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Mouse models demonstrating the role of stem/progenitor cells in gastric carcinogenesis

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1. ABSTRACT

Advances have been made in identifying the gastric epithelial stem cells and their immediate descendents which act as uncommitted or committed progenitors giving rise to cell lineages producing the various contents of the gastric juice and several hormones. New research suggests that these epithelial stem/progenitor cells also play an important role in the development of gastric cancer. In this review, we summarize results of examining three genetically manipulated mouse models in which the biological features and differentiation program of the gastric stem/progenitor cells were altered by three different approaches: 1) knockout of the trefoil factor 1 gene which is expressed initially in the partially committed pre-pit cell progenitors known to produce both mucus- and acid-secreting cell lineages, 2) expression of Simian virus 40 large T antigen gene in the acid-secreting parietal cell lineage, and 3) ablation of the parietal cells by using the attenuated diphtheria toxin A fragment. Systematic analysis of these animal models provided some clues to the role played by gastric stem/progenitor cells during carcinogenesis and to the cellular origin of gastric cancer.

2. INTRODUCTION

Many years ago, Stevens and Leblond (1) suggested that multipotent undifferentiated progenitor cells reside in the isthmus region of the gastric gland (Figure 1, epithelial unit on the left side). This concept was supported by subsequent studies demonstrating the morphological features of these stem/progenitor cells, their proliferation potential and subsequent differentiation into multiple epithelial cell lineages. In light of recent studies, the importance of these gastric stem/progenitor cells in physiological renewal and regeneration of all cell lineages forming the gastric epithelium is well established. Because gastric progenitor cells are few and thus not easily detectable in normal adult stomachs, development of methods to induce their amplification, transdifferentiation, and modulation in a reproducible fashion are essential for understanding the events that determine their ultimate fate, particularly during the course of gastric carcinogenesis. In this review, the identification of gastric stem cells, their biological features and these animal models generated to induce their alteration and, hence, suggest their possible role in carcinogenesis are discussed.

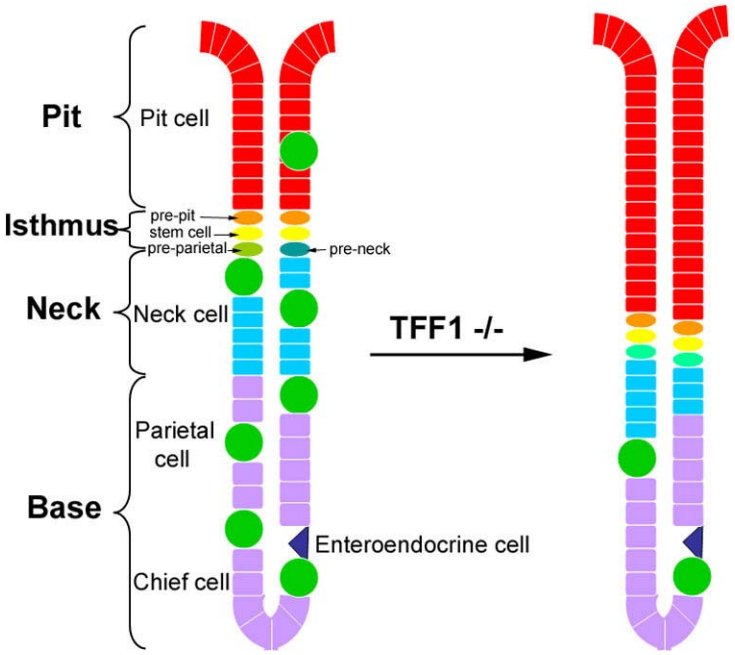

Figure 1. The cellular architecture of the gastric epithelial pit-gland units in the oxyntic mucosa of normal (left side) and TFF1-knockout mice. The normal gastric gland is made of isthmus, neck and base regions. Four cell types (prepit, preneck, preparietal and stem cells) are depicted in the isthmus region which give rise to pit cells in the pit, neck cells in the neck, chief cells in the base and parietal cells scattered throughout the unit. An enteroendocrine cell is also depicted in the base region. Note that TFF1 deficiency (right side) leads to an increase in the production of pit cells at the expense of parietal cells.

3. IDENTIFICATION OF GASTRIC EPITHELIAL STEM CELLS

Stem cells are generally defined by two main features: i) morphologically, they are undifferentiated and exhibit embryonic cell-like features, and ii) functionally, they are highly proliferative and capable of differentiation to replace other mature cells of the tissue while maintaining their own population (2). Following the observation of Stevens and Leblond (1) that some cells in the gastric glands are capable of mitosis, several investigators confirmed and demonstrated the incorporation of 3H-thymidine into these cells shortly after its injection (3-5). When electron microscopy was combined with high resolution radioautography at various time periods following administration of 3H-thymidine, both the morphological and functional features of the highly proliferative cells were identified in the oxyntic glands of the stomach. These mitotic cells were found to include undifferentiated granule-free cells which act as stem cells giving rise to uncommitted or committed progenitor cells (6, 7). While granule-free cells have neither secretory granules nor any other sign of differentiation, the pre-pit and pre-neck progenitor cells have a few membrane-bound secretion granules which appear similar to but smaller than those of mucus-secreting pit and neck cells, respectively (6). The third progenitor cell type, pre-parietal cell, exhibits little glycocalyx on the apical membrane with an increased number of microvilli which eventually elongate and become similar to but still fewer than those of the acid-secreting parietal cells (6-9). Dissection of the different levels of the oxyntic glands has revealed that these three cell types are all found in the isthmus region (Figure 1, left side). While granule-free cells can be seen at any level of the isthmus, pre-pit cells are found next to the isthmus-pit border and pre-neck cells next to the isthmus-neck border (6). In addition to pre-pit, pre-neck and pre-parietal cells, morphological and radioautographic evidences indicate that the undifferentiated granule-free stem cell also gives rise to pre-enteroendocrine and pre-caveolated cells, which in turn give rise to enteroendocrine and caveolated cell lineages (10).

Since one of the morphological features of undifferentiated stem cells is the presence of a primitive Golgi apparatus with no sign of secretory granule production, one should expect that the early sign of differentiation toward a secretory granule-producing cell lineage could be captured in the Golgi region. Accordingly, electron microscopic examination of the trans face of the Golgi region of granule-free cells revealed prosecretory vesicles which contain secretion material similar to those in the prosecretory vesicles of pre-pit and pre-neck cells, respectively. These subtypes of granule-free cells were referred to as pre-pit cell progenitor and pre-neck cell progenitor. In pre-pit cell progenitors, the contents of such vesicles appear uniform but at different stages of condensation. In pre-neck cell progenitors, the prosecretory vesicles have pale peripheral contents against darker background. In the third subtype of granule-free cells, the Golgi apparatus is very primitive and its trans face lacks any prosecretory vesicles indicating that this subtype is not involved in the production of secretory granules and therefore, represents the “undifferentiated

stem cell" (7). The ultrastructural features of the undifferentiated stem cell appear similar to those of undifferentiated embryonic cells: high nucleus-to-cytoplasm ratio, large reticulated nucleoli, many free ribosomes and few small organelles. In addition, 3H-thymidne labeling has indicated that granule-free cells are highly proliferative (7). This proliferative activity ensures the production of other cells and their own maintenance. In brief, granule-free cells are the least differentiated and most proliferative and, hence, fulfill the morphological and functional features of a stem cell (7).

3H-thymidine labeling of the granule-free cells in a pulse-chase experiment showed that the undifferentiated stem cells turn over very rapidly without retention of 3H-thymidine. The overall turnover time of the granule-free cells was estimated from a cumulative labeling experiment and averaged 2.6 days (7).

4. MOUSE MODELS OF ALTERED GASTRIC STEM/PROGENITOR CELLS SUGGESTING THEIR INVOLVEMENT IN CARCINOGENESIS

A fundamental question in cancer research is the nature of the cells which are capable of initiating and sustaining neoplasia. Nowell (11) initially proposed that cancer is monoclonal and originate from a single stem cell as a result of several genetic alterations. This hypothesis of the stem cell origin of cancer is supported by several studies (12-14). Our analysis of three different genetically engineered mouse models established in our collaborators' laboratories support this hypothesis of the stem or progenitor cell origin of cancer.

4.1. TFF1-knockout mice demonstrate amplification and invasiveness of gastric stem/progenitor cells

Trefoil factor 1 (TFF1) is a member of a group of small peptides which are synthesized and secreted with mucins of the gastric pit cells (15). TFF1 also functions with mucins in enhancing gastric mucosal protection and regeneration (16). In additions, several experimental studies suggested that TFF1 acts as a tumor suppressor that may be involved in development and/or progression of gastric cancer (17-19). Lack of TFF1 in a knockout mouse model was associated with a 5-fold increase of mitotic figures in the pyloric antrum and an elongation of the pit regions of the mucosa and finally the pit-gland units lost their tubular appearance and adenoma developed in all deficient mice. In many of these TFF1-knockout mice, the cells of the adenoma acquired malignant changes and a localized carcinoma *in situ* developed (20-22).

This remarkable role of TFF1 as a tumor suppressor is strongly supported by screening of different types of human gastric cancer which revealed an apparent down-regulation of TFF1 expression due to either allelic loss at the TFF1 gene locus, TFF1 promoter methylation, or TFF1 gene single point mutations (19, 23). Thus, the TFF1 knockout mouse provides an excellent model to look at the alterations that are associated with pre-cancerous lesions and to understand the development of gastric adenocarcinoma (21, 22).

Systematic cell lineage analysis in the oxyntic mucosa of the TFF1 knockout mice and their control littermates starting from birth up to more than 1 year of age demonstrated that TFF1 is localized initially in the Golgi saccules, prosecreory granules and secretion granules of pre-pit cells (24). In addition, with age, TFF1 knockout mice demonstrated a sequence of events starting with a gradual increase in the length of the gastric pits associated with a decrease in the number of acid-secreting parietal cells (Figure 1, right side). This was attributed to a change in the commitment program of pre-pit cells (24). In the pyloric antrum, the situation was more pronounced where nodular lesions and even carcinoma *in situ* in the basal portion of the mucosa were observed at around 6 months of age (20). In the 12-month-old knockout mice, some amplified glandular cells find their way through a gap in the muscularis mucosae and invade the submucosa (22) (Figure 2). These invading cells grow in the connective tissue of the submucosa and maintain some capacity to differentiate. This is in support of the concept of autocrine control of gastric stem cells and their capacity to differentiate outside their niche; so they are the source of instructions for their own commitment program (25). Thus, the TFF1 knockout mouse model recapitulates the classical chronological scheme of multi-step carcinogenesis including the initiation (due to the TFF1 deficiency), promotion and progression of the cancer cells (22).

Collectively, analysis of the TFF1-knockout mice at different age groups supports the hypothesis of the stem cell origin of gastric cancer (12, 13, 26-29). In the pyloric antrum of the TFF1-deficient mice, the cells which are responsible for the formation of early mucosal thickening, the carcinoma *in situ,* as well as the submucosal invasion with cyst-like structures are mainly epithelial progenitors. The fact that gastric progenitors including the undifferentiated granule-free stem cells are amplified in early stages of gastric tumorigenesis and formed the invasive cells in gastric adenocarcinomas raises a potential biological role of stem cells in the tumorigenesis cascade. Therefore, this mouse model could be taken as an evidence for the stem/progenitor cell origin of gastric cancer.

4.2. Mouse model of pre-parietal cell proliferation demonstrates their transdifferentiation during development of gastric neuroendocrine tumors

A lineage progenitor has typically been thought to be committed to the production of a mature cell type that performs a specific function. Thus, a pre-parietal cell gives rise to a parietal cell, not an enteroendocrine cell (8). A recent analysis of a transgenic mouse model of gastric cancer has provided some evidence for more plasticity for progenitor cell commitment and differentiation than previously considered possible.

In these mice, the transcriptional regulatory elements of the H,K-ATPase beta-subunit gene were used to deliver the product of Simian virus 40 large T antigen gene to preparietal cells. This forced expression of an oncoprotein in preparietal cells induced their proliferation from day 1 of postnatal life (9) and led to a massive (50- to 70-fold) expansion in their population by 1-2 months of age

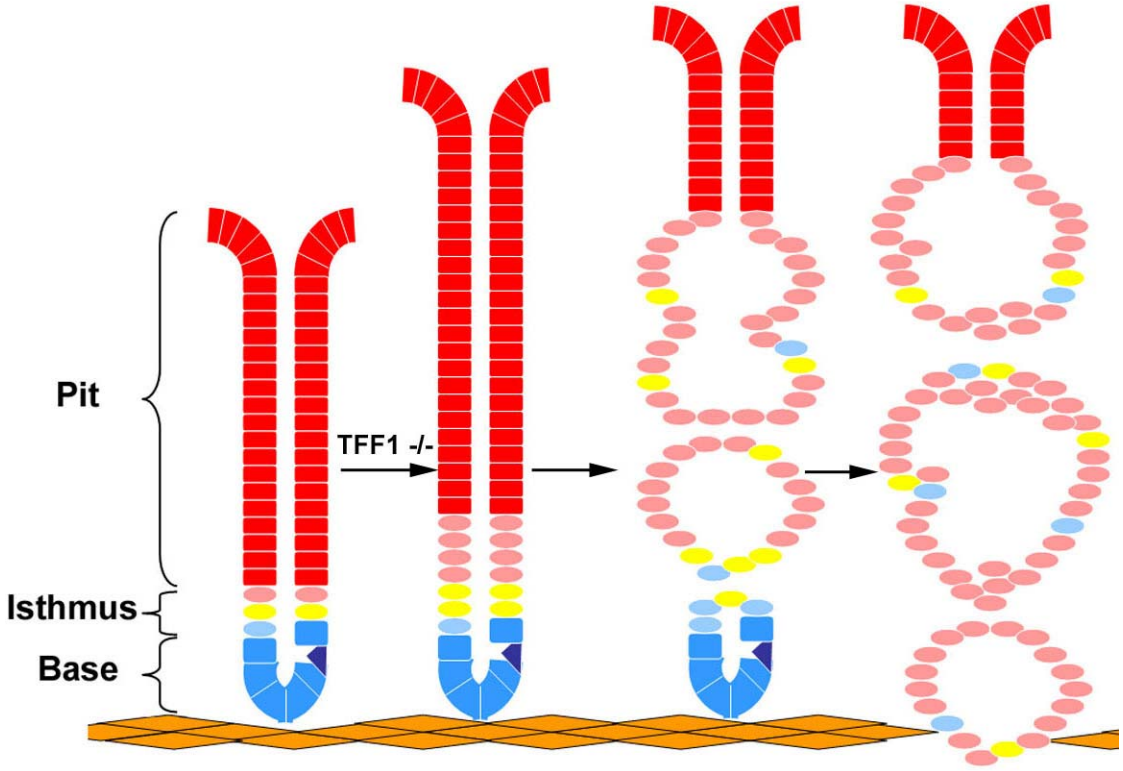

Figure 2. The effects of TFF1 deficiency on the cellular architecture of the gastric epithelial pit-gland units in the pyloric antral mucosa. Normal unit on the left side includes a pit and a gland made of isthmus and base regions. The isthmus includes the stem/progenitor cells which give rise to pit cells and gland cells, in the pit and base regions respectively. TFF1 deficiency leads to a sequence of events starting with an amplification of pit cells due to activation of progenitor cells. Eventually, stem/progenitor predominate in the unit which become dysplastic and eventually cells become invasive and cross the muscularis mucosa layer seen at the bottom.

(30). However, differentiation of pre-parietal cells to mature parietal cella and neck cells to zymogenic cells were blocked (Figure 3) (30).

When these mice became 3-6 months old, pre-parietal cell hyperplasia became associated with progressive mucosal thickening and glandular cyst formation. Areas of dysplasia were also developed. They were characterized by nuclear heterogeneity, loss of polarity, and stratification of glandular epithelial cells. In 10-month-old transgenic mice, areas with typical features of carcinoma *in situ* developed. These areas were characterized by complete loss of glandular architecture. Invasive epithelial cells formed loose trabeculae or ribbons. The cells had large nuclear-to-cytoplasmic ratio and much condensed heterochromatin. By 1 year of age invasive gastric cancer developed with local and distal (hepatic) metastases (31).

In this mouse model, immunohistochemical characterization of the gastric epithelial cells that form the invasive carcinoma revealed an interesting result. The transition from pre-parietal cell hyperplasia to neoplasia is marked by increased expression of neuroendocrine cell markers (chromogranin A and dopa decarboxylase) and loss of pre-parietal cell marker (H,K-ATPase). So, it seemed as if pre-parietal cell had switched their phenotype from H,K-ATPase synthesizing cells to enteroendocrine type synthesizing chromogranin A and dopa decarboxylase. Electron microscopic examination of these focal neoplastic areas demonstrated the transdifferentiation of pre-parietal cells into enteroendocrine cells (31). These findings may provide a possible explanation for the cellular origin of neuroendocrine cancer in the stomach which appears to be more common than generally thought (32).

4.3. Mouse model of parietal cell ablation demonstrates invasion and modulation of gastric stem/progenitor cells by *Helicobacter pylori*

H. pylori is a Gram-negative bacterium which colonizes the stomachs of more than half of the world's population. These *H. pylori*-positive individuals may remain asymptomatic throughout their life (33). On the other hand, some *H. pylori*-infected individuals may develop pathological changes leading to chronic atrophic gastritis (34) which is a pre-neoplastic condition characterized by loss of acid-producing parietal cells (35). In these individuals, *H. pylori* is found in the protective mucous layer of the stomach or closely attached to the cell membranes of the lining epithelium (36). Attachment of *H.*

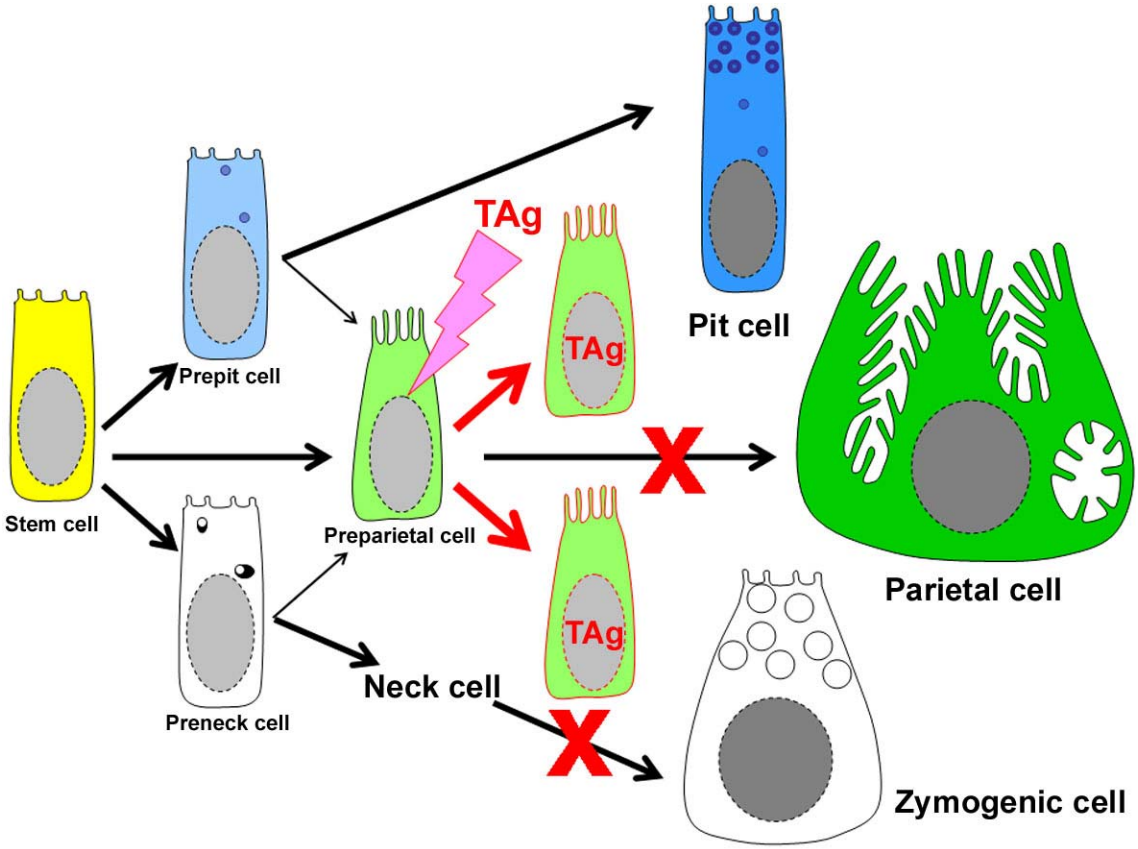

Figure 3. Diagramatic representation of the stem cell and differentiation pathways (black arrows) in the oxyntic mucosa. Induced expression of Simian virus 40 large T antigen in preparietal cells (TAg) induces their proliferation and blocks the differentiation of both parietal and zymogenic cells.

pylori is mediated via adhesin molecules which have affinity to NeuAcα2,3Galβ1,4-containing glycans (sialyl-Lewis x) on the apical plasma membranes of mucous cells (37).

When a transgenic mouse model of parietal cell ablation was generated by using the attenuated diphtheria toxin (DT) A fragment (*tox176*) and the lineage-specific promoter *Atp4b* (30), gastric epithelial stem/progenitor cell proliferation was stimulated leading to a progressive amplification of normally rare progenitors expressing NeuAcα2,3Galβ1,4 glycan (8, 38). Parietal cell loss and amplification of glycan-positive progenitors are features of humans with chronic atrophic gastritis (38).

Inoculating the stomachs of these DT-transgenic mice with *H. pylori* strains recovered from patients with or without chronic atrophic gastritis was associated with the growth and attachment of the bacteria to the amplified dividing and non-dividing epithelial progenitors expressing glycans specific to adhesins of *H. pylori* (39). Scanning confocal microscopy, combined with multilabel immunohistochemistry and electron microscopy, confirmed that a subset of gastric epithelial progenitors not only provided a surface for attachment of *H. pylori*, but also a habitat which supported formation of intracellular communities of *H. pylori* (Figure 4). The development of these intracellular bacterial communities in adult mammalian epithelial progenitors provides a new view of how *H. pylori* persists in some of its hosts, as well as an opportunity to consider how the biological features of these progenitors may not only support but also be influenced by intracellular bacterial communities.

To test the consequences of *H. pylori* invasion on gastric epithelial progenitors, an *in vitro* assay was developed by using a recently established mouse gastric epithelial progenitor (mGEP) cell line expressing the *H. pylori*-specific glycans (40). Incubating mGEP cells with *H. pylori* strains isolated either from chronic atrophic gastritis or from cancer patients showed that, while the former strain is adhesive to the progenitor cell membranes, the latter is invasive and capable of forming intracellular communities (41). Therefore, this intimate relationship between *H. pylori* and gastric epithelial progenitors as demonstrated both *in vivo* and *in vitro* is associated with changes in gene expression leading to carcinogenesis and provides another strong piece of evidence for the hypothesis of adult stem/progenitor cell origin of cancer.

Comparative studies of the gastritis and cancer strains of *H. pylori* support this concept. The cancer-strain

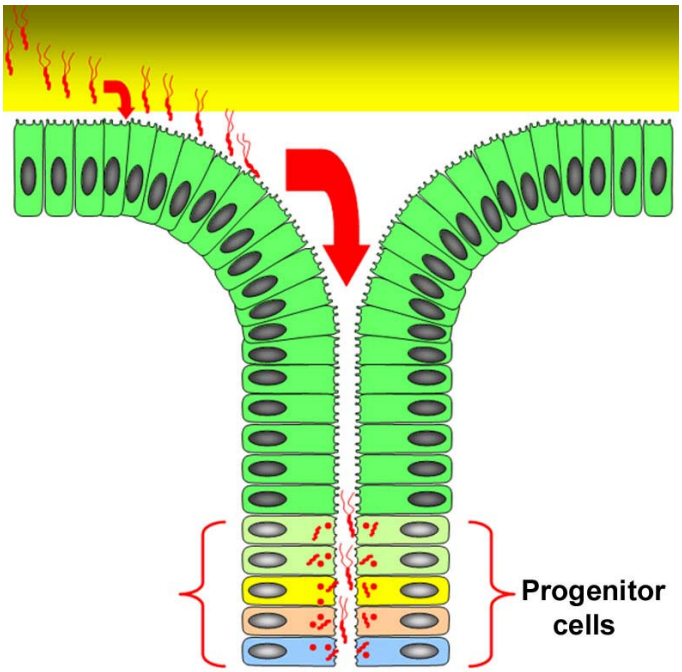

Figure 4. The pit and isthmus region of an epithelial unit in the oxyntic mucosa of DT-transgenic mouse which was inoculated with *H. pylori*. Note that the bacteria is seen in 3 main places: 1) floating in the mucus layer at the top, 2) attaching to luminal surface of pit cells and 3) invading the cytoplasm of progenitor cells and forming small communities.

induces higher levels of expression of ornithine decarboxylase and antizyme inhibitor (Azin1) in cultured mGEPs and up-regulates these transcripts in GEPs recovered by navigated laser capture microdissection from the stomachs of colonized gnotobiotic transgenic DT-mice. Thus, regulation of polyamine availability by intracellular *H. pylori* could affect the proliferative status of GEPs. Intriguingly, ornithine decarboxylase exhibits increased expression in gastric adenocarcinoma compared with tissue without metaplasia (42). Additional factors likely affect the outcome of this intimate association between *H. pylori* and gastric epithelial stem cells. Compared with the gastritis-associated *H. pylori* strain, infection of mGEP with the cancer-associated strain results in lower levels of expression of the elements of ephrin receptor signaling pathways known to control proliferation of gastrointestinal stem cells (43) and several tumor suppressors, including Kangai1. Down regulation of the latter correlates with poor prognosis of human gastric cancer (44).

Thus, results of TFF1-, SV40-, and DT-models together with the growing data about the stem cell origin of cancer strongly suggest that some members of the isthmal progenitor cells are involved in epithelial tumorigenesis and may have an early diagnostic, therapeutic, and/or prognostic clinical value.

5. CURRENT LIMITATIONS, FUTURE DIRECTIONS AND CONCLUSIONS

Although much progress has been made in developing animal models of gastric cancer to recapitulate its features, a continuing constraint in the generation of these models is the current lack of 'second-generation' promoters that can direct high-level transgene expression to all gastric epithelial cells. At present, there are no promoters that are capable of directing expression specifically to the undifferentiated granule-free cells, which would be advantageous for passing the transgene to all cell lineages. It is hoped that the extensive genomic analysis conducted on these cells and their immediate descendents using a combination of laser capture microdissection and Affymetrix GeneChip technology would facilitate the identification and use of such promoters (25).

The continued development of gastric-specific promoters will be essential for stomach-specific gene targeting, as the stomach phenotypes of many tumor suppressor genes are likely to be masked by lethality resulting from other causes. Also, the generation of stomach-specific inducible promoters should help the analysis of the sequential multistep order of genetic events in gastric carcinogenesis.

Knockout or transgenic mouse models of gastric cancer permit us not only to study the consecutive steps involved in its initiation and progression, but also to address questions like the cell of origin, and the role of stem cells in tumor maintenance. These genetically engineered mice need to be validated as suitable preclinical models for intervention studies in which questions with respect to therapy response and resistance can be addressed. Preclinical studies can be designed initially using inducible RNA interference technology to firmly establish the relevance of specific gene products for tumor maintenance and subsequently administering small

molecule inhibitors to impair the same pathway. Therefore, murine gastric cancer models could become a most valuable preclinical tool.

An intriguing aspect of the TFF1-knockout and Simian virus 40-transgenic murine models represents the early progenitor/stem cell hyperplastic lesions found along the gastric mucosae of the pyloric antrum or the oxyntic region, respectively. Even though there are many similarities between gastric epithelial cell lineages of humans and mice (45), no clear or well-defined precursor lesions for human gastric cancer have so far been characterized. If these hyperplastic progenitor/stem cells are, indeed, the precursor lesions of cancer, these lesions might guide us to find similar characteristic progenitor lesions in humans and help us to identify the target cell for transformation. Ultimately, specific protein expression patterns of murine hyperplastic lesions could then deliver early diagnosis markers for human gastric cancer.

Although current mouse models for gastric cancer need to be characterized in much more detail, they hold a substantial promise. They can help us 1) to recapitulate the pathophysiologic characteristics of this human disease in a ‘‘natural’’ manner, 2) to determine the cellular origin of gastric cancer, 3) to gain a detailed insight in basic gastric tumor biology, 4) to find markers for early gastric cancer diagnosis, finally and most importantly, 5) to test and validate new targeted anti-gastric-cancer therapies.

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Abbreviations: TFF1: trefoil factor 1; *H. pylori*: *Helicobacter pylori*; mGEP: mouse gastric epithelial progenitor

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