerate the malignancy of *Runx3*$^{-/-}$ gastric epithelial cells.

RUNX3 IN GASTRIC ONCOGENESIS

Chronic *Helicobacter pylori* (*H. pylori*) infection is commonly associated with pathological events leading to gastric cancer. RUNX3 is frequently inactivated by promoter hypermethylation; thus, one possible mechanism of RUNX3 inactivation to be considered is de novo DNA methylation induced by *H. pylori* [Maekita et al., 2006; Kitajima et al., 2008]. Additionally, the multiple growth-promoting signals elicited by *H. pylori* infection [Peek and Blaser, 2002] could stably inactivate the function of RUNX3. When the effects of *H. pylori* infection on Runx3 protein was examined in mouse, it was found that Runx3 is proteolytically degraded only by virulent *H. pylori* infection and not by less-virulent strains. It is now known that the CagA protein characteristic of virulent *H. pylori* is associated with a protease that degrades Runx3 [Tsang et al., 2010]. Moreover, it has been reported that Src kinase phosphorylates RUNX3 on tyrosine and that tyrosine-phosphorylated RUNX3 translocates to the cytoplasm [Goh et al., 2010]. RUNX3 in the cytoplasm is usually in an inactive form [Ito et al., 2005]. Since CagA is known to activate Src, it can be hypothesized that *H. pylori* infection leads to the phosphorylation of RUNX3 protein and its eventual inactivation by cytoplasmic sequestration. *H. pylori* infection, therefore, appears to inactivate RUNX3 by multiple mechanisms.

A well-documented stem cell marker of intestinal epithelial cells, *Lgr5* [Barker et al., 2007], has also been proposed as a possible marker of antral stem or progenitor cells in stomach [Barker et al., 2010]. Its potency as a marker of stem cells or long-lived progenitors in gastric epithelial glands, however, is arguable. Recently, TFF2/spasmolytic polypeptide-expressing cells were reported to be progenitors of mucus neck, parietal, and chief cells that entirely occupy the lower compartment of gastric glands [Quante et al., 2010] (Fig. 3A). As mentioned above, $Runx3^{-/-}$ gastric epithelia develop SPEM, which is thought to be a consequence of elevated oncogenic Wnt signaling. SPEM caused by chronic *H. pylori* infection can be considered to represent a sequential step in carcinogenesis [Weis and Goldenring, 2009], and thus the stepwise progression of (1) *RUNX3* inactivation, (2) upregulation of oncogenic Wnt, and (3) emergence of SPEM can be clearly identified in the axis from gastric epithelial stem or progenitor cells to gastric cancer initiation.

It is interesting that the emergence of SPEM in $Runx3^{-/-}$ mice occurs in the absence of significant inflammation. While various studies attribute SPEM induction to inflammation and oxyntic atrophy [Judd et al., 2004; Oshima et al., 2005; Nam et al., 2007; Tu et al., 2008], *Klf4* knockout mice exhibit oxyntic atrophy and SPEM with the notable absence of inflammation [Katz et al., 2005]. There are striking similarities between the functions of RUNX3 and KLF4 in gastrointestinal epithelial cells; namely, the upregulation of $p21^{WAF1/CIP1}$, interaction with β-catenin/TCF4, and attenuation of Wnt signaling [Ito et al., 2008; Evans et al., 2010]. These suggest a possible functional relationship between RUNX3 and KLF4 in gastrointestinal oncogenesis.

A MUTATION, R122C, IN *RUNX3*

Notably, the tumorigenicity of *RUNX3*-negative MKN28 and MKN74 gastric cancer cell lines is strongly inhibited by the exogenous expression of RUNX3 [Guo et al., 2002; Li et al., 2002]. However, the exogenous expression of a mutated form of RUNX3, RUNX3 (R122C), identified in a gastric cancer patient fails to inhibit tumor growth in nude mice, suggesting that this single amino-acid substitution within the DNA-binding Runt domain is sufficient to cause malfunction of RUNX3 [Li et al., 2002]. RUNX3 (R122C) reduces DNA binding affinity and also binds SMADs less efficiently [Chi et al., 2005]. The combination of these defects results in TGF-β signaling impairment, as observed by reduced $p21^{WAF1/Cip1}$ promoter activation by TGF-β [Chi et al., 2005]. More recently, it was found that RUNX3 (R122C) is defective in ternary complex formation with β-catenin/TCFs [Ito et al., 2011], which suggests that this mutation enhances tumorigenesis because of multiple defects in tumor suppressive functions in both Wnt and TGF-β signal pathways. Besides gastric cancer, mutations within the RUNX3 Runt domain as well as RUNX3 polymorphisms have also been isolated from bladder cancer tissue [Kim et al., 2005; Zhang et al., 2008]. Identification of such mutations or genetic variations provides further understanding of oncogenic and anti-oncogenic pathways mediated by RUNX3.

CONCLUSION

This "Prospect" has focused on and highlighted, in gastrointestinal cancers, the functional involvement of RUNX3 in oncogenic and anti-oncogenic signaling pathways pivotal to the growing number of studies of RUNX3 in the cancer research field. Tumor suppressive TGF-β/BMPs and oncogenic Wnt signaling are known to regulate the function and development of adult epithelial stem cells and so-called cancer stem cells. The results presented clearly suggest that $Runx3^{-/-}$ epithelium is cancer prone and this is consistent with the notion of Runx3 as a gatekeeper in gastrointestinal tumorigenesis. However, stem cell-specific RUNX3 functions in normal or cancerous epithelial cells are not yet fully understood, and require elucidation using conditional knock-out or transgenic animal lines under stem-cell-specific promoters. Furthermore, the identification of key cell- and stage-specific target genes of RUNX3 is an important step forward. If we are to harness our growing knowledge of RUNX3, targeting these gene combinations will improve the potential of RUNX3 as a biomarker for cancer diagnosis and for cancer therapeutics.

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