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BMI-1: a protein expressed in stem cells, specialized cells and tumors of the gastrointestinal tract

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Summary. Recently, BMI-1 was identified as a protein downregulating $p16^{ink4a}$ and mandatory for the continued existence of several stem cell compartments like hematopoietic and neural stem cells. In this study we investigated BMI-1 expression as a potential stem cell marker of the gastrointestinal tract. We found weak expression in the isthmus region of the stomach, and moderate expression in crypts of the intestines, whereas intestinal surface epithelial cells were weakly positive or negative for BMI-1. In addition, a variety of highly differentiated cells such as parietal cells, neuroendocrine cells of the pylorus, Paneth cells and a subset of goblet cells were moderately to strongly positive for BMI-1. Furthermore, we detected strong expression in gastrointestinal neoplasias. This expression pattern indicates a correlation of BMI-1 expression with gastrointestinal stem cells as well as numerous specialized cell types and points to a role of this protein in cellular differentiation in addition to that of stem cell maintenance. Besides, our results imply a role for BMI-1 in the tumorigenesis of gastrointestinal cancer.

Key words: Stem cells, Cancer, Gastrointestinal tract, BMI-1

Introduction

During tissue regeneration within the rapidly renewing gastrointestinal tract, complex patterns of differentiated cells are formed. These cells are derived from stem cells located in regenerating units that are either found in the region of the mucosal isthmus of the stomach or at the base of tubular invaginations in the small intestine and the colon (Brittan and Wright, 2002, 2004). Besides their role in renewal of the epithelial gastrointestinal surface and regeneration of its damage, stem cells are involved in gastrointestinal carcinoma formation by expansion of mutated clones.

Mechanisms regulating maintenance of these stem cells and regeneration of gastrointestinal epithelia are only incompletely understood. In addition, their exact location within regenerating units of the gastrointestinal tract remains unsolved, and there are different, partly conflicting hypotheses as to their number and location (Brittan and Wright, 2004), since stem cells are primitive cells lacking any definitive markers.

Polycomb genes are transcriptional repressors controlling development by the regulation of cell growth and differentiation genes (Mahmoudi and Verrijzer, 2001) and their dysregulation is linked to the aberrant proliferation of cancer cells (Glinsky et al., 2005; Raaphorst, 2005). Several polycomb genes, such as BMI-1 and Mel18, are also involved in the preservation of a variety of stem cell types (Iwama et al., 2005). Moreover; some of them have anti-senescent properties. Whereas the level of BMI-1 expression is independant of the growth state of human fibroblasts (Itahana et al., 2003), it is tightly linked to clonogenity and inversely correlated to senescence and its over-expression has been shown to extend their replicative lifespan. This effect is probably mediated by suppression of the INK4aARF locus (Jacobs et al., 1999; Itahana et al., 2003). BMI-1 can also activate human telomerase in mammary epithelial cells, but not in fibroblasts (Dimri et al., 2002). In addition, BMI-1 is also required for self renewal of hematopoietic (Lessard and Sauvageau, 2003; Park et al., 2003) and neural stem cells (Molofsky et al., 2003). BMI-1 deficiency leads to a strong reduction of hematopoietic stem cells in postnatal BMI-1-/- mice (Park et al., 2003) as well as a postnatal depletion of neural stem cells (Molofsky et al., 2003).

We hypothesized that BMI-1 may be an indicator of stem cell populations in the gastrointestinal tract. In contrast to many studies demonstrating a role of BMI-1 for hematopoietic and neural stem cell maintainance and for carcinogenesis, little is known about its expression pattern in healthy tissue. Thus we investigated the expression of this protein in a large number of samples of healthy, inflamed and neoplastic gastric and intestinal specimen and analysed its expression pattern in putative

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stem cell compartments.

Material and methods

Immunohistochemistry

Biopsies of normal mucosa of the antrum (n=8) and corpus (n=9), *Helicobacter-pylori*-associated gastritis (antrum (n=7) and corpus (n=10)), normal mucosa of the small (n=9) and large bowel (n=19) as well as surgical specimens including gastric carcinomas of the stomach (diffuse type: n=5, intestinal type: n=9), Morbus Crohn (10 samples) and carcinomas of the colon (11 samples) were obtained from the files of the Institute of Clinical Pathology / Medical University of Vienna.

Sections were stained with monoclonal BMI-1 antibody (anti-BMI-1, clone 229F6, Upstate Biotechnology, Lake Pacid, NY). Antigen retrieval was performed by boiling sections in EDTA (1 mM, pH 8.0) in an autoclave for 20 min. Avidin binding activity was blocked with an Avidin/Biotin Blocking Kit (Vector, Burlingame, CA). Subsequently, sections were incubated with BMI-1 at a dilution of 1:300 overnight. Bound antibodies were detected using biotinylated anti-mouse antibodies (Vector), a StreptABComplex/AP (Dako, Glostrup, Denmark) and Fast Red (Biogenex, San Ramon, CA). Negative controls were carried out using isotype-matched control reagents (IgG1, Coulter, Hialeah, FL).

Double stainings were performed combining immunohistochemical with immunofluorescent methods. A polyclonal rabbit anti-gastrin antibody (ICCB36-1, dilution 1:600, Accurate Chemical and Scientific Corporation, Westbury, NY) and a fluorophore labelled goat anti-rabbit antibody (Alexa Fluor® 488, dilution 1:300, Molecular Probes, Leiden, The Netherlands) were used for staining of the mucosa of the antrum (n=6). Samples were mounted with Fluoprep (BioMérieux, Marcy-l'Etoile, France) and analysed on an Olympus Provis AX70 microscope (Olympus, Vienna, Austria) with Meta Morph® Software Version 4.5 (Universal Imaging Corporation, West Chester, PA, USA).

Real time PCR

RNA was extracted from different human cell types (human umbilical venous endothelial cells (HUVEC, 5th passage), arterial smooth muscle cells (SMC, 5th passage), three different colonic carcinoma cell lines, i.e. PC3, HT29 and AQ) using TRIzole reagent (Invitrogen, Paisley, UK) according to the manufacturer´s instructions. First-strand cDNA synthesis was performed with 1 mg of total RNA, 60 U MuLV reverse transcriptase (Roche Applied Science, Vienna Austria), and 2.5 mM oligo (dT) primers in a 20-ml reaction volume. 1 ml cDNA was subjected to quantitative real-time PCR on a thermal cycler (LightCycler, Roche Applied Science) according to a standard protocol. Human BMI-1 gene was amplified with sense (5'- TTC ATT GAT GCC ACA ACC AT-3') and antisense (5'-CAG CAT CAG CAG AAG GAT GA-3') primers (MWG Biotech, Anzing, Germany). To normalize the quantity of cDNAs, the housekeeping gene ß-microglobulin was amplified in parallel with sense (5-GAT GAG TAT GCC TGC CGT GTG-3') and antisense (5-CAA TCC AAA TGC GGC ATC T-3') primers.

Results

Corpus

Both mucous epithelial cells of the pits and the isthmus expressed BMI-1 weakly to moderately (Fig. 1A), and this expression was exclusively nuclear. The staining intensity was sometimes weaker in pit cells, but most surface cells were stained with a similar intensity to isthmus mucous cells. Chief cells within the tubular glands were mostly negative or partly weakly positive for BMI-1 (Fig. 1B, bold arrows). By contrast, parietal cells expressed BMI-1 moderately to strongly (Fig. 1B, thin arrows).

Antrum

BMI-1 was weakly to moderately expressed in the neck regions of the glands. Similar to the corpus mucosa, expression intensity was sometimes weaker towards the surface of the pyloric mucosa. Expression of BMI-1 in cells of the pyloric glands was heterogenous. Whereas most cells were only weakly positive, single cells stained moderately to strongly for BMI-1. Double staining of mucosa specimen of the pylorus with gastrin identified most of these cells as neuroendocrine cells (Fig. 1C,D, arrows). By contrast, negative controls using isotype matched control antibodies did not result in nuclear staining (not shown).

Small bowel

Epithelial cells in the lower parts of the crypts were moderately positive for BMI-1. This expression was weaker in upper parts of the mucosa and epithelia at the foveolae were mostly negative (Fig. 2A). BMI-1 staining in Paneth cells at the base of the crypts was distinctly stronger than in adjacent glandular epithelial cells (Fig. 2B). BMI-1 expression of goblet cells was heterogeneous ranging from negative to distinctly positive, and sometimes goblet cells also in upper parts of the mucosa were moderately positive in contrast to adjacent mucinous cells (Fig. 2C). These irregularly distributed BMI-1 positive goblet cells were not obviously correlated with any location. Duodenal glands (of Brunner) were regularly positive for BMI-1 (not shown).

Large bowel

Epithelial cells of the lower part of the crypts

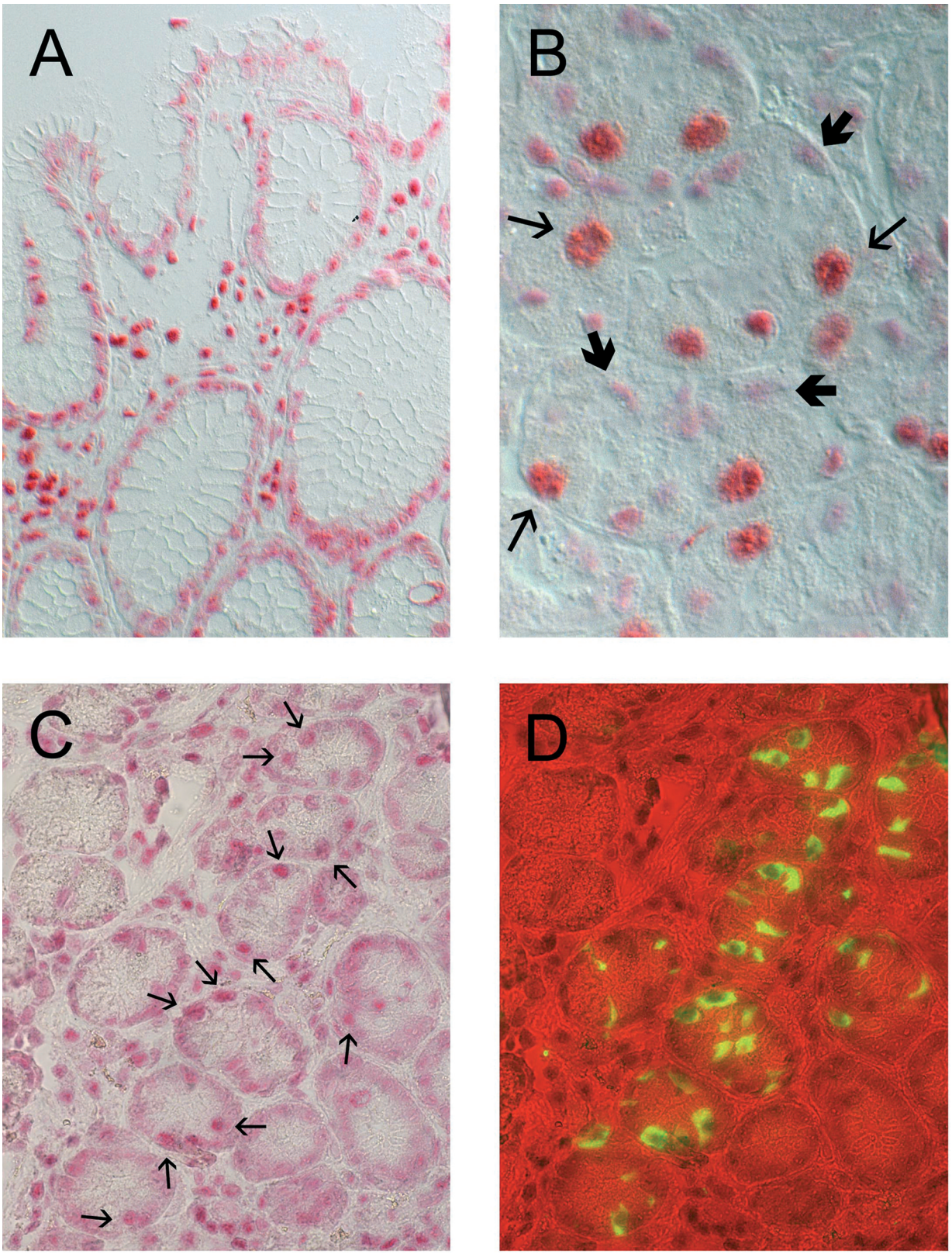

Fig. 1. BMI-1 is expressed in highly differentiated cells of the gastric mucosa. Weak to moderate nuclear staining for BMI-1 is found in mucous cells (**A**) of the body. Note that staining intensity decreases in the upper parts of the pits. Lymphatic cells within the lamina mucosa served as a positive control for BMI-1 staining. BMI-1 is strongly expressed in parietal cells (**B**, thin arrows), whereas chief cells (bold arrows) are negative or only weakly positive. Similarily neuroendocrine cells of the pylorus (**C**, arrows) that were identified by gastrin-expression (**D**, yellow cells) also stained strongly for BMI-1. In contrast, mucous cells of the pyloric glands were only weakly BMI-1 positive (panel **C**). A, C, D, x 100; B, x 400

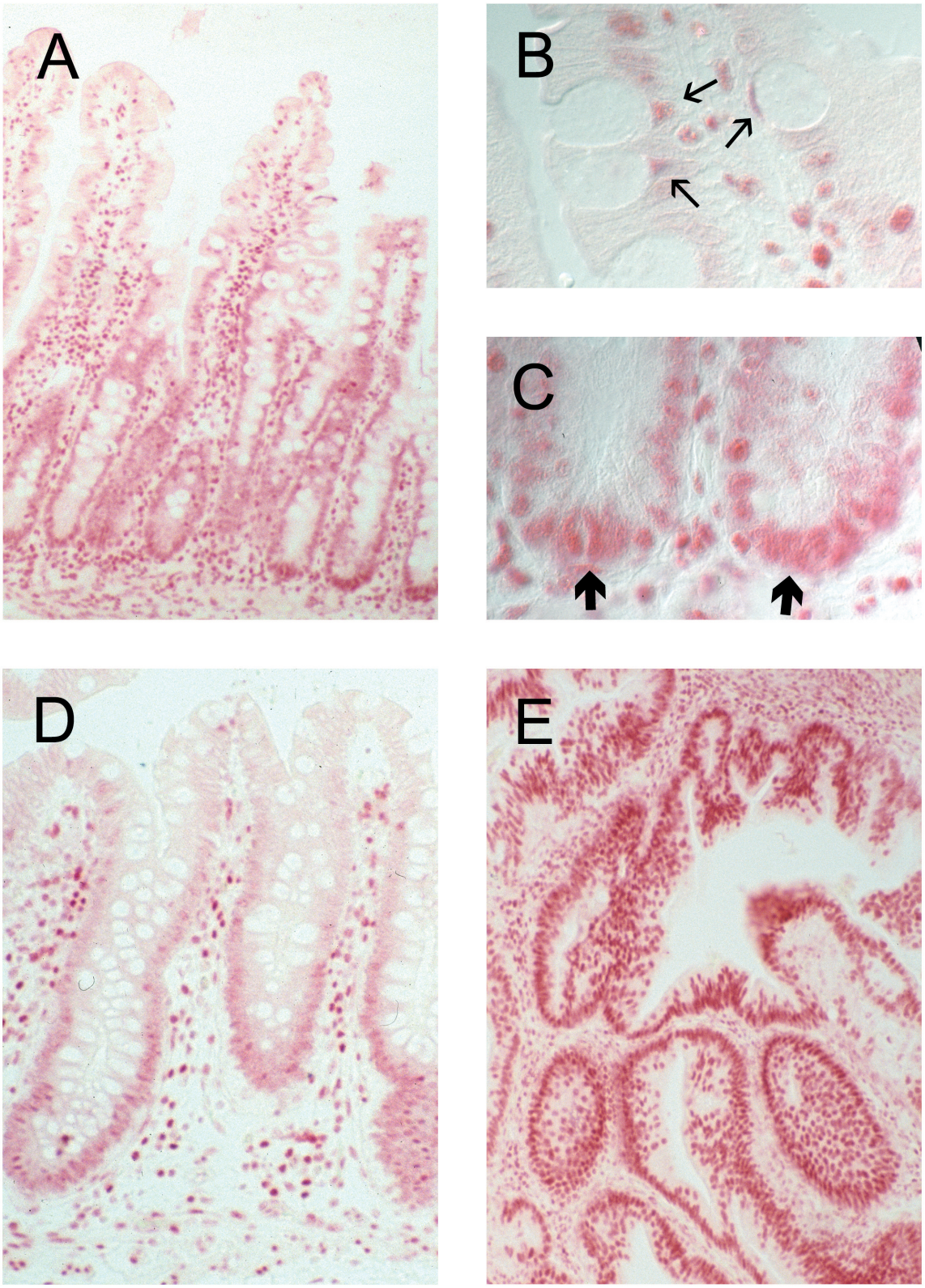

Fig. 2. BMI-1 is mainly expressed in crypts in the intestinal mucosa. Whereas columnar epithelial intestinal cells of the crypts stained distinctly for BMI-1, surface epithelial cells were mostly negative (**A**, small bowel, and **D**, large bowel). In addition, some goblet cells (**B**, arrows) and Paneth cells (**C**, arrows) also distinctly expressed BMI-1. The most prominent BMI-1 staining was found in adenocarcinomas (**E**, large bowel). Note that staining of tumor cells is much stronger than that of interstitial lymphocytes. A, D, E, x 50; B, C, x 400

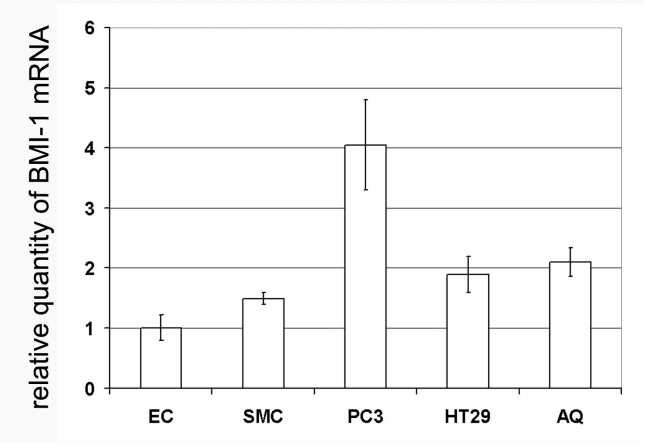

Fig. 3. BMI-1 mRNA can be detected in both stromal and gastrointestinal tumor cells. RT-PCR confirms the expression of BMI-1 in endothelial (EC), smooth muscle (SMC) and gastrointestinal tumor cells (PC3, HT29, AQ). The amount of endothelial BMI-1 mRNA was set as 1. Data are presented as mean value ± standard error. The degree of expression in different cell types is consistent with immunohistochemical results.

expressed BMI-1 weakly to moderately. BMI-1 expression decreased distinctly towards upper parts of the crypts and surface epithelia were mostly negative (Fig. 2D).

BMI-1 expression in inflamed gastrointestinal mucosa

When comparing acutely inflamed tissue of HP-gastritis and regular gastric tissue, no obvious up- or downregulation of BMI-1 in epithelial cells was found (not shown).

Adenocarcinomas of the gastrointestinal tract

BMI-1 expression was upregulated in 5 of 5 gastric carcinomas of the diffuse type and in 7 of 9 carcinomas of the intestinal type as compared to mucosal neck regions of adjacent healthy tissue. In 9 of these 14 tumors the staining intensity of BMI-1 was distinctly stronger in tumor cells than in lymphocytes, indicating a strong upregulation of this protein relative to normal tissue (Fig. 2E).

Similarly, in 9 of 11 colonic adenocarcinomas investigated BMI-1 expression was stronger than in crypts of adjacent regular tissue. In 6 cases this expression was even markedly stronger than in lymphocytes. No correlation was found between BMI-1 expression and grading or tumor type (not shown).

Stromal cells of the gastrointestinal tract

As described previously (Raaphorst et al., 2000) lymphocytes expressed BMI-1 strongly, and served as a positive control. Whereas endothelial cells were mostly negative or weakly positive for BMI-1, expression intensity of stromal spindle cells with the morphology of either fibroblasts or myofibroblasts was more heterogenous and ranged from negative to infrequently moderately positive (not shown). By contrast, smooth muscle cells of both vessels, lamina mucosa and muscularis propria moderately to strongly expressed BMI-1.

BMI-1 mRNA is expressed in stromal cells to a lower and in tumor cells to a higher degree

The specifity of BMI-1 immunohistochemical staining using a highly sensitive antigen retrieval method was further investigated by RT-PCR. BMI-1 mRNA was detected both in endothelial cells, arterial smooth muscle cells and three different colonic tumor cell lines, i.e. HT29, AQ and PC3. The expression levels correlated well with immunohistochemical results, i. e. endothelial cells contained less mRNA than smooth muscle cells, whereas colonic tumor cells contained the highest levels of BMI-1 mRNA (Fig. 3).

Discussion

The maintenance of stem cell compartments is critical for the function of the gastrointestinal tract. Due to the lack of specific molecular markers, these stem cells are still to be identified (Okamoto and Watanabe, 2004). Recent studies have shown that BMI-1 was indispensable for the self renewal of hematopoietic stem cells (Lessard and Sauvageau, 2003) and of stem cells in the central and peripheral nervous system (Molofsky et al., 2003). Therefore, we herein evaluated whether BMI-1 expression may indicate stem cell properties in the gastrointestinal tract.

We show that BMI-1 is constitutively expressed in both epithelial cells and the soft tissue of the human gastrointestinal tract. In the human stomach, weak BMI-1 expression was found at the pit-gland transition where progenitor cells of the gastric tract give rise to all other gastric epithelial cells in a bidirectional way. In the small intestine and the colon proliferating cells are located at the base of the crypts. These cells also stained distinctly for BMI-1, indicating that stem cell compartments of the gastrointestinal tract are BMI-1 positive. In contrast, in locations where cells are shed toward the lumen, such as near the villus tips of the small intestine, at the luminal end of the crypts and in the intercrypt tables of the large bowel, epithelia were mostly negative for BMI-1, showing that BMI-1 reactivity reflects the proliferative potential of intestinal epithelia to a certain extent.

In addition to these BMI-1 positive cells of the proliferative units, several types of differentiated cells were also BMI-1 positive. Whereas BMI-1 staining decreased only inconspicuously toward the apical pit regions of the corpus mucosa, this decrease was more distinct but still variable in the pyloric mucosa.

Furthermore, a moderate to strong staining was detected in a variety of specialized cell types such as parietal cells, neuroendocrine cells of the pylorus, Paneth cells and a subset of goblet cells. The expression intensity in these cells was partly distinctly stronger than in regions comprising gastrointestinal stem cells.

This finding is reminiscent of germinal centers in which proliferating cells were described to be BMI-1 negative in contrast to BMI-1 positive mantle zone cells (Raaphorst et al., 2000). For that reason it has been suggested that BMI-1 may contribute to differentiation of a variety of cell types by stabilization of cell type-specific gene expression (Raaphorst et al., 2000, 2001). Correspondingly, the BMI-1 expression profiles in the gastrointestinal tract also point to a role in the development of several epithelial cell types and therefore BMI-1 is not only a stem cell marker but can also be regarded as a differentiation marker of a variety of cell types within adult tissues. This interpretation is in accordance with the finding that other differentiated cells lacking stem cell features, such as smooth muscle cells also strongly express BMI-1. Thus BMI-1 expression can hardly be used to exactly demonstrate the distribution of stem and early progenitor cells in the gastrointestinal tract.

In our study, no change in BMI-1 expression was found in inflammatory conditions, suggesting that expression levels were independent of the growth state in normal tissue, which is in accordance with previous results (Itahana et al., 2003). However, similar to non-small cell lung cancer (Vonlanthen et al., 2001) and colon cancer (Kim et al., 2004) the majority of gastrointestinal carcinomas were strongly positive for BMI-1. Accordingly, carcinogenesis of the complete gastrointestinal tract is associated with upregulation of BMI-1 protein, further confirming the role of BMI-1 as an oncogenic protein (Valk-Lingbeek et al., 2004; Glinsky et al., 2005). Therefore BMI-1 expression apparently has a different significance in normal and malignant tissue.

There are few immunohistochemical investigations of BMI-1 expression in normal tissue. Our results are in contrast to previous reports in which smooth muscle cells (Vonlanthen et al., 2001) and colonic epithelial cells (Kim et al., 2004) were regularly negative for BMI-1. Since the specificity of our results could be confirmed by RT-PCR of human endothelial, smooth muscle and gastrointestinal tumor cells, these differences are most probably due to a more sensitive method of antigen retrieval. In addition, a distinct expression of BMI-1 protein has also been detected in bronchiolar epithelial cells (Breuer et al., 2004).

In summary, we show that BMI-1 expression is not specifically restricted to stem cells in the gastrointestinal tract. Moreover, our data indicate roles for BMI-1 not only in maintaining stem cell populations, but also in cellular differentiation of a variety of cell types, possibly by stabilization of cell type-specific gene expression and irreversibility of lineage choice (Raaphorst et al., 2001; Mahmoudi and Verrijzer, 2001). However, strong upregulation of BMI-1 in the majority of malignant gastrointestinal carcinomas corroborates its role in malignant tumor formation.

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