Tumorigenesis and Neoplastic Progression

The Loss of Sarco/Endoplasmic Reticulum Calcium Transport ATPase 3 Expression Is an Early Event during the Multistep Process of Colon Carcinogenesis

Jean-Philippe Brouland,* Pascal Gélébart,[†]
Tünde Kovács,[‡] Jocelyne Enouf,[†]
Johannes Grossmann,[§] and Béla Papp^{†¶}

From the Service d'Anatomie et Cytologie Pathologiques* et
Inserm U 348,[†] Hôpital Lariboisière, Paris, France; the National
Medical Center,[‡] Institute of Haematology and Immunology,
Budapest, Hungary; the Klinik und Poliklinik für Innere Medizin
I,[§] Klinikum Universität Regensburg, Regensburg, Germany; and
the Inserm U-718/EMI-00-03 Laboratoire de Biologie Cellulaire
Hématopoïétique,[¶] Institut Universitaire d'Hématologie, Hôpital
Saint-Louis, Paris, France

Calcium accumulation in the endoplasmic reticulum is accomplished by sarco/endoplasmic reticulum calcium transport ATPases (SERCA enzymes). To better characterize the role of SERCA3 in colon carcinogenesis, its expression has been investigated in colonic epithelium, benign lesions, adenomas, and adenocarcinomas. In addition, the regulation of SERCA3 expression was analyzed in the context of the adenomatous polyposis coli/ β -catenin/T-cell factor 4 (TCF4) pathway and of specificity protein 1 (Sp1)-like factor-dependent transcription. We report that SERCA3 expression increased along the crypts as cells differentiated in normal colonic mucosa and in hyperplastic polyps, was moderately and heterogeneously expressed in colonic adenomas with expression levels inversely correlated with the degree of dysplasia, was barely detectable in well and moderately differentiated adenocarcinomas, and was absent in poorly differentiated tumors. Inhibition of Sp1-like factor-dependent transcription blocked SERCA3 expression during cell differentiation, and SERCA3 expression was induced by the expression of dominant-negative TCF4 in colon cancer cells. These data link SERCA3 expression to the state of differentiation of colonic epithelial cells, and relate SERCA3 expression, already decreased in adenomas, to enhanced adenomatous polyposis coli/ β -catenin/TCF4-dependent signaling and

deficient Sp1-like factor-dependent transcription. In conclusion, intracellular calcium homeostasis becomes progressively anomalous during colon carcinogenesis as reflected by deficient SERCA3 expression. (Am J Pathol 2005, 167:233–242)

Calcium homeostasis of the endoplasmic reticulum (ER) is involved in several essential cell functions. Calcium stored in the ER is required for chaperone-assisted maturation of newly synthesized proteins transiting through the organelle.^{1–3} Moreover, second messenger-induced calcium release from the ER through inositol-*tris*-phosphate- and ryanodine receptor-type calcium channels constitutes an integral part of many intracellular signal transduction pathways and networks.^{4,5} Because ER calcium homeostasis is therefore involved in many constitutive or inducible cell functions, calcium accumulation into this organelle, assured by sarco/endoplasmic reticulum calcium transport ATPase (SERCA)-type calcium pumps is essential for numerous cellular activities such as secretion, neuronal plasticity, stress responses, proliferation, differentiation, or various forms of cell death.^{6–12} Three SERCA genes are known that code by alternative splicing several isoforms, the expression of which is tissue dependent and developmentally regulated.¹³

Supported by Inserm, by the Association pour la Recherche sur le Cancer, by the Fondation de France, and by the Hungarian Academy of Sciences (OTKA T 032766 and T 046814 IB2).

Accepted for publication March 29, 2005.

Current address of P.G.: Department of Biochemistry, 3-56 Medical Sciences Building, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

Current address of J.G.: Medizinische Klinik Ev. Krankenhaus Bethesda GmbH, Ludwig-Weber-Str. 15, 41061 Mönchengladbach, Germany.

Address reprint requests to Béla Papp, Inserm U-718/EMI-00-03 Laboratoire de Biologie Cellulaire Hématopoïétique, Institut Universitaire d'Hématologie, Hôpital Saint-Louis, 1 avenue Claude Vellefaux, 75010 Paris, France. E-mail: bela.papp@chu-stlouis.fr or bela.papp@stlouis.inserm.fr.

Calcium plays an important role in the physiology of intestinal epithelium. Cytosolic free calcium concentration increases during the ontogeny of intestinal epithelium,¹⁴ and the differentiation-inducing effect of increased cytosolic calcium levels has also been observed,¹⁵ suggesting that a cross-talk may exist between calcium homeostasis and the control of epithelial differentiation. In addition, extracellular free calcium concentration modulates the proliferation^{16,17} and the differentiation¹⁵ of enterocytic cells. Extracellular calcium has also been shown to inhibit, via calcium sensing receptor-mediated signaling, the β -catenin/T-cell factor 4 (TCF4) pathway in colon cancer cells,¹⁸ and to have a preventive effect on colon tumorigenesis.^{19,20} However, data about the biochemical mechanisms of colon epithelial calcium homeostasis and its defects in tumors are scarce.

We recently observed that whereas normal colon and gastric epithelial cells express simultaneously several SERCA-type enzymes, ie, the ubiquitous SERCA2b isoform and SERCA3, the expression of SERCA3 is selectively lost in colon and stomach cancer and that SERCA3 expression is induced when these cells undergo differentiation *in vitro*.²¹ Because the lack of SERCA3 expression therefore appears to be related to the malignant phenotype in colonic epithelium, to better define the role of the loss of SERCA3 expression during the multistep process of colon carcinogenesis, in the present work, we studied SERCA3 expression in normal fetal and adult colonic epithelium; hyperplastic polyps; adenomas; and well, moderately, and poorly differentiated adenocarcinomas. To link the regulation of SERCA3 expression to molecular mechanisms of colon tumorigenesis, we investigated, by using a dominant-negative form of TCF4, the effect of the inhibition of the adenomatous polyposis coli (APC)/ β -catenin/TCF4 pathway, a major oncogenic signal transduction mechanism in the colon, on SERCA3 expression in colon carcinoma cells. Moreover, we studied the role of specificity protein 1 (Sp1)-like factor-dependent gene expression in the induction of SERCA3 expression during differentiation of colon cancer cells. In addition, to study the involvement of SERCA function in the regulation of colon cancer phenotype, we investigated the effect of pharmacological SERCA inhibition on carcinoembryonic antigen synthesis in colon cancer cells.

Materials and Methods

Sampling

Samples of normal colon mucosa, colon adenomas, adenocarcinomas, and hyperplastic polyps were collected on surgical colon resection specimens after routine examination. Samples (27 adenocarcinomas, 8 adenomas, and 6 sporadic hyperplastic polyps) were snap-frozen in liquid nitrogen-cooled isopentane and stored until use at -80°C . Tumor type was confirmed by the histological analysis of formalin-fixed, paraffin-embedded, and cryosectioned samples.

Immunohistochemistry

Immunohistochemical staining for SERCA3 was performed using the PLIM430 mouse monoclonal antibody as previously described.²¹ Briefly, 5- μm -thick frozen tissue sections were dried on air, fixed in acetone for 15 minutes, dried, and stored at -80°C until use. After rehydration in Tris-buffered saline (150 mmol/L NaCl and 10 mmol/L Tris, pH 7.4) containing 0.1% Tween-20 (Tris-buffered saline (TBS)/Tween), nonspecific protein fixation was inhibited by incubation in TBS/Tween supplemented with 5% nonfat dry milk (TBS/Tween/milk) for 30 minutes. Purified PLIM430 antibody was thereafter applied at 1 $\mu\text{g}/\text{ml}$ concentration in TBS/Tween/milk for 90 minutes at room temperature in a humidified chamber. After three rinses with distilled water, slides were incubated for 10 minutes with TBS/Tween/milk. After a second wash by distilled water and TBS/Tween/milk, slides were incubated with biotinylated anti-mouse IgG antibody (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) for 1 hour, rinsed, and incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit) for 45 minutes according to the instructions of the manufacturer. Signal was revealed with 3,3'-diaminobenzidine, and slides were counterstained with hematoxylin. Omission of primary antibody and replacement by isotype-matched irrelevant antibody were used as negative controls and gave no staining. In addition, staining of vascular endothelial cells and lymphocytes (cell types known to express SERCA3^{22,23}) by PLIM430 was used as internal positive control.

Cell Culture and Treatments

The KATO-III gastric carcinoma, as well as the DLD-1, COLO-205, and Caco-2 colon carcinoma cell lines were obtained from American Type Culture Collection (Manassas, VA) and were cultured as previously described.²¹ Cells were seeded at 2 to 4×10^4 cells/ cm^2 in 20-cm^2 cell culture Petri dishes as described previously.²¹ Postconfluent Caco-2 cultures were re-fed by renewal of medium with or without SERCA inhibitors every 2 days. LsL8 cells have been obtained²⁴ by stably transfecting Ls174T colon carcinoma cells that carry mutated β -catenin with the tetracycline repressor and with dominant-negative TCF4 within the context of the T-REx system developed by Invitrogen for the inducible expression of cDNAs in eukaryotic cells. In these cells, doxycycline treatment leads to the expression of dominant-negative TCF4. As negative control, cells transfected with the tetracycline repressor alone (LsTR4 cells) were used. The establishment and the characterization of these cells have been published previously.²⁴

Mithramycin-A and the SERCA inhibitors thapsigargin, cyclopiazonic acid, and 2,5-di-*tert*-butyl-1,4-benzohydroquinone (tBHQ) were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). The drugs were dissolved in dimethylsulfoxide and added to the cells from concentrated stock solutions kept at -20°C . To avoid oxidation, tBHQ solutions were kept in the vapor phase of liquid nitrogen in small aliquots and these were used only

once. The concentration of dimethylsulfoxide vehicle in cell cultures was less than 0.1% was included in control experiments and did not interfere with the assays.

After treatments, cells were harvested, and Western blot analysis was carried out as described previously.²¹ Briefly, cells were washed with ice-cold 150 mmol/L NaCl and precipitated with cold 5% trichloroacetic acid. The obtained total cellular protein precipitate was pelleted by centrifugation, and the amount of precipitated protein was determined. The volume of modified Laemmli-type sample buffer (90 mmol/L Tris, 5 mmol/L EDTA, 100 mmol/L dithiothreitol, 2 mol/L urea, 2% SDS, 0.02% bromophenol blue, and 10% glycerol, pH 7.89, supplemented prior use with 1 mmol/L phenylmethylsulfonyl fluoride) in which precipitated protein was dissolved was adjusted to obtain lysates of 3 mg/ml protein concentration. Electrophoresis (100 μ g protein/well) followed by transfer onto nitrocellulose was performed as described previously.²¹ The presence of equal amounts of total protein per lane was controlled by Ponceau S staining of blots and densitometry as described previously.²¹ SERCA2, SERCA3, and carcinoembryonic antigen expression levels were detected by Western blotting with appropriate antibodies, and signal intensity was evaluated by scanning luminograms of the Western blots as described in detail previously.²¹ Data shown in this work are representative of at least three independent experiments.

Results

SERCA3 Expression in Colonic Epithelium and Colon Tumors

In normal colon mucosa, (Figure 1B), surface epithelium and crypt cells displayed a marked staining for SERCA3. In goblet cells, the labeling was strong and predominantly located at the basal area of the cell and surrounding mucus vacuoles. In cylindrical absorptive cells, the labeling was more diffuse in the cytoplasm. Along the crypts, the intensity of staining increased gradually, and the strongest staining was observed at the surface, whereas the deeper one-third of the crypt was labeled somewhat more weakly. In fetal colonic mucosa (17 and 20 weeks; Figure 1C), the labeling was of the same type as in adult tissue, albeit with a less pronounced gradient of staining intensity along the crypts.

In hyperplastic polyps (Figure 1D), SERCA3 labeling was similar to that seen in normal mucosa. However, the gradient of staining intensity along the hyperplastic crypts was more pronounced than in normal crypts. Thus, at the base of hyperplastic crypts, which corresponded to approximately one-fifth of their full length, only a faint staining could be observed, whereas more distal, mature cells were strongly labeled.

In adenomas (Figure 1E), the intensity of SERCA3 labeling was globally lower than in normal colonic mucosa. Moreover, heterogeneity of labeling could be observed between different lesions and also between different fields within the same lesion. Staining was rather pre-

served in adenomatous structures (ie, glands and villousities) that exhibited low-grade dysplasia (characterized by well-maintained glandular architecture, regular or minimally enlarged nuclei with inconspicuous nucleoli, nuclear location in the lower one-half of the epithelium, very rare mitoses, and persistence of cytoplasmic mucus vacuoles), whereas labeling was decreased in adenomas with moderate to high grade dysplasia (characterized by enlarged hyperchromatic nuclei, nuclear location in the upper one-half of the epithelium, prominent nucleoli, loss of mucosecretion, and increased nuclear-to-cytoplasmic ratio).

In adenocarcinomas (Figure 1F), the cytoplasmic labeling was globally null or very weak in the neoplastic cells. The variations of staining intensity were dependent on tumor differentiation. In well-differentiated areas of adenocarcinomas, a very weak labeling could be seen, whereas in moderately and poorly differentiated carcinomas, staining was barely detectable or absent. In some moderately differentiated cases, staining was heterogeneous with very faint labeling alternating with areas displaying no labeling, and this staining pattern correlated with the state of differentiation of the various regions of the tumor as judged by morphological criteria. Normal endothelial cells and lymphocytes present in the specimens (Figure 1A), as well as regions containing residual normal mucosa (Figure 1F) exhibited easily discernible labeling for SERCA3 identical to that seen in normal tissue.

Induction of SERCA3 Expression by Dominant-Negative TCF4

To explore the involvement of the APC/ β -catenin/TCF4-dependent signaling pathway in the control of SERCA3 expression, the effect of the expression of a dominant-negative form of the TCF4 transcription factor on SERCA3 protein levels was investigated in the Ls174T mucus-secreting colon cancer cell line as outlined in Materials and Methods. Oncogenic mutations in APC or β -catenin inhibit the degradation of β -catenin by the proteasome and are involved in the formation of a majority of colon carcinomas. These mutations allow the accumulation in the cell of β -catenin, which will complex with the TCF4 transcription factor leading to aberrant gene expression and tumorigenesis. The expression of a dominant-negative TCF4 transgene disrupts the APC/ β -catenin/TCF4 oncogenic pathway by blocking the function of TCF4 in colon carcinoma cells. Expression of dominant-negative TCF4 in colon cancer cells therefore permits the investigation of the role of the APC/ β -catenin/TCF4 pathway in the control of the cellular phenotype in a specific manner.

Cells stably transfected with the tetracycline repressor only (LsTR4 cells) or with a vector coding for a dominant-negative version of TCF4 (LsL8 cells) were treated with doxycycline or vehicle control, and SERCA3 expression was determined by Western immunoblotting using the SERCA3-specific PLIM430 monoclonal antibody. In addition, as a positive control, LsL8 cells and the KATO-III cell line were treated with butyrate in the absence of

Butyrate	-	+	-	-	-	-	+
Doxicycline	-	-	-	+	-	+	-
SERCA3							
	KATO-III	LsTR4	LsL8	LsL8			

Figure 2. Induction of SERCA3 expression in colon cancer cells by dominant-negative TCF4. Ls174T cells transfected with repressor only (LsTR4 cells) or a doxycycline-inducible vector coding for dominant-negative TCF4 (LsL8 cells) were grown in the absence or presence of 2 μ mol/L doxycycline for 3 days. After loading equal amounts of total cellular protein on SDS-polyacrylamide gels, electrophoresis, and transfer, SERCA3 expression was determined by Western immunoblotting using the SERCA3-specific PLIM430 monoclonal antibody. In parallel, as positive controls for SERCA3 induction, LsL8 cells and KATO-III cells were treated with 2 mmol/L butyrate for the same period. Expression of dominant-negative TCF4 leads to SERCA3 expression.

doxycycline. As shown in Figure 2, expression of dominant-negative TCF4 resulted in the induction of SERCA3 protein expression in these cells (LsL8). Induction of SERCA3 expression could not be observed in cells devoid of dominant-negative TCF4 (LsTR4 cells) or in cells carrying an inducible vector coding for p21^{CIP/WAF} (not shown).

Inhibition of SERCA3 Expression by Mithramycin-A

By binding to GC-rich DNA sequences, the antibiotic mithramycin-A inhibits Sp1-like factor-dependent gene expression.^{25–27} Because Sp1-like factors are involved in the expression of SERCA3 in normal vascular endothelial cells,²² the effect of mithramycin-A on short chain fatty-acid-induced SERCA3 expression in colon and gastric cancer cell lines was studied by Western blotting. As shown in Figure 3A, the induction of SERCA3 expression in the DLD-1 colon cancer cell line by sodium valerate could be inhibited by mithramycin-A in a dose-dependent manner in the submicromolar concentration range.

Figure 1. Immunohistochemical detection of SERCA3 protein in normal adult and fetal colonic epithelium and in colon tumors. **A:** Internal positive controls for PLIM430 monoclonal antibody labeling. Endothelial cells (1) and lymphocytes (2) exhibit a strong cytoplasmic staining. **B:** PLIM430 labeling of normal adult colonic mucosa. At low magnification (1), epithelial cells exhibit a strong labeling with a progressive increase along the crypt toward the upper part (2) from the deeper part (3). **C:** PLIM430 labeling of normal fetal colonic mucosa. At low magnification, staining of epithelial cells is strong (1) and located at the basal area of cells (3). The gradient of labeling along the crypts is less pronounced than in normal adult colonic mucosa (2). **D:** PLIM430 labeling in hyperplastic polyps. At low magnification, crypts are elongated, tortuous, and covered by strongly labeled epithelial cells (1). The gradient of labeling along the crypts is more pronounced than in normal adult colonic mucosa, toward the upper part (2) from the deeper part (3). **E:** PLIM430 labeling of adenomas. At low magnification (1 and 4), the intensity of adenoma staining is globally lower than in normal colonic mucosa. Between different fields, labeling is heterogeneous (2, 3, 5, and 6). In adenomatous structures with low grade dysplasia, epithelial staining is more preserved than in adenomatous structures with higher grade dysplasia (arrows). These regions are generally located in the upper part of adenomas (5). **F:** PLIM430 labeling of adenocarcinomas. Whereas adjacent normal epithelium is strongly labeled, the staining of adenocarcinomatous tissue is very weak or null (1 to 3). The weak variations of labeling of the neoplastic cells are related to the differentiation of the tumor. In well-differentiated adenocarcinoma, cells are weakly labeled (4 and 5), whereas in poorly differentiated adenocarcinoma, tumor cells do not show any staining (9). In moderately differentiated cases, labeling is more heterogeneous (6) with alternating areas of weak (7) and null (8) staining according to local differentiation of tumor cells.

A : DLD-1

B : KATO III

C : COLO-205

Figure 3. Inhibition of SERCA3 expression by mithramycin-A. **A:** Inhibition of valerate-induced SERCA3 expression by various concentrations of mithramycin-A: DLD-1 colon cancer cells were treated with valerate in the absence or the presence of various concentrations of mithramycin-A for 2 days, and SERCA3 protein was quantitated by Western blotting. Untreated cells expressed undetectable levels of SERCA3 protein. Induction of SERCA3 expression was obtained by treatment with valerate, and valerate-induced SERCA3 expression was inhibited by mithramycin-A in a dose-dependent fashion in the high nanomolar concentration range. SERCA2 expression was not significantly affected by the treatments. **B:** Inhibition by mithramycin-A of SERCA3 expression induced by various concentrations of butyrate: KATO-III cells were treated for 2 days with various concentrations of butyrate in the absence or in the presence of 10 μ M/L mithramycin-A, and SERCA2 as well as SERCA3 protein levels were determined. Butyrate-induced SERCA3 expression was abolished by mithramycin-A. **C:** Inhibition of constitutive SERCA3 expression in COLO-205 cells by various concentrations of mithramycin-A: COLO-205 colon carcinoma cells were treated for 3 days with vehicle or various concentrations of mithramycin-A, and SERCA2 as well as SERCA3 expression was determined. Although mithramycin-A did not affect SERCA2 expression, SERCA3 expression was inhibited by the drug in a concentration-dependent manner.

Inhibition was specific, because mithramycin-A did not inhibit the expression of SERCA2 in the cells. Inhibition of sodium butyrate-induced SERCA3 expression by mithramycin-A could be observed in the KATO-III gastric carcinoma cell line as well (Figure 3B). In addition, expression of SERCA3 in COLO-205 cells, which express low levels of this enzyme constitutively, could also be dose-dependently inhibited by mithramycin-A (Figure 3C).

Induction of Carcinoembryonic Antigen Expression by SERCA Inhibitors

When cultured postconfluently, Caco-2 colon carcinoma cells differentiate spontaneously toward an absorptive

A

B

Figure 4. Induction of carcinoembryonic antigen expression in Caco-2 cells by SERCA inhibitors. Caco-2 cells at day 1 postconfluence were treated for 7 days with vehicle or with 10 μ M/L tBHQ, 6 μ M/L cyclopiazonic acid (CPA), or 0.125 nmol/L thapsigargin (Tg), and cellular carcinoembryonic antigen was detected by Western blotting. Carcinoembryonic antigen levels were increased by all three structurally unrelated SERCA inhibitors when compared with untreated cells.

enterocytic phenotype, reflected by the apparition of morphological (brush border membrane), functional (transcellular electrolyte transport, growth arrest), and immunophenotypic markers, including, among others, carcinoembryonic antigen (CEA) expression.²¹ Because CEA is a well-established marker of differentiation for Caco-2 cells, to study the involvement of cellular calcium homeostasis in the differentiation process of colon cancer cells, we investigated the expression of this marker by Western blotting in early postconfluent cultures of Caco-2 cells in the absence and in the presence of specific SERCA inhibitors. As shown in Figure 4, tBHQ, cyclopiazonic acid, and thapsigargin, three structurally unrelated SERCA inhibitors, enhanced cellular CEA production when compared with untreated cells, indicating that diminished calcium accumulation in the ER leads to phenotypic changes suggestive of enhanced differentiation.

Discussion

Colon carcinoma is one of the most frequent types of human malignancies, representing about 8.5% of new cancers. Benign colon tumors are the most frequently

adenomas. Because colon carcinogenesis proceeds through the sequential accumulation of genetic lesions that activate oncogenes or inactivate tumor suppressor genes,^{28–30} adenomas are precancerous lesions, which, by the acquisition of further mutations, can acquire a fully malignant phenotype.

Adenomas form sessile or pedunculated polyps and are histologically characterized by various degrees of glandular or villous complexity, hypercellularity with enlarged, hyperchromatic nuclei, various degrees of nuclear stratification and loss of polarity. According to the importance of epithelial modifications, intraepithelial neoplasia can be classified as low grade or high grade (dysplasia). Adenoma formation is most frequently due to the constitutive activation of the APC/ β -catenin/TCF4 transcriptional regulatory pathway, which initiates the proliferation of dysplastic epithelium to the luminal surface,³¹ whereas in normal epithelium, proliferation is confined to the base of the crypt. Polyps thus appear to grow as a consequence of accelerated crypt fission and maintained proliferation.³² The expression of endoplasmic reticulum-associated calcium transport ATPases of the SERCA3 type is lost or markedly decreased in fully malignant colon cancer cells.²¹ To locate and quantify the defect of SERCA3 expression along the multistep process of colon carcinogenesis, in the present study, we compared the expression of SERCA3 in low-grade and high-grade intraepithelial neoplasia (dysplastic lesions) with that of normal adult and fetal mucosa, adenocarcinoma of various degrees of differentiation, and hyperplastic lesions. We show that SERCA3 expression is modulated along normal colonic crypts with lower expression in the less differentiated, regenerative, more deeply located parts of the crypts. In colon adenocarcinomas, SERCA3 expression is markedly decreased in well-differentiated lesions and immunohistochemically undetectable in poorly differentiated adenocarcinomas. In intraepithelial neoplasia, SERCA3 expression is globally decreased when compared with normal mucosa, and the decrease of SERCA3 expression is more pronounced as the degree of dysplasia (loss of differentiation) increases. These observations taken together indicate for the first time, that decreased SERCA3 expression is an early event in the multistep process of colon carcinogenesis and that its degree follows the sequence of loss of cell differentiation that occurs along the low-grade and high-grade intraepithelial neoplasia and well, moderately, and poorly differentiated adenocarcinoma sequence.

Sporadic hyperplastic polyps are non-neoplastic lesions of the colon, the distinction of which from adenomas can sometimes be difficult. Hyperplastic polyps are morphologically characterized by elongated and serrated crypts lined on the luminal surface, as well as the upper part of the mucosa, by infolded epithelial tufts, enlarged goblet cells, and a proliferative basal epithelium. Unlike adenomas, sporadic hyperplastic polyps are considered to have no significant malignant potential.^{33,34} However, the histogenesis of hyperplastic polyps is unclear. They are thought to arise as a result of aberrant differentiation of epithelial cells of the crypt with hypermaturation of surface epithelium³⁵ resulting from

decreased apoptosis in the upper part of the crypts.³⁶ Although clonality, ras mutations, and *Bcl-2* overexpression have been detected,³⁷ the APC/ β -catenin pathway is not involved, and sporadic hyperplastic polyps are not considered to be premalignant lesions. The high expression of SERCA3 in hyperplastic polyps observed in this work is in accordance with the notion of hypermaturation taking place in these lesions and further confirms that the physiopathogeny of these lesions and their phenotype differs from those of the classical adenoma/adenocarcinoma sequence. The distinct expression pattern of SERCA3 expression of sporadic hyperplastic polyps (ie, similar to that of normal epithelium) when compared with that of adenomas (decreased SERCA3 expression) observed in this work also indicates that the intracellular calcium homeostasis of these two types of lesions is different. This may contribute to the distinct biological behavior of these lesions. In addition, our work indicates that the immunohistochemical analysis of SERCA3 expression may be useful for the better distinction of these lesions by the pathologist.

Because no data are currently available regarding the molecular mechanisms or their defects involved in the regulation of SERCA3 expression in colon cancer, we explored the involvement of Sp1-like factor-dependent transcription and of the APC/ β -catenin/TCF4 pathway in this process.

The antibiotic mithramycin-A intercalates into GC-rich DNA sequences.^{25–27} Because Sp1-like factor-binding DNA sequences, found in many TATA-less promoters are GC-rich, mithramycin-A is widely used as an inhibitor for the study of the implication of Sp1-like transcription factors in gene expression. Mithramycin-A has been shown to inhibit Sp1-like factor-dependent expression of several genes, such as C-MYC, C-HA-RAS, MUC-2, MDR1, and others.^{38–49} The TATA-less human and mouse SERCA3 promoters contain several Sp1-binding sequences, which have been shown, along with ETS-binding sites, to direct basal SERCA3 expression in mouse vascular endothelial cells.^{22,50} The inhibition by mithramycin-A of the constitutive residual expression of SERCA3 in COLO-205 cells, which is quantitatively similar to that observed in well-differentiated colon carcinoma tissue, suggests that Sp1-like factor-dependent transcription drives SERCA3 expression observed also in well differentiated tumors. Importantly, short chain fatty acid (ie, butyrate or valerate)-induced expression of SERCA3 in cells that initially express undetectable amounts of SERCA3 protein was also inhibited in a dose-dependent manner by mithramycin-A. This indicates that in these cells, butyrate, a physiological differentiation-inducing agent in the colon, acts on SERCA3 expression via mechanisms that involve Sp1-like factor-dependent transcription, deficient in colon carcinomas. Variations of mithramycin concentrations required for inhibition observed in COLO-205 versus DLD-1 cells may reflect cell type-specific differences such as drug penetration or efflux. In addition, a constitutive, fully established, and operational Sp1-like factor-dependent transcriptional machinery (such as in COLO-205 cells) may be inhibited less efficiently by mithramycin than one that builds up *de novo*, such as in valerate-treated DLD-1

cells, for example, for reasons related to target accessibility. However, this requires further investigation.

Regulation of gene expression by Sp1-like factors is complex. This family comprises more than 20 members, including Sp1, Sp2, Sp3, Sp4, and various Krüppel-like factors including the gut-enriched Krüppel-like factor, GKLF.⁵¹ Sp1-like factors are involved in epithelial differentiation in the colon,^{52–54} and their expression has been shown to be perturbed in dysplastic lesions and carcinoma.^{53,55} Sp1-like factors possess distinct transcriptional activities and may associate with other transcription factors and histone modifying enzymes.^{51,56} Their activity may be modulated by enzymatic modifications including acetylation and has been shown to be altered by histone deacetylase inhibitors such as butyrate.^{56–66} Because of the complex and combinatorial nature of the interactions that take place between various transcription factors and because of the intricate mechanisms that regulate chromatin remodelling including histone acetylation/deacetylation, the elucidation of the precise molecular mechanisms that governs Sp1-like factor-dependent expression of SERCA3 requires further work. However, our data indicate, for the first time, that Sp1-like factor-dependent transcriptional mechanisms are involved in the control of SERCA3 expression in colon carcinoma.

The APC/ β -catenin/TCF4 signaling pathway plays an important role in colon carcinogenesis. Whereas in normal cells, APC targets β -catenin to proteasome-dependent degradation, oncogenic mutations in either partner lead to impaired β -catenin degradation. β -Catenin thus accumulated in the cell complexes with the TCF4 transcription factor, leading to aberrantly enhanced expression of TCF4 target genes.⁶⁷ The β -catenin/TCF4 signaling complex is considered a key switch that induces proliferation and blocks growth arrest and differentiation of colon tumors. Its constitutive activation is thought to be responsible for the undifferentiated “progenitor-like” phenotype of colon cancer cells,²⁴ and the inhibition of the APC/ β -catenin/TCF4 pathway by the expression of dominant-negative TCF4 has been reported to be accompanied by growth inhibition and differentiation of colon cancer cells.²⁴ Induction of SERCA3 protein expression following expression of a dominant-negative form of TCF4 suggests that decreased SERCA3 expression observed in this work in adenomatous polyps may be due to hyperactive β -catenin signaling, because in these benign lesions, oncogenic mutations principally target this pathway, on which mutations in other oncogenes are added during transition to carcinoma. In our hands, induction of SERCA3 expression by dominant-negative TCF4 was, however, moderate when compared with that obtained by butyrate treatment of the same cells or with SERCA3 levels observed in normal primary colonic epithelium. These observations and the further decrease of SERCA3 expression during transition from adenoma to carcinoma taken together suggest that additional regulatory mechanisms may also be involved in the loss of SERCA3 expression during colon carcinogenesis. Because of the considerable complexity of transcriptional regulation by Sp1-like factors and because complex interactions may exist between GKLF-dependent gene expression and the

APC/ β -catenin pathway,⁶⁸ the identification of the exact molecular mechanisms that lead to the loss of SERCA3 expression during the multistep process of colon carcinogenesis will require further work. However, our data link, for the first time, SERCA3 expression in cancer to the β -catenin pathway and to Sp1/Krüppel-like factor-dependent transcription, which are key factors in colon carcinogenesis and differentiation, respectively. Decreased SERCA3 expression observed in adenomas indicates that anomalous SERCA3 expression is an early event during colon tumorigenesis and that the extent of the deficiency of SERCA3 expression correlates with the degree of the loss of cell differentiation in benign and malignant tumors. Therefore, SERCA3 immunostaining may constitute a useful new tool for the analysis of the phenotype of various colon tumors.

Inhibition of calcium sequestration into the ER by specific SERCA inhibitors or calcium ionophores has been shown to lead to cell differentiation/activation in several lymphoid or myeloid leukemia cell types,^{23,69–71} and the enhancement of the cell differentiation-inducing effect of retinoids by SERCA inhibitors has also been observed in myeloid leukemia cells.⁷² In addition, SERCA3 expression is induced during the differentiation of several myeloid⁷³ and colon cancer cell types, including Caco-2 cells.²¹ The calcium affinity of SERCA3 is inferior to that of the ubiquitous SERCA2b isoform.^{74–77} Therefore, the replacement of SERCA2b by SERCA3 during differentiation may lead to decreased intra-ER calcium content and higher resting cytosolic calcium levels²¹ as well as to more pronounced cytosolic calcium signals on cell stimulation. The pharmacological inhibition of SERCA activity with thapsigargin and other SERCA inhibitors may lead to an analogous situation and thus mimic the effects of the up-regulation of SERCA3 expression. In addition, it has been proposed previously⁷⁸ that in some cell types, SERCA3 may be specifically associated with the IP3-mobilisable part of the ER calcium pool involved in signaling, whereas SERCA2 may pump calcium into IP3-insensitive ER subcompartments involved in homeostatic/constitutive ER functions such as protein neo-synthesis and maturation. These observations taken together allowed us to postulate that a SERCA isoform-specific cross-talk exists between cellular calcium homeostasis and various signaling pathways involved in the control of cell differentiation and that the modulation of the expression of various SERCA isoenzymes, which possess distinct calcium transport characteristics, contributes to the fine tuning of ER calcium homeostasis, required for normal differentiation and the function of the mature cell.⁷⁹ When the spontaneous differentiation of postconfluent Caco-2 cells was conducted in the presence of inhibitors of SERCA-dependent calcium uptake into the ER, the expression of carcinoembryonic antigen, a widely used marker of differentiation of this cell type, was markedly increased. This observation suggests that, similarly to leukemia, ER calcium homeostasis and, in particular, SERCA3 expression may be involved also in the control of the phenotype of colon cancer cells.

SERCA-dependent calcium sequestration may be involved in the control of the phenotype of other tissue and

tumor types as well. For example, in rat thyroid tissue in which only SERCA2 was detected, the amount and enzymatic activity of this isoenzyme has been shown to be decreased in highly malignant cells when compared with normal tissue and non-tumorigenic cell lines.⁸⁰

Taken together, our work indicates for the first time that the ER calcium homeostasis of colon tumors becomes anomalous already at an early, premalignant stage (ie, in adenomas), shows that this defect further deepens during transition to carcinoma, and identifies SERCA3 as a useful new marker for the study of the formation and phenotype of colon tumors.

Acknowledgments

We are indebted to Dr. M. van de Wetering (Hubrecht Laboratory, Center for Biomedical Genetics, Utrecht, The Netherlands) for the doxycycline-inducible cell lines used in this study and to Prof. P. Valleur (Service de Chirurgie Générale et Digestive, Hôpital Lariboisière, Paris, France) and Prof. Jacqueline Mikol, Dr. Judith Németh, Dr. Jacqueline Ferrand, and Mr. Patrice Castagnet (Service d'Anatomie et Cytologie Pathologiques, Hôpital Lariboisière, Paris, France) for support and help with obtaining specimens.

References

1. Michalak M, Mariani P, Opas M: Calreticulin, a multifunctional Ca^{2+} binding chaperone of the endoplasmic reticulum. *Biochem Cell Biol* 1998, 76:779–785
2. Michalak M, Robert Parker JM, Opas M: Ca^{2+} signaling and calcium binding chaperones of the endoplasmic reticulum. *Cell Calcium* 2002, 32:269–278
3. Molinari M, Helenius A: Chaperone selection during glycoprotein translocation into the endoplasmic reticulum. *Science* 2000, 288:331–333
4. Schulz I, Krause E: Inositol 1,4,5-trisphosphate and its co-players in the concert of Ca^{2+} signalling: new faces in the line up. *Curr Mol Med* 2004, 4:313–322
5. Patterson RL, Boehning D, Snyder SH: Inositol 1,4,5-trisphosphate receptors as signal integrators. *Annu Rev Biochem* 2004, 73:437–465
6. Fitzjohn SM, Collingridge GL: Calcium stores and synaptic plasticity. *Cell Calcium* 2002, 32:405–411
7. Brewer JW, Diehl JA: PERK mediates cell-cycle exit during the mammalian unfolded protein response. *Proc Natl Acad Sci USA* 2000, 97:12625–12630
8. Liu CY, Kaufman RJ: The unfolded protein response. *J Cell Sci* 2003, 116:1861–1862
9. Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC: Regulation of apoptosis by endoplasmic reticulum pathways. *Oncogene* 2003, 22:8608–8618
10. Rao RV, Peel A, Logvinova A, del Rio G, Hermel E, Yokota T, Goldsmith PC, Ellerby LM, Ellerby HM, Bredesen DE: Coupling endoplasmic reticulum stress to the cell death program: role of the ER chaperone GRP78. *FEBS Lett* 2002, 514:122–128
11. Mattson MP, Chan SL: Calcium orchestrates apoptosis. *Nat Cell Biol* 2003, 5:1041–1043
12. Hajnóczky G, Davies E, Madesh M: Calcium signaling and apoptosis. *Biochem Biophys Res Commun* 2003, 304:445–454
13. Wuytack F, Raeymaekers L, Missiaen L: Molecular physiology of the SERCA and SPCA pumps. *Cell Calcium* 2002, 32:279–305
14. Black BL, Rogers JO: Development of Ca^{2+} homeostasis in epithelial cells from embryonic and neonatal intestine. *Am J Physiol* 1992, 263:G371–G379
15. Lipkin M, Reddy B, Newmark H, Lamprecht SA: Dietary factors in human colorectal cancer. *Annu Rev Nutr* 1999, 19:545–586
16. Kállay E, Kifor O, Chattopadhyay N, Brown EM, Bischof MG, Peterlik M, Cross HS: Calcium-dependent c-myc proto-oncogene expression and proliferation of Caco-2 cells: a role for a luminal extracellular calcium-sensing receptor. *Biochem Biophys Res Commun* 1997, 232:80–83
17. Kansas GS, Muirhead MJ, Dailey MO: Expression of the CD11/CD18, leukocyte adhesion molecule 1, and CD44 adhesion molecules during normal myeloid and erythroid differentiation in humans. *Blood* 1990, 76:2483–2492
18. Chakrabarty S, Radjendirane V, Appelman H, Varani J: Extracellular calcium and calcium sensing receptor function in human colon carcinomas: promotion of E-cadherin expression and suppression of beta-catenin/TCF activation. *Cancer Res* 2003, 63:67–71
19. Bonithon-Kopp C, Kronborg O, Giacosa A, Rath U, Faivre J: Calcium and fibre supplementation in prevention of colorectal adenoma recurrence: a randomised intervention trial. *European Cancer Prevention Organisation Study Group. Lancet* 2000, 356:1300–1306
20. Mobarhan S: Calcium and the colon: recent findings. *Nutr Rev* 1999, 57:124–126
21. Gélébart P, Kovács T, Brouland JP, van Gorp R, Grossmann J, Rivard N, Panis Y, Martin V, Bredoux R, Enouf J, Papp B: Expression of endomembrane calcium pumps in colon and gastric cancer cells: induction of SERCA3 expression during differentiation. *J Biol Chem* 2002, 277:26310–26320
22. Hadri L, Ozog A, Soncin F, Lompre AM: Basal transcription of the mouse sarco(endo)plasmic reticulum Ca^{2+} -ATPase type 3 gene in endothelial cells is controlled by Ets-1 and Sp1. *J Biol Chem* 2002, 277:36471–36478
23. Launay S, Bobe R, Lacabartz-Porret C, Bredoux R, Kovács T, Enouf J, Papp B: Modulation of endoplasmic reticulum calcium pump expression during T lymphocyte activation. *J Biol Chem* 1997, 272:10746–10750
24. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Battle E, Coudreuse D, Haramis AP, Tjonn-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R, Clevers H: The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 2002, 111:241–250
25. Miller DM, Polansky DA, Thomas SD, Ray R, Campbell VW, Sanchez J, Koller CA: Mithramycin selectively inhibits transcription of G-C containing DNA. *Am J Med Sci* 1987, 294:388–394
26. Cons BM, Fox KR: High resolution hydroxyl radical footprinting of the binding of mithramycin and related antibiotics to DNA. *Nucleic Acids Res* 1989, 17:5447–5459
27. Carpenter ML, Cassidy SA, Fox KR: Interaction of mithramycin with isolated GC and CG sites. *J Mol Recognit* 1994, 7:189–197
28. Aaltonen LA: The multistep process of colon carcinogenesis. *Cytokines Mol Ther* 1996, 2:111–114
29. Takami K, Yana I, Kurahashi H, Nishisho I: Multistep carcinogenesis in colorectal cancers. *Southeast Asian J Trop Med Public Health* 1995, 26(Suppl 1):190–196
30. Kountouras J, Boura P, Lygidakis NJ: New concepts of molecular biology for colon carcinogenesis. *Hepatogastroenterology* 2000, 47:1291–1297
31. Jen J, Powell SM, Papadopoulos N, Smith KJ, Hamilton SR, Vogelstein B, Kinzler KW: Molecular determinants of dysplasia in colorectal lesions. *Cancer Res* 1994, 54:5523–5526
32. Araki K, Ogata T, Kobayashi M, Yatani R: A morphological study on the histogenesis of human colorectal hyperplastic polyps. *Gastroenterology* 1995, 109:1468–1474
33. Frazin G, Zamboni G, Scarpa A, Dina R, Iannucci A, Novelli P: Hyperplastic (metaplastic) polyps of the colon: a histologic and histochemical study. *Am J Surg Pathol* 1984, 8:687–698
34. Goldman H, Ming S, Hickock DF: Nature and significance of hyperplastic polyps of the human colon. *Arch Pathol* 1970, 89:349–354
35. Hayashi T, Yatani R, Apostol J, Stemmermann GN: Pathogenesis of hyperplastic polyps of the colon: a hypothesis based on ultrastructure and in vitro cell kinetics. *Gastroenterology* 1974, 66:347–356

36. Tateyama H, Li W, Takahashi E, Miura Y, Sugiura H, Eimoto T: Apoptosis index and apoptosis-related antigen expression in serrated adenoma of the colorectum: the saw-toothed structure may be related to inhibition of apoptosis. *Am J Surg Pathol* 2002, 26:249–256
37. Kikuchi Y, Dinjens WN, Bosman FT: Proliferation and apoptosis in proliferative lesions of the colon and rectum. *Virchows Arch* 1997, 431:111–117
38. Campbell VW, Davin D, Thomas S, Jones D, Roesel J, Tran-Patterson R, Mayfield CA, Rodu B, Miller DM, Hiramoto RA: The G-C specific DNA binding drug, mithramycin, selectively inhibits transcription of the C-MYC and C-HA-RAS genes in regenerating liver. *Am J Med Sci* 1994, 307:167–172
39. Aslam F, Palumbo L, Augenlicht LH, Velcich A: The Sp family of transcription factors in the regulation of the human and mouse MUC2 gene promoters. *Cancer Res* 2001, 61:570–576
40. Tagashira M, Kitagawa T, Isonishi S, Okamoto A, Ochiai K, Ohtake Y: Mithramycin represses MDR1 gene expression in vitro, modulating multidrug resistance. *Biol Pharm Bull* 2000, 23:926–929
41. Nehls MC, Brenner DA, Gruss HJ, Dierbach H, Mertelsmann R, Herrmann F: Mithramycin selectively inhibits collagen-alpha 1(I) gene expression in human fibroblast. *J Clin Invest* 1993, 92:2916–2921
42. Ryuto M, Ono M, Izumi H, Yoshida S, Weich HA, Kohno K, Kuwano M: Induction of vascular endothelial growth factor by tumor necrosis factor alpha in human glioma cells: possible roles of SP-1. *J Biol Chem* 1996, 271:28220–28228
43. Vigneswaran N, Thayaparan J, Knops J, Trent J, Potaman V, Miller DM, Zacharias W: Intra- and intermolecular triplex DNA formation in the murine c-myc proto-oncogene promoter are inhibited by mithramycin. *Biol Chem* 2001, 382:329–342
44. Arredouani A, Guioit Y, Jonas JC, Liu LH, Nenquin M, Pertusa JA, Rahier J, Rolland JF, Shull GE, Stevens M, Wuytack F, Henquin JC, Gilon P: SERCA3 ablation does not impair insulin secretion but suggests distinct roles of different sarcoendoplasmic reticulum Ca^{2+} pumps for Ca^{2+} homeostasis in pancreatic beta-cells. *Diabetes* 2002, 51:3245–3253
45. Marinovic AC, Zheng B, Mitch WE, Price SR: Ubiquitin (UbC) expression in muscle cells is increased by glucocorticoids through a mechanism involving Sp1 and MEK1. *J Biol Chem* 2002, 277:16673–16681
46. Dubbink HJ, Cleutjens KB, van der Korput HA, Trapman J, Romijn JC: An Sp1 binding site is essential for basal activity of the human prostate-specific transglutaminase gene (TGM4) promoter. *Gene* 1999, 240:261–267
47. Steiner E, Holzmann K, Pirker C, Elbling L, Micksche M, Berger W: SP-transcription factors are involved in basal MVP promoter activity and its stimulation by HDAC inhibitors. *Biochem Biophys Res Commun* 2004, 317:235–243
48. Arinze IJ, Kawai Y: Sp family of transcription factors is involved in valproic acid-induced expression of Galphai2. *J Biol Chem* 2003, 278:17785–17791
49. Kim YK, Han JW, Woo YN, Chun JK, Yoo JY, Cho EJ, Hong S, Lee HY, Lee YW, Lee HW: Expression of p21(WAF1/Cip1) through Sp1 sites by histone deacetylase inhibitor apicidin requires PI 3-kinase-PKC epsilon signaling pathway. *Oncogene* 2003, 22:6023–6031
50. Dode L, De Greef C, Moutan I, Attard M, Town MM, Casteels R, Wuytack F: Structure of the human sarco/endoplasmic reticulum Ca^{2+} -ATPase 3 gene: promoter analysis and alternative splicing of the SERCA3 pre-mRNA. *J Biol Chem* 1998, 273:13982–13994
51. Black AR, Black JD, Azizkhan-Clifford J: Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer. *J Cell Physiol* 2001, 188:143–160
52. Shie JL, Chen ZY, Fu M, Pestell RG, Tseng CC: Gut-enriched Kruppel-like factor represses cyclin D1 promoter activity through Sp1 motif. *Nucleic Acids Res* 2000, 28:2969–2976
53. Shie JL, Chen ZY, O'Brien MJ, Pestell RG, Lee ME, Tseng CC: Role of gut-enriched Kruppel-like factor in colonic cell growth and differentiation. *Am J Physiol Gastrointest Liver Physiol* 2000, 279:G806–G814
54. Chen X, Whitney EM, Gao SY, Yang VW: Transcriptional profiling of Kruppel-like factor 4 reveals a function in cell cycle regulation and epithelial differentiation. *J Mol Biol* 2003, 326:665–677
55. Dang DT, Bachman KE, Mahatan CS, Dang LH, Giardiello FM, Yang VW: Decreased expression of the gut-enriched Kruppel-like factor gene in intestinal adenomas of multiple intestinal neoplasia mice and in colonic adenomas of familial adenomatous polyposis patients. *FEBS Lett* 2000, 476:203–207
56. Xiao H, Hasegawa T, Isobe K: p300 collaborates with Sp1 and Sp3 in p21(waf1/cip1) promoter activation induced by histone deacetylase inhibitor. *J Biol Chem* 2000, 275:1371–1376
57. Nakano K, Mizuno T, Sowa Y, Orita T, Yoshino T, Okuyama Y, Fujita T, Ohtani-Fujita N, Matsukawa Y, Tokino T, Yamagishi H, Oka T, Nomura H, Sakai T: Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. *J Biol Chem* 1997, 272:22199–22206
58. Mikkelsen IM, Huseby NE, Visvikis A, Moens U: Activation of the gamma-glutamyltransferase promoter 2 in the rat colon carcinoma cell line CC531 by histone deacetylase inhibitors is mediated through the Sp1 binding motif. *Biochem Pharmacol* 2002, 64:307–315
59. Zhang W, Bieker JJ: Acetylation and modulation of erythroid Kruppel-like factor (EKLf) activity by interaction with histone acetyltransferases. *Proc Natl Acad Sci USA* 1998, 95:9855–9860
60. Ammanamanchi S, Freeman JW, Brattain MG: Acetylated sp3 is a transcriptional activator. *J Biol Chem* 2003, 278:35775–35780
61. Yang J, Kawai Y, Hanson RW, Arinze IJ: Sodium butyrate induces transcription from the G alpha(i2) gene promoter through multiple Sp1 sites in the promoter and by activating the MEK-ERK signal transduction pathway. *J Biol Chem* 2001, 276:25742–25752
62. Sowa Y, Orita T, Minamikawa S, Nakano K, Mizuno T, Nomura H, Sakai T: Histone deacetylase inhibitor activates the WAF1/Cip1 gene promoter through the Sp1 sites. *Biochem Biophys Res Commun* 1997, 241:142–150
63. Sowa Y, Orita T, Hiranabe-Minamikawa S, Nakano K, Mizuno T, Nomura H, Sakai T: Histone deacetylase inhibitor activates the p21/WAF1/Cip1 gene promoter through the Sp1 sites. *Ann NY Acad Sci* 1999, 886:195–199
64. Sowa Y, Orita T, Minamikawa-Hiranabe S, Mizuno T, Nomura H, Sakai T: Sp3, but not Sp1, mediates the transcriptional activation of the p21/WAF1/Cip1 gene promoter by histone deacetylase inhibitor. *Cancer Res* 1999, 59:4266–4270
65. Lu Y, Lotan R: Transcriptional regulation by butyrate of mouse galectin-1 gene in embryonal carcinoma cells. *Biochim Biophys Acta* 1999, 1444:85–91
66. Zhang W, Kadam S, Emerson BM, Bieker JJ: Site-specific acetylation by p300 or CREB binding protein regulates erythroid Kruppel-like factor transcriptional activity via its interaction with the SWI-SNF complex. *Mol Cell Biol* 2001, 21:2413–2422
67. Bright-Thomas RM, Hargest R: APC, beta-catenin and hTCF-4: an unholy trinity in the genesis of colorectal cancer. *Eur J Surg Oncol* 2003, 29:107–117
68. Stone CD, Chen ZY, Tseng CC: Gut-enriched Kruppel-like factor regulates colonic cell growth through APC/beta-catenin pathway. *FEBS Lett* 2002, 530:147–152
69. Toyokawa Y, Sawami H, Uchino H, Ito K: Calcium ionophore A23187 induces differentiation of HL-60 cells into macrophage-like cells. *Nippon Ketsueki Gakkai Zasshi* 1988, 51:746–751
70. Koski GK, Schwartz GN, Weng DE, Czerniecki BJ, Carter C, Gress RE, Cohen PA: Calcium mobilization in human myeloid cells results in acquisition of individual dendritic cell-like characteristics through discrete signaling pathways. *J Immunol* 1999, 163:82–92
71. Engels FH, Kreisel D, Faries MB, Bedrosian I, Koski GK, Cohen PA, Czerniecki BJ: Calcium ionophore activation of chronic myelogenous leukemia progenitor cells into dendritic cells is mediated by calcineurin phosphatase. *Leuk Res* 2000, 24:795–804
72. Launay S, Gianni M, Diomedea L, Machesky LM, Enouf J, Papp B: Enhancement of ATRA-induced cell differentiation by inhibition of calcium accumulation into the endoplasmic reticulum: cross-talk between RAR alpha and calcium-dependent signaling. *Blood* 2003, 101:3220–3228
73. Launay S, Gianni M, Kovács T, Bredoux R, Bruel A, Gélébart P, Zassadowski F, Chomienne C, Enouf J, Papp B: Lineage-specific modulation of calcium pump expression during myeloid differentiation. *Blood* 1999, 93:4395–4405
74. Lytton J, Westlin M, Burk SE, Shull GE, MacLennan DH: Functional comparisons between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps. *J Biol Chem* 1992, 267:14483–14489
75. Dode L, Vilsen B, Van Baelen K, Wuytack F, Clausen JD, Andersen JP: Dissection of the functional differences between sarco(endo)plas-

- mic reticulum Ca^{2+} -ATPase (SERCA) 1 and 3 isoforms by steady-state and transient kinetic analyses. *J Biol Chem* 2002, 277: 45579–45591
76. Dode L, Andersen JP, Leslie N, Dhitavat J, Vilsen B, Hovnanian A: Dissection of the functional differences between sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) 1 and 2 isoforms and characterization of Darier disease (SERCA2) mutants by steady-state and transient kinetic analyses. *J Biol Chem* 2003, 278:47877–47889
77. Wuytack F, Dode L, Baba-Aissa F, Raeymaekers L: The SERCA3-type of organellar Ca^{2+} pumps. *Biosci Rep* 1995, 15:299–306
78. Papp B, Pászty K, Kovács T, Sarkadi B, Gardos G, Enouf J, Enyedi A: Characterization of the inositol trisphosphate-sensitive and insensitive calcium stores by selective inhibition of the endoplasmic reticulum-type calcium pump isoforms in isolated platelet membrane vesicles. *Cell Calcium* 1993, 14:531–538
79. Papp B, Brouland JP, Gélébart P, Kovács T, Chomienne C: Endoplasmic reticulum calcium transport ATPase expression during differentiation of colon cancer and leukaemia cells. *Biochem Biophys Res Commun* 2004, 322:1223–1236
80. Pacifico F, Ulianich L, De Micheli S, Treglia S, Leonardi A, Vito P, Formisano S, Consiglio E, Di Jeso B: The expression of the sarco/endoplasmic reticulum Ca^{2+} -ATPases in thyroid and its down-regulation following neoplastic transformation. *J Mol Endocrinol* 2003, 30:399–409