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Polyamine and Methionine Adenosyltransferase 2A Crosstalk in Human Colon and Liver Cancer

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Abstract

Methionine adenosyltransferase (MAT) is an essential enzyme that is responsible for the biosynthesis of S-adenosylmethionine (SAdMe), the principal methyl donor and precursor of polyamines. *MAT1A* is expressed in normal liver and *MAT2A* is expressed in all extrahepatic tissues. *MAT2A* expression is increased in human colon cancer and in colon cancer cells treated with mitogens whereas silencing *MAT2A* resulted in apoptosis. The aim of the current work was to examine the mechanism responsible for *MAT2A*-dependent growth and apoptosis. We found that in RKO (human adenocarcinoma cell line) cells, *MAT2A* siRNA treatment lowered cellular SAdMe and putrescine levels by 70 to 75%, increased apoptosis and inhibited growth. Putrescine supplementation blunted significantly *MAT2A* siRNA-induced apoptosis and growth suppression. Putrescine treatment (100 pmol/L) raised *MAT2A* mRNA level to 4.3-fold of control, increased the expression of c-Jun and c-Fos and binding to an AP-1 site in the human *MAT2A* promoter and the promoter activity. In human colon cancer specimens, the expression levels of *MAT2A*, ornithine decarboxylase (ODC), c-Jun and c-Fos are all elevated as compared to adjacent non-tumorous tissues. Overexpression of ODC in RKO cells also raised *MAT2A* mRNA level and *MAT2A* promoter activity. ODC and *MAT2A* are also overexpressed in liver cancer and consistently, similar *MAT2A*-ODC-putrescine interactions and effects on growth and apoptosis were observed in HepG2 cells. In conclusion, there is a crosstalk between polyamines and *MAT2A*. Increased *MAT2A* expression provides more SAdMe for polyamines biosynthesis; increased polyamine (putrescine in this case) can activate *MAT2A* at the transcriptional level. This along with increased ODC expression in cancer all feed forward to further enhance the proliferative capacity of the cancer cell.

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Keywords

methionine adenosyltransferase 2A; putrescine; AP-1; colon cancer; liver cancer

INTRODUCTION

Colorectal cancer is a major cause of morbidity and mortality as the third most common cancer worldwide and the fourth most common cause of cancer death¹. In the past 20 years, tremendous progress has been made on understanding the molecular pathogenesis of this common cancer. Four signaling pathways commonly aberrant in colorectal cancer are: 1) Wnt, 2) K-ras, 3) transforming growth factor β , and 4) p53². Mutations in these pathways can lead to inactivation of tumor suppressor function and/or activation of proto-oncogenes. In addition, aberrant signaling in several growth factors such as insulin-like growth factor receptor-1 (IGF-1R), epidermal growth factor receptor (EGFR) and leptin has also been shown to be important in colon cancer growth, invasion and metastasis³⁻⁶.

Methionine adenosyltransferase (MAT) is an essential enzyme that catalyzes the formation of S-adenosylmethionine (SAME), the principal biological methyl donor and precursor for polyamine biosynthesis⁷. Two genes encode for the catalytic subunit of MAT, *MAT1A* encodes for $\alpha 1$ that is expressed predominantly by normal liver as dimer (MATIII) and tetramer (MATI), and *MAT2A* encodes for $\alpha 2$ that is expressed by all extrahepatic tissues as the MATII isoenzyme⁷. We reported that *MAT2A* expression is induced in human colorectal cancer and in colon cancer cells treated with IGF-1, EGF and leptin⁸. Importantly, mitogenic effect of these growth factors was abolished if *MAT2A* induction was prevented⁸. In addition, knockdown of *MAT2A* by siRNA in colon cancer cells resulted in increased apoptosis⁸. Thus, *MAT2A* expression is a key factor that regulates growth and apoptosis in colon cancer cells.

Given the importance of SAME in polyamine biosynthesis, a logical hypothesis is that increased *MAT2A* expression would provide an increased supply of SAME to enhance polyamine biosynthesis. Polyamines are organic cations with multiple functions that are essential for the cell's survival⁹. Mammalian cells have three polyamines: putrescine, spermidine and spermine. They interact with DNA (causing changes in chromatin structure), RNA and proteins and have antioxidant properties^{10,11}. They regulate cell cycle progression and inhibition of polyamine synthesis leads to cell cycle arrest and apoptosis¹⁰. Of interest, the expression of ornithine decarboxylase (ODC), the rate-limiting enzyme responsible for the initial step in polyamine biosynthesis, namely formation of putrescine, is often induced and polyamine levels are higher in colon cancer¹¹. However, whether *MAT2A* regulates growth and apoptosis via polyamines has not been examined. In this work we uncovered a crosstalk between *MAT2A* and polyamines that has not been previously recognized which can further enhance the growth of the cancer cell.

MATERIALS AND METHODS

Materials

Antibodies used for Western blot analyses of MATII and c-Jun were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), ODC was from Abcam (Cambridge, MA) and β -actin was from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade and obtained from commercial sources.

Cell Culture and Putrescine Treatment

RKO and HepG2 cells were obtained from the Cell Culture Core of the USC Research Center for Liver Diseases at Keck School of Medicine. Cells were grown according to instructions provided by American Type Cell Collection (Rockville, MD). RKO and HepG2 cells were grown to 50 to 60% confluence on six-well plates, and changed to media containing 1% for 24 hours before treatment with 50 to 200 pmol/L or 50 to 200 μ mol/L of putrescine (Sigma), respectively, for 24 hours.

Tissue Specimens

Thirteen colon cancers and adjacent non-tumorous tissues were obtained by Prof. Giordano (Whipps Cross University Hospital, London, UK) during surgical resection for primary colon cancer. These tissues were immediately frozen in liquid nitrogen for subsequent RNA and protein extraction. Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by Keck School of Medicine University of Southern California's human research review committee.

RNA Extraction and Real-Time Polymerase Chain Reaction (PCR) Analysis

Total RNA isolated from cells and colons as described⁸ was subjected to reverse transcription by using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA). One microliter of reverse transcription product was subjected to quantitative real-time PCR analysis. The primers and TaqMan probes for *MAT2A*, *ODC*, *c-Jun*, *c-Fos*, and Universal PCR Master Mix were purchased from ABI (Foster City, CA). Hypoxanthine phosphoribosyl-transferase 1 and 18S rRNA were used as housekeeping genes as described⁸. The delta Ct (Δ Ct) obtained was used to find the relative expression of genes according to the following formula: relative expression = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_t$ of respective genes in experimental groups $-\Delta C_t$ of the same genes in control group.

RNA Interference and Overexpression Experiments

The pre-designed small interfering RNA (siRNA) targeting human *MAT2A* (sense sequence: ACACAUUGGAUAUGAUGAUTT) (Invitrogen, Carlsbad, CA) was as described⁸, *ODC* (sc-43982, Santa Cruz, CA) and negative control siRNA (Ambion, Austin, TX) were purchased commercially. RKO and HepG2 cells were cultured in six-well plate (3×10^5 cells/well) and transfected using RNAiMax (5 μ L/well) from Invitrogen with *MAT2A*, *ODC* siRNA (10 nM) or negative control siRNA for 48 hours following the manufacturer's instructions.

ODC overexpression vector was purchased from Origene (Rockville, MD). RKO and HepG2 cells were cultured in 12-well plates (0.2×10^6 cells/plate), transfected using 7.5 μ L of Superfect from Qiagen (Valencia, CA) and 1 μ g of target plasmid per well. After 4 hours, the transfection medium was changed to normal medium. Effects of *ODC* overexpression on mRNA levels and *MAT2A* promoter activity (see below) were examined 24 hours later.

Cell Proliferation and Apoptosis Assays

Cell proliferation in *MAT2A* silenced cells and/or treated with putrescine (100 pmol/L for RKO, 100 μ mol/L for HepG2) for 24 hours was measured by the incorporation rate of 5-bromo-2'-deoxyuridine (BrdU) into DNA using a BrdU assay kit (CalBiochem, San Diego, CA) as described¹² with 3,000 cells per well in 96 well plates and 1 hour of BrdU incorporation time.

For apoptosis, cells were grown on coverslips and treated with siRNA targeting human *MAT2A* or/and putrescine for 24 hours, and cells were fixed with paraformaldehyde and stained with 8 µg/mL Hoechst 33258 dye for 30 minutes. Cells with bright, fragmented, condensed nuclei were identified as apoptotic cells using the Nikon Eclipse TE300 fluorescent microscope (Melville, NY). At least 5 random fields (at 300X) were counted.

Protein Isolation and Western Blot Analysis

Protein extracts from cells and colon specimens were isolated and subjected to Western blot analyses as described⁸ and resolved by electrophoresis on 12–13% SDS-polyacrylamide gel. Western blotting was performed following standard protocols (Amersham BioSciences, Piscataway, NJ) using primary antibodies for anti-MATII, ODC, c-Jun and actin. Blots were developed using enhanced chemoluminescence.

MAT2A Promoter Construct and Transient Transfection Assays

The human –571/+60 *MAT2A* promoter construct was previously described¹³. Effect of putrescine treatment (100 pmol/L for 24 hours) and ODC overexpression (for 24 hours) on *MAT2A* promoter activity was examined by measuring luciferase activity driven by the promoter using transient transfection analysis in RKO cells as we previously described¹³. Each experiment was done in triplicate samples and results are reported as fold of vector control.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was carried out following the ChIP assay kit protocol provided by Upstate (Waltham, MA). RKO cells were treated with putrescine (100 pmol/L for 24 hours) or vehicle control and processed for ChIP assay as we described using anti-c-Jun antibody¹⁴. PCRs of the human *MAT2A* promoter region –571 to –60 used forward primer 5′-AGCACAAAACCTCCGCGATTC-3′ and reverse primer 5′-CTCCAGAGACCGTTCAGCTAT-3′. All PCR products were run on 2% agarose gels and stained with ethidium bromide for 15–30 min.

SAME and Polyamine Levels

SAME and polyamine levels from *MAT2A* siRNA treated RKO cells were determined as previously described⁸.

Statistical analysis

Data are given as mean±SEM. Statistical analysis was performed using ANOVA and Fisher's test. For mRNA and protein levels, ratios of respective housekeeping densitometric values were compared. Significance was defined by $p < 0.05$.

RESULTS

Effects of MAT2A Silencing on SAME and Polyamine Levels, Apoptosis and Proliferation

Treatment of RKO cells with *MAT2A* siRNA (siMAT2A) for 48 hours lowered cellular SAME level by 70%, increased % apoptosis more than 3-fold and lowered proliferation (Fig. 1A–E). At this time point cellular putrescine level fell by 75% but spermidine and spermine levels were unchanged (Fig. 1F). However, both spermidine and spermine fell with more prolonged treatment (72 hours, data not shown) but there was also extensive cell death at that time point (>40% apoptosis).

To evaluate the role of putrescine depletion on *MAT2A* siRNA-mediated increase in apoptosis and growth suppression, RKO cells were treated with scrambled siRNA (Sc)

control or *MAT2A* siRNA with or without putrescine. Addition of putrescine caused a dose-dependent lowering of apoptosis from 50 to 100 pmol/L but this protective effect was lost at 200 pmol/L concentration (Fig. 2A). Putrescine at 100 pmol/L also blunted the growth suppressive effect of si*MAT2A* (Fig. 2C).

MAT2A expression is also induced in hepatocellular carcinoma (HCC)⁷ and its induction is required for mitogen-induced growth in HepG2 cells¹⁵. In HepG2 cells, knockdown of *MAT2A* resulted in a 71% reduction in cellular SAME level¹⁵. We next examined whether putrescine might also exert a protective effect in *MAT2A* knocked down HepG2 cells. Figure 2B shows that in HepG2 cells, treatment with *MAT2A* siRNA increased apoptosis nearly 6-fold and putrescine supplementation protected against this. A much higher putrescine dose was required in HepG2 cells, which is consistent with previous reports that used 100 μ M to 1 mM of putrescine in this cell line^{16,17}. Putrescine also blunted the growth suppressive effect of si*MAT2A* in HepG2 cells (Fig. 2D).

Effects of Putrescine on *MAT2A*, ODC, AP-1 Expression

In si*MAT2A* treated RKO cells, putrescine supplementation (100 pmol/L) increased *MAT2A* mRNA level over that of Sc control (Fig. 3A). At the protein level, putrescine raised *MAT2A* protein level above that of si*MAT2A* treated RKO cells by 81% (Fig. 3B). Putrescine (100 μ mol/L) exerted less of an effect on *MAT2A* mRNA level in HepG2 cells (did not raise it over that of Sc control) (Fig. 3C) but was equally effective in raising *MAT2A* protein level (Fig. 3D).

In both RKO and HepG2 cells, knockdown of *MAT2A* had no influence on the mRNA levels of *ODC*, *c-Fos* or *c-Jun* but addition of putrescine raised the mRNA levels of all three genes over that of Sc controls (Fig. 4A,C). Interestingly, although si*MAT2A* had no effect on *ODC* mRNA level, it lowered ODC protein level by 79% and 62% in RKO and HepG2 cells, respectively, and putrescine supplementation blunted this effect (Fig. 3B,D). In both cell types, overexpression of ODC also raised *MAT2A*, *c-Fos* and *c-Jun* mRNA levels similar to putrescine treatment (Fig. 4B,D). To see if induction of ODC is required for putrescine to raise *MAT2A* mRNA level, cells were treated with siODC or putrescine (100 pmol/L for RKO, 100 μ mol/L for HepG2) alone or together. Putrescine was able to raise *MAT2A* mRNA level in both cell types in the presence of ODC knockdown (Fig. 4E,F). In both cell types, ODC knockdown lowered *MAT2A* mRNA level.

Effects of Putrescine and ODC overexpression on *MAT2A* Promoter Activity

The human *MAT2A* promoter construct -571/60 contains a functional AP-1 *cis*-acting element¹³. Overexpression of ODC and putrescine treatment both increased the *MAT2A* promoter activity to comparable extent (Fig. 5A) and putrescine treatment increased c-Jun binding to the region of the *MAT2A* promoter that contains the putative AP-1 site (Fig. 5B).

Expression of *MAT2A*, ODC, AP-1 in Human Colon Cancer

Since ODC overexpression raised *MAT2A*, *c-Fos* and *c-Jun* mRNA level, we examined whether there is any correlation in the expression level of ODC with the expression levels of these genes in colon cancer specimens. Table I shows clinical and pathologic features of the colon cancer specimens. Comparison was made between cancerous to adjacent non-tumorous tissues. Majority of the cancer specimens have higher mRNA levels of all four genes (Table I and Figure 6A). Protein levels of *MAT2A*, ODC and c-Jun also show significant induction in colon cancer specimens (Fig. 6B). Consistently, there is a significant correlation between the expression level of ODC and *MAT2A* in colon cancer specimens (Fig. 6C).

DISCUSSION

Colorectal cancer remains one of the most common malignancies afflicting the world today. Although tremendous progress has been made toward understanding the molecular mechanisms involved, colorectal cancers accounted for 608,000 deaths in 2008 worldwide (World Health Organization Fact sheet N°297, February 2012). Despite improvement treatments, it remains important to identify new paradigms that may lead to additional targets for therapy. We reported that *MAT2A* expression is increased in colorectal cancer and that the induction of *MAT2A* was required in order for IGF-1, EGF and leptin to exert their mitogenic effect in colon cancer cells⁸. In that work we also found that knockdown of *MAT2A* in colon cancer cells resulted in apoptosis⁸. Given the fact that MAT is the sole enzyme that catalyzes the biosynthesis of SAME, which is a precursor for polyamines⁷, the most likely explanation is that varying *MAT2A* expression affects growth and apoptosis via changes in polyamine levels since these molecules can modulate growth and apoptosis^{9,10}. However, this link has not been conclusively demonstrated to our knowledge.

We began this investigation by measuring SAME and polyamine levels following MAT2A siRNA treatment. Consistent with our previous report⁸, knockdown of MAT2A lowered SAME level, cell growth and induced apoptosis after 48 hours. However, to our surprise, only putrescine level was lowered at this time point. Putrescine is formed from the decarboxylation of ornithine, catalyzed by ODC, the rate-limiting step in polyamine biosynthetic pathway¹¹. Putrescine combines with decarboxylated SAME (derived from SAME via enzymatic reaction catalyzed by SAME decarboxylase) to generate spermidine via spermidine synthase, and spermine via spermine synthase⁹. The catabolism of polyamines is essentially a reverse route of their biosynthesis and is catalyzed by spermidine and spermine N'-acetyltransferase and polyamine oxidase¹⁸. Regulation occurs at multiple levels involving many of these enzymatic steps. ODC expression is often induced and polyamine levels are higher in colon cancer and loss of APC (Adenomatous polyposis coli) function causes an increase in ODC activity and polyamine levels¹¹. Since SAME feeds into spermidine and spermine synthesis, we expected SAME depletion would lower the levels of these two polyamines. However, putrescine depletion occurred before spermidine and spermine levels fell. ODC is regulated at multiple levels, including transcription, translation, and protein degradation¹⁹. Although *ODC* mRNA level was not affected by MAT2A knockdown, ODC protein level was markedly reduced. At the present time we do not know how MAT2A knockdown lowered ODC protein level but this can explain the fall in putrescine level. To our knowledge, SAME depletion has not been shown to regulate ODC protein level.

Human spermidine synthase and spermine synthase have very low K_m s for decarboxylated SAME (dcSAME, 7 μ M for spermidine synthase and 1 μ M for spermine synthase)²⁰. While the intracellular SAME level is estimated to be around 60 μ M²¹, the intracellular dcSAME level is low to not detectable in cells under normal conditions⁷. Thus, both spermidine synthase and spermine synthase are probably working at a concentration of dcSAME close to or below their K_m s. Therefore, biosynthesis of spermidine and spermine may proceed unaffected until cellular dcSAME level is severely depleted. In addition, SAME decarboxylase in eukaryotes is activated by putrescine, which lowers the K_m for SAME^{22,23}. When putrescine level falls, SAME decarboxylase activity is reduced due to an increase in the K_m for SAME and this will lead to a lower dcSAME content and decreased spermidine/spermine synthesis. These two observations help to explain why putrescine depletion precedes spermidine and spermine.

Putrescine depletion played a key role in both increased apoptosis and growth suppression induced by MAT2A knockdown as supplementation with putrescine in the medium

attenuated these effects. However, there is a dose-dependent effect so that higher putrescine concentration (200 pmol/L) lost the protection. This is consistent with reports that excess putrescine can also trigger apoptosis²⁴. Interestingly, we found that putrescine at the optimum dose induced the expression of MAT2A and ODC, in addition to two key AP-1 family members c-Jun and c-Fos. The latter finding is consistent with previous report that increase in intracellular putrescine level induced the expression of c-Jun and c-Fos at the mRNA level²⁵. Increasing intracellular putrescine level by overexpressing ODC also raised *MAT2A*, *c-Jun* and *c-Fos* mRNA levels. *MAT2A* mRNA level was reduced when ODC was knocked down but exogenous putrescine was able to still raise it, indicating putrescine's effect on *MAT2A* expression is independent of ODC. We reported that the human *MAT2A* promoter contains a functional AP-1 site that is activated by treatment with tumor necrosis factor α and IGF-1^{13,26}. This prompted us to examine whether raising putrescine by overexpressing ODC or exogenous treatment can activate the *MAT2A* promoter activity. Indeed we found that both treatments increased the *MAT2A* promoter activity and putrescine treatment increased c-Jun binding to the *MAT2A* promoter region that contains the functional AP-1 site.

Previous studies have shown overexpression of ODC in colon cancer^{11,25}. Our in vitro studies suggest this could increase *MAT2A* expression at least via AP-1 activation. We next examined whether this crosstalk occurs in colorectal cancer specimens. Consistent with our in vitro results, *MAT2A*, *ODC*, *c-Jun* and *c-Fos* mRNA levels are higher in cancer specimens as compared to adjacent non-tumorous tissues and the expression level of ODC correlates nicely with that of *MAT2A* in the cancer specimens. Higher *MAT2A*, ODC and c-Jun protein levels were confirmed in the same colon cancer specimens.

Since the expression of *MAT2A*^{26,27} and *ODC*^{28,29} is induced in HCC, we also examined whether there is an interplay among *MAT2A*, ODC and putrescine in liver cancer cells. We used HepG2 cells because we had previously observed in these cells induction of *MAT2A* was required for leptin to increase growth¹⁵ and *MAT2A* promoter activity was activated by AP-1²⁶. Indeed, essentially the same findings were observed in HepG2 cells, where knockdown of *MAT2A* induced apoptosis and growth suppression, which were protected by putrescine. Knockdown of *MAT2A* also lowered ODC protein level, and this was prevented by exogenous putrescine. Finally, putrescine treatment also induced the expression of both *MAT2A* and ODC. Thus, the same interplay also occurs in liver cancer. The only difference is that the dose of putrescine required was much higher in HepG2 cells, which is consistent with previous reports^{16,17}. The reason for this difference in putrescine dose response between RKO and HepG2 cells is not clear at present.

Taken together, our current study describes a crosstalk between *MAT2A* and polyamines in colorectal and liver cancer where increased ODC expression can activate the *MAT2A* gene via AP-1 and increased *MAT2A* expression in turn provides an increased supply of SAME for polyamine biosynthesis. In normal cells, ODC expression is tightly regulated so that increased polyamine levels will exert a feedback regulation to lower ODC activity by multiple mechanisms³⁰. However, dysregulation of ODC in colorectal and liver cancer allows this crosstalk to perpetuate and further enhance cancer cell growth.

CONCLUSIONS

There is a crosstalk between *MAT2A* and polyamine where increased *MAT2A* expression that occurs in human colon and liver cancer can enhance polyamine biosynthesis and higher polyamine level can increase AP-1 binding and *trans*-activation of the *MAT2A* gene. This along with higher ornithine decarboxylase expression that also occurs in colon and liver

cancer all feed forward to induce growth. This work is the first to identify this crosstalk and the impact it has on enhancing growth.

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Abbreviations used (alphabetical order)

AP-1	activator protein-1
BrdU	5-bromo-2'-deoxyuridine
ChIP	chromatin immunoprecipitation
dcSAmE	decarboxylated SAmE
EGF	epidermal growth factor
HCC	hepatocellular carcinoma
HPLC	high-performance liquid chromatography
IGF-1	insulin-like growth factor-1
MAT	methionine adenosyltransferase
ODC	ornithine decarboxylase
PCR	polymerase chain reaction
SAmE	S-adenosylmethionine
Sc	scrambled siRNA
siRNA	small interfering RNA

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Highlights

- MAT 2A knockdown depletes putrescine and leads to apoptosis.
- Putrescine attenuates MAT2A knockdown-induced apoptosis and growth suppression.
- Putrescine induces AP-1, which activates MAT2A promoter to increase its expression.
- Putrescine increases ornithine decarboxylase expression, which induce MAT2A promoter.
- Expression of MAT2A correlates with that of ornithine decarboxylase in colon cancer.

Fig. 1. Effects of MAT2A siRNA treatment on SAME and polyamine levels, apoptosis, and cell growth

(A) RKO cells were treated with MAT2A siRNA (siMAT2A, 12 pM) or scrambled siRNA (Sc), and MAT2A mRNA levels were measured by real-time PCR after 48 hours. (B) SAME levels were measured by high-performance liquid chromatography (HPLC). Results are expressed as nmol of SAME per milligrams of protein. Apoptosis (C, D) and cell growth (E) were determined by Hoechst staining and BrdU incorporation after 48 hours, respectively. (F) Polyamine levels were measured by HPLC. Results are expressed as nmol of putrescine, spermidine and spermine per milligrams of protein. Results represent mean $\pm$ SEM from 3 independent experiments performed in triplicate for all variables. * $p < 0.001$ vs. Sc.

Fig. 2. Role of putrescine on MAT2A knockdown-induced apoptosis and growth suppression
 RKO (A and C) and HepG2 (B and D) cells were treated with Sc or siMAT2A (12 pM for 48 hours) $\pm$ putrescine in varying doses for the last 24 hours. For A and B, results from 3 experiments are presented as mean percentage of apoptotic cells $\pm$ SEM. * $p < 0.05$ vs. Sc, † $p < 0.05$ vs. siMAT2A. For C and D, cell growth was analyzed by BrdU incorporation assay. Mean values $\pm$ SD are shown for 3 independent experiments. * $p < 0.05$ vs. Sc, † $p < 0.05$ vs. siMAT2A.

Fig. 3. Effects of MAT2A knockdown and putrescine on MAT2A and ODC expression
 RKO (A and B) and HepG2 (C and D) cells were treated with Sc or siMAT2A (12 pM for 48 hours) ± putrescine in varying doses for the last 24 hours. MAT2A and ODC expression was determined by real-time PCR (A and C) and Western blotting (B and D). MAT2A mRNA results represent mean±SEM from 3 to 4 independent experiments performed in duplicate. * $p < 0.04$ vs. Sc, † $p < 0.03$ vs. siMAT2A. Densitometric changes in protein levels are summarized in the boxes adjacent to blots. Results are expressed as % of Sc from 3 to 4 independent experiments, * $p < 0.01$ vs. Sc, † $p < 0.05$ vs. siMAT2A.

Fig. 4. Effects of MAT2A knockdown, putrescine and ODC on MAT2A, c-Fos and c-Jun expression

RKO (A, B, E) and HepG2 (C, D, F) cells were treated with siMAT2A (48 hours) or Sc alone, or siMAT2A plus putrescine (100 pM for RKO, 100 μM for HepG2, last 24 hours), ODC overexpression vector (24 hours), empty vector (EV) control or putrescine (same dose as above for 24 hours) and *MAT2A*, *ODC*, *c-Fos* and *c-Jun* mRNA levels were determined by real-time PCR. In parts E and F, cells were treated with ODC siRNA or putrescine alone or in combination and *ODC* and *MAT2A* mRNA levels were determined. All results are mean±SEM from 3 to 4 independent experiments performed in duplicates. *p<0.05 vs. Sc (A, C, E and F) or EV (B and D), †p<0.05 vs. siODC (E and F).

Fig. 5. Effects of ODC overexpression and putrescine on MAT2A promoter activity
 (A) RKO cells were transfected transiently MAT2A promoter construct -571/+60-LUC or pGL-3 enhancer vector and co-transfected with ODC overexpression vector or treated with putrescine (100 pM) for 24 hours. Results represent mean $\pm$ SEM from 3 independent experiments performed in triplicate. Data are expressed as relative luciferase activity to that of pGL-3 enhancer vector control. * p <0.001 vs. pGL-3, † p <0.002 vs. -571/+60-LUC construct control. (B) Cells were treated with putrescine (100 pM) for 24 hours and processed for ChIP using an anti-c-Jun antibody and a ChIP assay kit according to the manufacturer's protocol. The AP-1 element in MAT2A promoter fragment from c-Jun immunoprecipitates was amplified by PCR using the corresponding primers. The regulatory

fragment was directly amplified by PCR of genomic DNA from each group as input. Value are average of 3 separate experiments $\pm$ SEM. * $p < 0.001$ vs. untreated control.

Fig. 6. Expression of MAT2A, ODC, c-Fos and c-Jun in human colon cancer

(A) 13 human colon cancer specimens and respective matched adjacent non-tumorous (NT) tissues were processed to measure mRNA levels of MAT2A, ODC, c-Fos and c-Jun by real-time PCR. Results are expressed as fold of matched NT colon tissue (mean±SEM). * $p < 0.05$ vs. NT colon tissue. (B) Western blotting in select paired colon cancer specimens (N for non-tumorous, T for tumor) show increased protein levels of MAT2A, ODC and c-Jun. Densitometric changes are expressed as % of NT tissues and summarized in the box next to the blot. * $p < 0.04$ vs. NT. (C) The direct correlation between the levels of ODC and MAT2A mRNA levels was tested with a Pearson correlation test ($r = 0.72$).

Table 1

MAT2A, ODC, c-Fos and c-Jun Expression in Colon Cancer Specimens.

				Grade			mRNA level in CA, fold over NT				
#	Gender	Age	Site	T	M	N	Histology	MAT2A	ODC	c-Fos	c-Jun
1	F	70	Rectum	T3N0	0	0	Moderate	1.43	1.61	2.12	1.93
2	M	79	Sigmoid	T3N0	0	0	Moderate	1.78	1.10	0.90	0.75
3	F	54	Ascending colon	T3N1	0	1	Moderate	4.45	6.94	2.40	2.60
4	F	73	Ascending colon	T3N0	0	0	Moderate	1.98	3.92	4.33	2.53
5	M	73	Ascending colon	T3N0	0	0	Poor	1.05	0.34	0.53	0.92
6	M	78	Sigmoid	T3N0	0	0	Moderate	1.25	1.40	1.30	1.47
7	M	55	Cecum	T3N0	0	0	Moderate	4.79	10.51	1.31	1.22
8	M	68	Descending colon	T1N0	0	0	High	1.24	5.62	4.26	1.50
9	F	64	Ascending colon	T3N0	0	0	Moderate	1.64	8.40	1.76	2.42
10	M	76	Sigmoid	T2N0	0	0	Moderate	1.24	5.01	1.61	1.46
11	M	68	Sigmoid	T2N1	0	1	Moderate	1.69	3.34	1.46	0.85
12	M	70	Sigmoid	T3N1	0	1	Moderate	1.12	1.45	1.38	1.05
13	M	56	Rectum	T4N2	1	1	Poor	0.79	0.40	1.03	1.39

CA = cancer, NT = adjacent non-tumorous tissue