Potent Modulation of Intestinal Tumorigenesis in Apc<sup>min/+</sup> Mice by the Polyamine Catabolic Enzyme Spermidine/Spermine N<sup>1</sup>-acetyltransferase


Abstract

Intracellular polyamine pools are homeostatically maintained by processes involving biosynthesis, catabolism, and transport. Although most polyamine-based anticancer strategies target biosynthesis, we recently showed that activation of polyamine catabolism at the level of spermidine/spermine N<sup>1</sup>-acetyltransferase-1 (SSAT) suppresses tumor outgrowth in a mouse prostate cancer model. Herein, we examined the effects of differential SSAT expression on intestinal tumorigenesis in the Apc<sup>Min/+</sup> (MIN) mouse. When MIN mice were crossed with SSAT-overproducing transgenic mice, they developed 3- and 6-fold more adenomas in the small intestine and colon, respectively, than normal MIN mice. Despite accumulation of the SSAT product, N<sup>1</sup>-acetylspermidine, spermidine and spermine pools were only slightly decreased due to a huge compensatory increase in polyamine biosynthetic enzyme activities that gave rise to enhanced metabolic flux. When MIN mice were crossed with SSAT knock-out mice, they developed 75% fewer adenomas in the small intestine, suggesting that under basal conditions, SSAT contributes significantly to the MIN phenotype. Despite the loss in catabolic capability, tumor spermidine and spermine pools failed to increase significantly due to a compensatory decrease in biosynthetic enzyme activity giving rise to a reduced metabolic flux. Loss of heterozygosity at the Apc locus was observed in tumors from both SSAT-transgenic and -deficient MIN mice, indicating that loss of heterozygosity remained the predominant oncogenic mechanism. Based on these data, we propose a model in which SSAT expression alters flux through the polyamine pathway giving rise to metabolic events that promote tumorigenesis. The finding that deletion of SSAT reduces tumorigenesis suggests that small-molecule inhibition of the enzyme may represent a nontoxic prevention and/or treatment strategy for gastrointestinal cancers. (Cancer Res 2005; 65(12): 5390-8)

Introduction

Cell proliferation, differentiation and cell death depend on or are affected by a sustained supply of intracellular polyamine pools. Enzymes such as ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) contribute to the de novo biosynthesis of polyamines, whereas catabolic enzymes such as spermidine/spermine N<sup>1</sup>-acetyltransferase-1 (SSAT) and polyamine-directed oxidases play important roles in lowering polyamine pools by export and in catalyzing their interconversion (1–4). Alterations in polyamine homeostasis, whether pharmacologically- or genetically induced, lead to changes in intracellular polyamine pools that have important ramifications in cell physiology and cell growth. For example, increased ODC and SAMDC activity, and the associated elevations in intracellular polyamines, have been implicated in certain cancers, including those of the gastrointestinal tract (3, 5–10). As such, polyamines and their key biosynthetic enzymes have been viewed as attractive targets for the treatment and prevention of cancer. Findings to be presented here suggest that catabolic enzymes such as SSAT may represent even more compelling targets in gastrointestinal cancers.

SSAT, which is encoded by the X-linked Sat1 gene, is the rate-limiting enzyme of polyamine catabolism. It catalyzes the acetylation of spermine and spermidine in response to cell stress (4) and to excess polyamines (11). Acetylated polyamines are either oxidized by polyamine oxidase, which leads to the back-conversion of higher polyamines (i.e., spermine) to lower polyamines (i.e., spermidine and putrescine or more typically, they are efficiently excreted out of the cell as a means of lowering intracellular polyamine pools (1, 2). Whether achieved genetically (12, 13) or pharmacologically with polyamine analogues (14, 15), induction of SSAT typically gives rise to growth inhibition or apoptosis, depending upon the cell type and the extent of enzyme overexpression. In such experiments, growth inhibition has been closely linked to depletion of intracellular polyamine pools (12) and disturbances in polyamine metabolism (13), whereas apoptosis has been associated with downstream events emanating from polyamine oxidase–mediated oxidation of acetylated polyamines and the associated release of oxidatively reactive by-products such as hydrogen peroxide and the aldehyde, 3-acetamidopropanal (4, 16, 17).

The relationship between SSAT and cancer is only now being defined. Because maintenance of cell proliferation requires a constant supply of polyamines (1–3), it might be expected that tumor cells would keep polyamine biosynthetic activity high and catabolic activity low, so as to create a selection pressure favoring polyamine availability. Several studies showing induced apoptosis in tumor cells overexpressing SSAT are consistent with this paradigm (1, 13, 16, 17). Conditional overexpression of SSAT in LNCaP prostate carcinoma cells has been shown to bring about near total growth inhibition (13). Extending this finding to an in vivo system, we recently reported that cross-breeding SSAT-overexpressing transgenic mice with prostate cancer–predisposed TRAMP [transgenic adenocarcinoma of mouse prostate; ref. (18)]
mice inhibits tumor outgrowth (19). This provocative finding suggests quite clearly that high levels of SSAT expression can exert tumor-suppressive effects. However, in contrast to expectations, polyamine pools were not significantly depleted by SSAT in either LNCaP cells or in prostate tumors of TRAMP mice (13, 19). Rather, the antitumor effect seems to be directly related to a compensatory up-regulation of polyamine biosynthesis in response to SSAT overproduction. This, in turn, leads to heightened metabolic flux through the biosynthetic and catabolic arms of the pathway and an associated depletion of metabolic precursors such as the SSAT cosubstrate acetyl-CoA. On the assumption that this somewhat unexpected metabolic response may be unique to prostate-derived tumors, we sought to determine the consequences of SSAT overexpression in a murine model of intestinal cancer.

Apc^{Min/+} mice (MIN) carry a truncation mutation (the Min allele) within the adenomatous polyposis coli (Apc) tumor suppressor gene (20, 21). The same gene defect is frequently seen in both inherited and spontaneous colon cancer in humans (22). Heterozygous Apc^{Min/+} animals are predisposed to the development of multiple adenomas in the small intestine and, to a much lesser extent, in the colon (20, 21). The formation of these polypos is initiated by loss of heterozygosity (LOH) at the Apc locus (23), which leads to stabilization of the transcription factor β-catenin, constitutive activation of the WNT signaling pathway, and induced expression of target genes, such as those encoding cyclin D1, c-MYC, and others (24). Of relevance to the current study, polyamine metabolism is known to be directly related to β-catenin expression via c-MYC-mediated transactivation of ODC (25). Indeed, ODC mRNA levels and polyamine pools have been shown to be increased in the small intestine and colon of MIN mice (26). Furthermore, treatment of MIN mice with the specific ODC inhibitor α-difluoromethylornithine causes a reduction in intestinal polyamines and a significant decrease in tumor number (26, 27). Thus, there is a strong rationale for examining the consequences of altered SSAT expression in this particular model system. Herein, we report that transgenic overexpression of SSAT markedly increases tumor development in the MIN mouse, whereas genetic depletion of the enzyme has the opposite effect.

Materials and Methods

Animals. Apc^{Min/+} mice (MIN) in the C57BL/6J background (21) were obtained from the Jackson Laboratories (Bar Harbor, ME), and maintained by breeding MIN males to C57BL/6J females. Heterozygous progeny were identified by PCR analysis of tail DNA using allele-specific primers (20). These animals express elevated levels of SSAT enzyme activity in most copies of a full-length murine Sat1 gene was described earlier (29). These animals were mated to C57BL/6J females and used as carriers of the transgene. Heterozygous MIN mice (26). Furthermore, treatment of MIN mice with the specific ODC inhibitor α-difluoromethylornithine causes a reduction in intestinal polyamines and a significant decrease in tumor number (26, 27). Thus, there is a strong rationale for examining the consequences of altered SSAT expression in this particular model system. Herein, we report that transgenic overexpression of SSAT markedly increases tumor development in the MIN mouse, whereas genetic depletion of the enzyme has the opposite effect.

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Generation of embryonic stem cells carrying a targeted inactivating mutation within the X-linked Sat1 gene was described earlier (29). These cells were injected into C57BL/6J blastocysts to produce chimeric mice that were bred to yield SSAT knockout mice (SSAT-ko, genotypes Sat1^{−/−} and Sat1^{−/−}). Male MIN/SSAT-ko mice (genotype Apc^{Min/+}/Sat1^{−/−}) contain the inactive SSAT-ko allele within the Apc^{Min/+} background, and were produced by mating female SSAT-ko mice to male MIN mice. The presence of the SSAT-ko allele was determined by PCR using three primers: 20 pmol each of two allele-specific forward primers (wild-type primer, 5′-CTTCCTCCTGCTTCTCAAGTA-3′; null primer, 5′-TACCTGCCCATTCC-GACC-3′) and 40 pmol of a reverse primer that recognizes both the wild-type and null alleles (5′-CAGTTCCTGGGGGAC-GACG-3′). Reaction mixtures containing all three primers were subjected to 34 cycles of standard PCR conditions with a 56°C annealing temperature. Products were resolved using 5% acrylamide gel electrophoresis and stained with ethidium bromide for the presence of wild-type SSAT allele (408 bp) or the null allele (615 bp).

Tumor scoring. Tumor burdens were determined as described previously (30). Following cervical dislocation, the small and large intestines were removed, flushed free of debris, sliced longitudinally, and fixed flat between sheets of filter paper for 3 hours in 10% buffered formalin. Fixed tissues were stained with 0.002% methylene blue. Adenomatous polyps were counted under a dissecting microscope by a single observer who was blinded to the genotypes of the animals. Upon counting, each tumor was classified as being >1 or <1 mm in diameter using an ocular micrometer. All data are reported as mean ± SE, except where noted. Student’s t test was used to compare the means of each group; differences were considered to be significant if P values were <0.05.

Enzyme and polyamine analyses. Biochemical analyses were done on mice that were different from those used for intestinal tumor counts but which were derived from the same litter. Tissues were excised from 9-week-old mice under a dissecting microscope, and tumor tissue was snap-frozen and stored at −70°C. Following removal of tumors, the normal small intestinal and colonic mucosa was scraped from the muscularis externa layer with a glass slide and snap-frozen. Frozen mucosa and tumor tissues were crushed into a fine powder using a Bio-Pulverizer (BioSpec Products, Inc., Bartlesville, OK). Tris/EDTA (pH 7) breaking buffer was added to ~50 to 100 mg of sample for sonication and centrifugation to obtain the soluble supernatant extracts for enzyme and polyamine analyses.

SSAT activity was assayed radiochemically as described previously (31) and expressed as picomoles of N⁴-[¹⁴C]acetylspermidine generated per minute per millgram of protein. Both ODC and SAMDC activities were determined by a CO₂ trap assay as previously described (32), and reported as nanomoles of radiolabeled CO₂ per hour millgram of protein. For intracellular polyamine pool analysis, buffered extracts were further extracted with 1.2 N perchloric acid, and dansiylated prior to assessment by high-performance liquid chromatography using methods described elsewhere (12).

Histologic analysis of tumors. Tumor tissues for histologic analysis were removed and fixed for a minimum of 3 hours in 10% formalin, after which they were rolled, embedded in paraffin, sectioned, and stained with H&E. Stained sections were observed at 100× magnification under a light microscope.

Assessment of loss of heterozygosity. Intestinal epithelial cells from tumors or from adjacent histologically normal regions were collected using a PixCell Ie Laser Capture Microdissection System (Arcturus, Mountain View, CA). The cells were incubated for 12 hours at 50°C in 0.5 mg/mL proteinase K, 10 mmol/L Tris-HCl (pH 8.0), 50 mmol/L KCl, 0.45% NP40, and 0.45% Tween 20 (33) and genomic DNA was extracted by standard methods. LOH at the Apc locus was determined using PCR-based methods (23, 34). A 155-bp fragment of the Apc gene was amplified in the presence of [³²P]dCTP; the forward primer was 5′-TCTCGTTCTGGAAAG-GACAGAAAGCT-3′, and the reverse primer was 5′-TCTACCTCCTTC-AACAAGTGGTGCTAT-3′. The resulting product was digested with HindIII, resolved by gel electrophoresis in 8% acrylamide, and visualized by autoradiography. The wild-type Apc allele yields a 123 bp product, whereas the Min allele yields a 144 bp product. Quantitation of relative band intensities was carried out by densitometry (Storm 860 Imager and Image Quant 5.2 software; Molecular Dynamics, Sunnyvale, CA).

Results

SSAT overproduction enhances tumor development in MIN mice. To test the effects of elevated SSAT expression on intestinal tumorigenesis in the MIN mouse model, we intercrossed MIN (genotype Apc^{Min/+}) and SSAT-tg (genotype Sat1^{tg/0}) mice. The latter were produced and characterized in an earlier study (28), and were...
shown to express increased levels of SSAT mRNA and enzyme activity in most tissues, including the small intestine and colon. The genotypes of the progeny of the cross of MIN females and SSAT-tg males were determined at weaning and were found to segregate in a non-Mendelian fashion. The number of MIN/SSAT-tg progeny (Apc^{Min/+}/Sat1tg{+/0}) was only about 1/3 of that expected (Table 1; \( P = 4.6 \times 10^{-9} \)). Furthermore, SSAT-tg segregants (Apc^{+/+}/Sat1tg{+/0}) were also reduced in number relative to mice with wild-type (Apc^{+/+}) or MIN (Apc^{Min/+}) genotypes (Table 1; \( P = 0.013 \)), although the deficiency of this genotype was not as dramatic as that for MIN/SSAT-tg progeny. Thus, overproduction of SSAT seems to be deleterious to survival, particularly in combination with APC haploinsufficiency. Although the basis for this phenomenon is unknown, it is likely manifested during gestation because there were no noticeable increases in postnatal mortality. Normal Mendelian segregation of genotypes was observed previously in a cross of SSAT transgenic and TRAMP mice (13, 19). The deficiency of MIN/SSAT-tg progeny in the current cross may be related to the report of Jacoby et al. (26, 27), who observed that treatment with the ODC inhibitor α-difluoromethylornithine during embryonic development is selectively lethal to fetuses bearing a heterozygous mutation in the Apc gene.

Previous work showed that SSAT mRNA and enzyme concentrations in the small intestine and the colon are ~10-fold higher in SSAT transgenic as compared with nontransgenic mice (28). We carried out similar activity measurements on MIN and MIN/SSAT-tg tissues (Fig. 1A). We consistently observed higher SSAT levels in normal small intestinal mucosa (\( P = 0.003 \)) and in colonic tumors (\( P = 0.01 \)) of the MIN/SSAT-tg progeny. There was a tendency toward higher SSAT activities in MIN/SSAT-tg intestinal tumors and in normal colon tissue, but the differences did not reach statistical significance. SSAT enzyme activities were significantly higher in colonic tumors relative to normal mucosa in MIN/SSAT-tg mice (\( P = 0.01 \)), but not in MIN mice. Small intestinal tumors exhibited slightly higher activities relative to normal tissues in both MIN and MIN/SSAT-tg progeny, but the increases were not statistically significant. One difficulty with these assays is that SSAT-specific activities are masked by the high levels of nonspecific acetyltransferases in tissue extracts (35), leading to an underestimation of the magnitude of change in SSAT expression. Thus, we consider the accumulation of the SSAT reaction product 1-acetylspermidine (see below) to be a more reliable indicator of increased SSAT enzyme activity in MIN/SSAT-tg mice.

Development of adenomatous polyps in the MIN/SSAT-tg mice was determined at 9 weeks of age over the full length of the small and large intestines, and compared with that in their non-transgenic MIN littermates. Total number of tumors in the small intestines of MIN mice (Fig. 1B) were similar to previously reported values (30). However, the MIN/SSAT-tg mice developed ~3-fold more tumors in the small intestine (\( P < 0.01 \)), including ~2-fold increase in tumors <1 mm in diameter and a 4-fold increase in tumors >1 mm in diameter (Fig. 1B). Although tumor incidence was lower in colon than small intestine (Fig. 1B), all of the colonic polyps were >1 mm and the MIN/SSAT-tg mice contained about 6-fold more tumors than nontransgenic MIN mice (\( P < 0.0001 \)). On average, tumor sizes in either the small intestine or the colon were not significantly different between MIN and MIN/SSAT-tg mice. These data indicate that the presence of the SSAT transgene potently enhances tumorigenesis in both the small intestine and colon of MIN mice.

SSAT overexpression is accompanied by compensatory increases in polyamine biosynthetic activity in MIN mice. To gain insight into the mechanisms underlying increased tumor development in MIN/SSAT-tg mice, we measured the activities of the two key polyamine biosynthetic enzymes ODC and SAMDC. Enzyme activities in normal mucosa and in tumors of the small intestine and colon were determined, and found to be dramatically different between MIN/SSAT-tg and MIN progeny. ODC levels in MIN/SSAT-tg mice were about 3- to 5-fold higher in normal mucosa of both the small intestine (\( P < 0.0005 \)) and the colon (\( P < 0.00001 \)) relative to the MIN mice (Fig. 2). Increases of 7- to 10-fold were observed in tumors of the small intestine (\( P < 0.0005 \)) and the colon (\( P < 0.003 \); Fig. 2). Similarly, SAMDC activities in MIN/SSAT-tg mice were significantly increased relative to MIN mice in the normal mucosa of both the small intestine (i.e., ~20-fold; \( P < 0.002 \)) and colon (i.e., >10-fold; \( P < 0.01 \)). SAMDC in MIN/SSAT-tg tumors were also higher than MIN tumors in the small intestine (i.e., >40-fold; \( P < 0.04 \)) and in the colon (i.e., >50-fold; \( P < 0.001 \); Fig. 2).

These results indicate that the presence of the SSAT transgene is associated with increases in both ODC and SAMDC activities in normal tissues and in polyps of the gastrointestinal tract. Thus, the increase in polyamine catabolic activity mediated by SSAT overexpression is metabolically offset by a large compensatory increase in biosynthetic activity. This finding, which is consistent with earlier characterization of various normal tissues of the SSAT transgenic mouse (19, 28), implies that metabolic flux through the polyamine metabolic pathway is heightened in both tumors and the intestinal epithelium of MIN/SSAT-tg mice.

**Overexpression of SSAT in MIN mice differentially alters polyamine pools.** To examine the effects of increased SSAT expression on polyamine pools, the concentrations of the SSAT product N^1-acetylspermidine, as well as the primary polyamines putrescine, spermidine, and spermine were measured and compared in MIN and MIN/SSAT-tg mice (Fig. 3). In normal small intestinal tissues, MIN/SSAT-tg mice exhibited a ~30-fold increase in N^1-acetylspermidine levels (\( P < 0.0002 \)), and an 18-fold increase in putrescine pools (\( P < 0.00001 \)) relative to MIN tissues. By comparison, spermidine concentrations decreased by only 29% (\( P < 0.0002 \)), whereas spermine levels were not significantly affected. Similar increases in N^1-acetylspermidine and putrescine were seen in the MIN/SSAT-tg normal colon, with no change in spermidine or spermine levels (Fig. 3).

Even larger increases in both N^1-acetylspermidine and putrescine levels were observed in tumors of MIN/SSAT-tg mice relative to MIN mice (Fig. 3). N^1-acetylspermidine levels were elevated by >70-fold in small intestinal tumors (\( P < 0.01 \)), and by >200-fold in colonic tumors (\( P < 0.02 \)). Putrescine concentrations were ~9-fold

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**Table 1. Genotypes of progeny resulting from crossing MIN females (Apc^{Min/+}) to SSAT-tg males (Sat1tg{+/0})**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Observed*</th>
<th>Predicted</th>
</tr>
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<tbody>
<tr>
<td>Apc^{+/+}</td>
<td>109 (37.7%)</td>
<td>72 (25%)</td>
</tr>
<tr>
<td>Apc^{+/+}/Sat1tg{+/0}</td>
<td>63 (21.8%)</td>
<td>72 (25%)</td>
</tr>
<tr>
<td>Apc^{Min/+}</td>
<td>95 (32.9%)</td>
<td>72 (25%)</td>
</tr>
<tr>
<td>Apc^{Min/+}/Sat1tg{+/0}</td>
<td>22 (7.6%)</td>
<td>72 (25%)</td>
</tr>
</tbody>
</table>

*Observed progeny distribution (percentage of total based on 289 mice).
higher in small intestinal polyps \((P < 0.0003)\), and ~8-fold higher in colonic polyps \((P < 0.03)\). As seen in normal tissues, tumor spermidine levels decreased by a modest 23% of MIN/SSAT-tg mice \((P < 0.02)\), whereas spermine levels remained unchanged.

Overall, the results for normal tissues agree well with published data indicating that putrescine and \(N^1\)-acetylspermidine levels are dramatically higher in tissues of MIN/SSAT-tg mice as compared with normal MIN mice, whereas spermidine and spermine pools are essentially unchanged \((28)\), presumably due to the compensatory increase in biosynthetic activity. The increase in putrescine is probably due to the combined effects of polyamine biosynthesis (resulting from high ODC and SAMDC levels) and back-conversion (due to SSAT overexpression). Expansion of the \(N^1\)-acetylspermidine pool is a strong indicator that SSAT is overproduced at the level of enzyme activity \((28)\).

SSAT deficiency inhibits intestinal tumor development in MIN mice. The finding that overexpression of SSAT augments tumorigenesis in the MIN mouse model predicts that MIN mice lacking SSAT will exhibit reduced tumor development. To test this prediction, we used a SSAT-ko mouse that contains a targeted, inactivating insertion within the X-linked \(Sat1\) gene \((29)\). SSAT-ko mice are viable, fertile, and show no obvious phenotype under the conditions of the current studies.

We crossed MIN males with \(Sat1^{++}\) females, and observed that all expected genotypes segregated in a normal Mendelian fashion (data not shown). SSAT activities were examined in the small intestinal tumors from MIN/SSAT-ko progeny (all of which are males) and MIN progeny (Fig. 4A). The difference in total activity between MIN and MIN/SSAT-ko tumors was small and not statistically significant, due to low levels of basal SSAT activity, as well as the presence of nonspecific acetylating enzymes \((35)\). Indirect evidence for loss of SSAT was apparent in a significant decrease in ODC activity, as noted below.

Tumor numbers were determined in the MIN/SSAT-ko mice, and compared with those in normal male MIN progeny. Total tumor counts in the small intestine were reduced by about 75% in MIN/SSAT-ko mice \((P < 0.02)\); such a decrease was observed for both small (\(<1\) mm diameter) and large (\(>1\) mm diameter) tumors (Fig. 4B). Interestingly, there were no significant effects on the number or size of colonic tumors, despite the fact that overexpression of SSAT increased tumor number in this organ by ~6-fold (see Fig. 1).

ODC activity in intestinal tumors was significantly different between MIN/SSAT-ko and normal MIN mice \((P = 0.016; \text{Fig. } 4A)\). Thus, loss of SSAT expression in the knock-out mice results in a 66% compensatory decrease in ODC activity. SAMDC activity was not measured due to the small size of the MIN/SSAT-ko tumor samples. Analysis of polyamine pools in small intestinal tumors of the two genotypes revealed modest changes in putrescine, spermidine, and spermine concentrations in MIN/SSAT-tg mice \((\text{Fig. } 4C)\). Putrescine fell by 45% \((P = 0.017)\), whereas the 18% increase in spermidine and the 6% decrease in spermine were not statistically significant. The SSAT product \(N^1\)-acetylspermidine was undetectable in tumors from either MIN or MIN/SSAT-ko mice (data not shown). The relative lack of change among the higher polyamines was somewhat unexpected because deletion of SSAT is expected to increase polyamine pools due to reduced catabolic activity \((29)\). We attribute this lack of change to the decrease in ODC activity that accompanied loss of SSAT and thus, decreased metabolic flux through the polyamine pathways. It is likely that this also accounts for the reduction in putrescine pools.

Tumor histology and loss of heterozygosity at the \(Apc\) locus in SSAT-overexpressing and in SSAT-deficient MIN mice. No discernible changes in gross morphology of tumors were noted in either MIN/SSAT-tg or MIN/SSAT-ko mice (data not shown). Tumor sections from both strains exhibited a typical adenomatous histology that was very similar to that found in MIN mice (data not shown).

Several studies have indicated that tumor development in \(Apc^{Min/+}\) mice results from LOH at the \(Apc\) locus \((20, 23, 36)\), leading to the loss of the wild-type \(Apc\) allele and a deficiency in expression of the full-length \(Apc\) protein. To determine if LOH is the predominant mechanism of \(Apc\) inactivation in mice with altered expression of SSAT, we isolated tumor epithelial cells from both MIN/SSAT-tg and MIN/SSAT-ko mice by laser capture microdissection, and assessed loss of the wild-type \(Apc\) allele in these cells.

The wild-type and \(Min\) alleles of the \(Apc\) gene differ by an A/T substitution at codon 850, resulting in gain/loss of a \textit{HindIII} restriction site \((23)\). \textit{HindIII} digestion of a 155-bp PCR-generated fragment spanning this site results in 123 and 144 bp products, representing the wild-type and \(Min\) alleles, respectively. Both PCR fragments were evident in \textit{HindIII}-digested DNA extracted from normal villus of MIN mice (Figs. 5A and B, both on lane 2). In contrast, epithelial cell DNA from small intestinal SSAT-tg tumors (Fig. 5A, lanes 3-15) exhibited the 144 bp \textit{HindIII} fragment, with little evidence of the 123 bp fragment, indicating loss of the wild-type allele. Quantitation of band intensities showed that the ratio...
of the wild-type to the Min allele was 0.80 ± 0.06 in DNA from normal villus and 0.25 ± 0.05 in DNA from adenoma epithelial cells (P < 0.001). The residual amount of the 123 bp wild-type band in some lanes is due to unavoidable contamination by infiltrating normal cells such as inflammatory cells, lymphocytes, and fibroblasts.

Very similar results were obtained upon analysis of MIN/SSAT-ko mice (Fig. 5B). DNA from normal villus exhibited both bands (lane 2), whereas that from microdissected tumor epithelial cells predominantly showed the Min-specific band. Thus, as in normal MIN mice, tumorigenesis in MIN/SSAT-tg and MIN/SSAT-ko mice is associated with loss of APC expression due to LOH at the Apc locus. Despite profound effects on tumorigenesis, genetic manipulation of SSAT expression does not change the underlying oncogenic mechanism, namely, APC loss during tumor initiation, indicating that SSAT contributes in some way to the process of LOH.

**Discussion**

The primary finding of this study is that variations in SSAT expression modulate intestinal tumorigenesis in the MIN mouse model. The ability of SSAT overexpression to increase intestinal polyps shows the tumorigenic potential of SSAT under exaggerated, although perhaps, nonphysiologic conditions. However, the observation that SSAT deficiency reduces intestinal polyps implies a significant role for the enzyme under basal conditions, a finding perhaps more relevant to understanding the actual role of SSAT in carcinogenesis. Similar to normal MIN mice, LOH at the Apc locus occurs in tumors of both the SSAT-overproducing as well as the SSAT-deficient MIN mice, suggesting that genetic manipulation of SSAT expression alters the frequency of LOH-type events that are involved in the initiation of tumor development.

Of additional significance to the current findings is the demonstration that SSAT-mediated effects are context-dependent, i.e., the impact of dysregulated SSAT expression varies among tumor types. We have recently shown that overexpression of SSAT in the TRAMP mouse model of prostate cancer markedly suppresses tumor outgrowth (19), an effect opposite to that of the present study. Thus, the impact of SSAT differs between the prostate and the gastrointestinal tract. Despite this disparity, the findings with both models strongly indicate a role for SSAT in tumor development, whereas at the same time reflecting the ambiguities associated with our current understanding of polyamine catabolism and its relationship to cell proliferation (4). There is no indication in our experience that SSAT-overproducing transgenic mice are more prone to developing spontaneous tumors in any tissue.4

A number of studies conducted over the past few years substantiate the complexity and context-dependency of SSAT and its role in tumorigenesis. Several reports, based on studies of cultured cells and animal models, are consistent with the notion that SSAT suppresses cell growth. For example, studies with polyamine analogues that potently induce SSAT indicate that apoptotic indices are increased when the enzyme is up-regulated, and are decreased when it is not (17). Conditional overexpression of SSAT causes growth inhibition in both MCF-7 breast carcinoma cells (12) and LNCaP prostate cancer cells (13). In vivo studies have shown that relative to normal mice, SSAT-overproducing transgenic mice are resistant to carcinogen-induced skin papilloma formation (37). As noted above, transgenic overexpression of SSAT in prostate cancer–predisposed TRAMP mice markedly reduces outgrowth of tumors (19). Taken as a whole, ample evidence indicates that SSAT exerts a decidedly negative effect on cell growth and tumor development.

In contrast to these findings, studies in other systems are consistent with the idea that SSAT stimulates, rather than inhibits, tumorigenesis. Enzyme levels are higher in breast tumors than in normal tissues, and correlate positively with tumor size (38). Gene expression profiling has identified SSAT mRNA as an up-regulated transcript in rectal tumors (39). Treatment of colon cancer cells with the chemopreventive agent resveratrol reduces levels of SSAT activity while causing growth arrest (40). Perhaps most convincingly, targeted overexpression of the enzyme in epidermal keratinocytes of mice increases the susceptibility to skin tumors in response to carcinogens (41). This is consistent with the present findings that SSAT overexpression promotes, whereas SSAT deficiency inhibits, tumor development in the MIN mouse.

In considering the mechanism by which SSAT modulates intestinal tumorigenesis in the MIN mouse model, we have taken into account the rather important observation that alterations in SSAT levels exert only minor effects on spermidine and spermine pools. It is well known that polyamine metabolism is highly dynamic and subject to compensatory responses designed to conserve intracellular polyamine pools (13, 19, 28, 29, 41, 42). Thus,

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4 Unpublished results.
as seen here and elsewhere (13, 28), activation of polyamine catabolism at the level of SSAT gives rise to a compensatory increase in polyamine biosynthetic enzymes (i.e., ODC and SAMDC). We consider it unlikely that the relatively minor changes in spermidine and spermine pools seen in tumors of MIN/SSAT-tg and in MIN/SSAT-ko mice (Figs. 3 and 4) are responsible for altered tumorigenesis. Rather, we submit that changes in tumor development more likely arise from deregulated metabolic flux through the polyamine biosynthetic and/or catabolic pathways. This flux can be viewed as a metabolic ratchet (Fig. 6) driven by spermidine and spermine homeostasis. When SSAT is overexpressed as in transgenic mice, spermidine and spermine pools decrease, triggering a sustained increase in biosynthetic activity. This results in heightened metabolic flux and maintenance of polyamine pools which, in turn, permit continued polyamine oxidation. Changes in the flux can have dramatic effects on the concentrations of a number of metabolites. On the biosynthetic side, substrates such as ornithine, methionine, and S-adenosylmethionine are used, whereas products such as putrescine, methylthioadenosine and carbon dioxide are generated. On the catabolic side, substrates such as acetyl-CoA and FAD are used, whereas products and by-products such as putrescine, N1-acetylspermidine, 3-acetamidopropanal, hydrogen peroxide, and FADH2 are liberated (Fig. 6). The rate at which the metabolic ratchet turns determines how rapidly substrates are depleted and products are produced. We propose that at some point, either a substrate becomes rate-limiting or a product becomes toxic, causing a phenotypic change. It is interesting to note that the ratchet model also applies to the SSAT-ko mice where we showed that deletion of SSAT results in a lowering of ODC activity and polyamine pools (Fig. 4) and thus, a decrease in metabolic flux.

The metabolic ratchet model provides a useful explanation for the effects of SSAT on intestinal polyp formation, as observed in the present study. Although the particular metabolites mediating these responses are not yet known, we believe that certain oxidatively reactive by-products of polyamine catabolism may play a significant role. SSAT is rate-limiting in polyamine catabolism. When deregulated, the enzyme’s activity gives rise to unlimited acetylated polyamines which are readily oxidized by polyamine oxidase to lower polyamines; hydrogen peroxide and aldehydes are released as by-products. Thus, when SSAT expression and polyamine catabolism are high, such as in MIN/SSAT-tg mice, a compensatory increase in polyamine biosynthesis heightens metabolic flux, giving rise to a sustained release of reactive by-products, an increased frequency of LOH events at the Apc locus, and thus, increased polyp formation. Conversely, when metabolic flux is low, such as in the MIN/SSAT-ko mice, there is minimal release of reactive by-products and thus, a much lower probability of LOH events and tumor formation. The correlative changes in LOH status at the Apc locus observed in normal MIN mice, as well as in MIN/SSAT-tg and MIN/SSAT-ko mice, is highly supportive of the above paradigm. The idea that the LOH may be due to increased DNA damage caused by reactive by-products of polyamine oxidase is quite consistent with several studies showing that MIN mice having deficiencies in DNA repair manifest similar increases in intestinal tumor development (43–46).

Figure 3. Polyamine pools in normal intestinal tissues and polyps of MIN and MIN/SSAT-tg mice. The concentrations of N1-acetylspermidine, putrescine, spermidine, and spermine were determined for normal regions of small intestine and colon, and for polyps from both tissues. Values for MIN mice (gray columns) and MIN/SSAT-tg mice (black columns) represent averages of three to nine animals per group.
The consequences of altered metabolic flux through the polyamine pathways are obviously very different between the intestinal tract and the prostate of the mouse. SSAT suppression of prostate tumor growth has been associated both \textit{in vitro} and \textit{in vivo} with depletion of acetyl-CoA and/or S-adenosylmethionine pools (19). Although applicable to the TRAMP model, depleted acetyl-CoA pools and the attendant interference with fatty acid synthesis hardly seems relevant to tumorigenesis in the MIN

![Figure 4](image)

**Figure 4.** A, SSAT and ODC activity levels in small intestinal polyps of MIN/SSAT-ko mice. Activities were measured in tumor extracts and compared with those in normal MIN mice. Values for MIN/SSAT-ko mice (black columns) and MIN mice (gray columns) represent the averages of five to seven animals per group. The similar SSAT activity in MIN versus MIN/SSAT tumors is due to the low basal enzyme levels relative to nonspecific acetylating enzymes that are also detected in the assay. B, tumor numbers in SSAT-ko mice. Polyps were counted in the small intestine and the colon of MIN/SSAT-ko mice ($n = 10$, black columns), and compared with those found in MIN mice ($n = 14$, gray columns). For the small intestine, tumors $<1$ and $>1$ mm in diameter were counted separately (indicated above the columns). C, polyamine concentrations in small intestinal polyps of MIN/SSAT-ko mice. Polyamine pools were measured in tumor extracts of MIN/SSAT-ko mice (black columns) relative to MIN mice (gray columns). In all samples, N1-acetylspermidine levels were below the level of detection (0.5 nmol/mg protein). Note that the scale of the y axis differs between polyamine graphs. Values represent the averages of seven to eight animals per group.

![Figure 5](image)

**Figure 5.** LOH at the \textit{Apc} locus in intestinal tumors. Epithelial cells were isolated from tumor tissues and normal villus from MIN intestine by laser capture microdissection. DNA was extracted and the appropriate region within the \textit{Apc} gene was amplified by PCR using radioactive nucleotide substrates; the amplified DNA was digested with HindIII, and observed by gel electrophoresis and autoradiography (see Materials and Methods for details). A, undigested DNA from normal MIN villus (lane 1), HindIII-digested DNA from normal MIN villus (lane 2), and HindIII-digested DNA from microdissected tumors of MIN/SSAT-tg mice (lanes 3-15). B, undigested DNA from normal MIN villus (lane 1), HindIII-digested DNA from normal MIN villus (lane 2), and HindIII-digested DNA from microdissected MIN/SSAT-ko tumors (lanes 3-8). Sizes of the various DNA bands are shown (126 kb fragment represents wild-type allele, 144 kb fragment represents MIN allele).
Figure 6. Metabolic ratchet model for polyamine homeostasis. Large arrows, the primary pathways for polyamine biosynthesis (left) and catabolism (right). Various metabolites are shown which are either substrates or products of these pathways. Substrates and precursors used in polyamine biosynthesis include ornithine (Orn), methionine (Met) and the SAMDC aminopropyl donor, S-adenosylmethionine (SAM), whereas substrates used in polyamine catabolism include spermidine (Spd), spermine (Spm), acetyl-CoA (AcCoA), and FAD. Compounds produced during polyamine biosynthesis include the natural polyamines (PA), the SAMDC by-product decarboxylated S-adenosylmethionine (dcSAM), the spermidine and spermine synthase by-product, methylthioadenosine (MTA) and the decarboxylase by-product, CO₂. Compounds produced during catabolism include the acetylated polyamines (AcPA), the polyamine oxidase product putrescine (Put) and the by-products hydrogen peroxide (H₂O₂), the aliphatic aldehyde 3-acetamidopropanal (RCHO), and FADH₂. In response to SSAT-induced decreases in spermidine and spermine, polyamine biosynthesis increases, giving rise to a sustained increase in metabolic flux. As flux through the pathway increases (such as that occurring in SSAT-tg mice), substrate utilization and product accumulation increase; conversely, as flux decreases, such as that seen in the SSAT-ko mice, substrate utilization and product accumulation decrease. The cellular response to alterations in flux depends upon the particular metabolites that change and how effectively the cell is able to react to that change. The direct correlation between SSAT expression levels and intestinal tumorigenesis suggests that a product of SSAT or a downstream enzyme such as polyamine oxidase is facilitating the tumorigenic process.

mouse because high fat content is known to be causally related to tumor development in the MIN mouse model (47); thus, depletion of acetyl-CoA would be expected to decrease rather than increase tumorigenesis. Similarly, it is also unlikely that depletion of the methyl donor S-adenosylmethionine is relevant because it has been shown that reduction of hypermethylation suppresses, rather than promotes, polypl formation in the MIN mouse (48).

In further consideration of the disparity in SSAT effects between MIN and TRAMP mice, it must be realized that the consequences of altered flux through the polyamine pathway will depend upon (a) the nature of the metabolite effector, (b) how well the cell is prepared to respond to that metabolite, and (c) the overall metabolic milieu of the cell. All of these are likely to vary significantly between prostate epithelium and the intestinal mucosa. Because SSAT is systemically overexpressed in all cells and tissues of SSAT-tg mice, these same variables are also likely to differ in the stromal environment of tumor cells in the two tissues. The tumor stroma has been widely implicated in both tumorigenesis and drug responses (49), and could be a critical determinant of the SSAT effects.

Another contextual factor unique to these particular models is the driving cancer gene. Tumorigenesis in the MIN mouse is driven by a mutated Apc suppressor gene (20) and its downstream effectors, whereas that in the TRAMP mouse is driven by the SV40 large T antigen and its various target molecules (18). These are two entirely different and nonconverging pathways. For example, ODC, which is potently up-regulated when SSAT is overexpressed in both models, is transactivated by c-MYC (25), and thus, serves as an effector in the WNT signaling pathway that drives tumorigenesis in the MIN mouse (24). In contrast, ODC is not among the SV40-large T antigen targets of the TRAMP mouse.

Although daunting, the task of determining which of the above factors is most critical in determining tumor outcome is likely to shed new insights into the overall process of tumor development, the role of polyamine catabolism as a modulator of that process, and possible new strategies for cancer prevention or treatment. In this regard, the observation that by promoting metabolic flux, SSAT would seem to behave as a promoter of intestinal tumorigenesis, raises the interesting possibility that pharmacologic inhibitors of the enzyme or the downstream enzyme polyamine oxidase might be a useful targeted anticancer strategy. This notion is reinforced by the observation that within the context of the present experiments, the SSAT-ko mice exhibited no apparent phenotype suggesting that such an inhibitor of this enzyme, if specific, would have minimal host effects. Lastly, our findings tend to discount previous reports in which suppression of SSAT by activated Ki-ras has been implicated in intestinal tumorigenesis (50), or in which induction of SSAT by sulindac sulfone has been implicated in colorectal chemoprevention (10), at least in so far as these observations might apply to the MIN mouse model.

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Potent Modulation of Intestinal Tumorigenesis in $Apc^{min/+}$ Mice by the Polyamine Catabolic Enzyme Spermidine/Spermine $N^1$-Acetyltransferase

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