AD_____

Award Number: DAMD17-03-1-0024

TITLE: Activation of Polyamine Catabolism as a Novel Strategy for Treating and/or Preventing Human Prostrate Cancer

PRINCIPAL INVESTIGATOR: Carl W. Porter, Ph.D.

CONTRACTING ORGANIZATION: Roswell Park Cancer Institute
Buffalo, NY 14263

REPORT DATE: March 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2004	Annual (1 Mar 2003 - 28 Feb 2004)

4. TITLE AND SUBTITLE

Activation of Polyamine Catabolism as a Novel Strategy for Treating and/or Preventing Human Prostate Cancer

5. FUNDING NUMBERS

DAMD17-03-1-0024

6. AUTHOR(S)

Carl W. Porter, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Roswell Park Cancer Institute
Buffalo, NY 14263

E-Mail: Carl.porter@roswellpark.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The relationship of polyamines to the prostate is unique among all tissues since, in addition to synthesizing these molecules for cell growth, the gland produces massive quantities for export into semen. It might, therefore, be expected that prostatic tumors could exhibit atypical polyamine-related regulatory responses. We propose that activation of polyamine catabolism may have unique therapeutic potential against prostate carcinoma. Thus far, we have validated this hypothesis by demonstrating that (a) conditional overexpression of the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT) causes growth inhibition in LNCaP prostate carcinoma cells and (b) cross-breeding of SSAT transgenic mice with prostate cancer prone TRAMP mice markedly suppresses genitourinary tumor development via an apparent depletion of the SSAT cofactor acetyl-CoA. Depletion of the critical metabolite, acetyl-CoA, appears to be responsible for a selective effect on the prostate and prostate proliferative disease. The utimate goal of these studies is to genetically validate the concept that small molecule induction of SSAT represents a promising anticancer strategy against prostate cancer and that it is worthy of small molecule discovery and development.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Acetyl-CoA, LNCaP cells, polyamines, prostate, SSAT,TRAMP mice, transgenic mice	29
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

_✓__ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature 03/20/04 Date

TABLE OF CONTENTS

DAMD17-03-1-0024

REPORT BODY ***(Tables & Figures located in Appendix 1)***

A. Introduction:

Polyamines are organic cations found in all cells and known to be essential for the initiation and maintenance of cell growth. Intracellular levels are sensitively maintained by a series of regulatory responses. The relationship of polyamines to the prostate is unique among all tissues since in addition to synthesizing these molecules for cell growth, the gland produces massive quantities for export into semen. It might, therefore, be expected that prostatic tumors could exhibit atypical polyamine-related regulatory responses. We have recently observed that in contrast to other normal and tumor cell types, two of three prostate carcinoma lines displayed an altered ability to regulate polyamine transport in responses to polyamine analog or inhibitors (1). More specifically, two lines could not down-regulate uptake in response to analogs and one could not up-regulate it in response to polyamine depletion caused by inhibitors. In recognition of the unique physiology of the prostate gland, Heston and collaborators were among the first to propose that targeting polyamine biosynthesis may be particularly effective against prostate cancer (2). Although most of these efforts have focused on inhibition of polyamine biosynthesis, we propose that activation of polyamine catabolism may have unique therapeutic potential against prostate carcinoma. The concept derives from our studies with polyamine analogs where, for example, we have shown that polyamine analogs such as N^1,N^{11}-diethylnorspermine (DENSPM) down-regulate polyamine biosynthesis at the level of ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) and at the same time, potently (i.e. >200-fold) up-regulate polyamine catabolism at the level of spermidine/spermine N^1-acetyltransferase (SSAT, 3). Several lines of evidence support the idea that analog induction of SSAT and hence, activation of polyamine catabolism, is a critical determinant of DENSPM drug action. For example, DENSPM growth inhibition among tumor cell lines correlates with the extent to which SSAT is induced (4, 5), and analogs that differentially induce SSAT inhibit cell growth in a correlative manner (6, 7). As more direct evidence for this relationship, McCloskey *et al.* (8) showed that DENSPM-resistant CHO cells are unable to induce SSAT. Recently, we (9, 10) reported that siRNA interference with DENSPM induction of SSAT prevented polyamine pool depletion and at the same time, blocked analog-induced apoptosis in human melanoma cells.

The aforementioned studies relate to SSAT induction in the context of analog treatment but they do not address what happens when SSAT is selectively induced in cells. In the absence of a specific SSAT inducer, this could only be evaluated using genetic systems such as conditional overexpression as a means to mimic small molecule induction of the enzyme. In an earlier report (11), we showed that conditional overexpression of SSAT leads to polyamine pool depletion and growth inhibition in MCF-7 breast carcinoma cells. The growth inhibition, however, was gradual in onset and incomplete in effect. On the basis of rationale suggesting that prostate carcinoma may be more sensitive to perturbations in polyamine homeostasis, we have now investigated the metabolic and antiproliferative consequences of conditional SSAT overexpression in *in vitro* and *in vivo* systems. *The bottom-line goal of these studies is to genetically validate the concept that small molecule induction of SSAT is a useful anticancer strategy against prostate cancer and thus, worthy of small molecule discovery and development.*

B. *Originally Proposed Tasks*

Task 1. To evaluate the cellular and metabolic consequences of conditional SSAT over-expression in cultured LNCaP cells as a means to genetically validate the antiproliferative potential of this strategy .

Task 2. To determine the generality of cellular and metabolic consequences seen in Task 1 on representative prostate cancer cell lines in which SSAT has been adenovirally transduced.

Task 3. To confirm that responses to SSAT over-expression seen *in vitro* (as determined in Tasks 1 and 2) are translatable to antitumor activity using three novel *in vivo* approaches. These include (**a**) conditional regulation of SSAT in LNCaP tumors to evaluate therapeutic potential, (**b**) adenoviral based SSAT therapy of human prostate tumor xenografts to test therapeutic generality and (**c**) cross-breeding SSAT over-expressing transgenic mice (12) with prostate carcinoma prone (TRAMP, 13) mice to evaluate chemopreventive potential.

C. *Progress:*

Task 1. *To evaluate the cellular and metabolic consequences of conditional SSAT over-expression in cultured LNCaP cells as a means to genetically validate the antiproliferative potential of this strategy .*

On the basis of findings with polyamine analogs, we hypothesize that activation of the polyamine catabolism at the level of spermidine/spermine N^1-acetyltransferase (SSAT) will also inhibit cell growth. The hypothesis was first confirmed in MCF-7 breast carcinoma cells where conditional SSAT overexpression led to polyamine pool depletion and gradual growth inhibition (11). In Task 1, we investigated the possibility that due to unique levels of polyamine biosynthesis in the prostate gland, tumors derived from it may be particularly sensitive to genetic activation of polyamine catabolism. Thus, SSAT was conditionally overexpressed in LNCaP prostate carcinoma cells via a tetracycline-regulatable (Tet-off) system (Figure 1). Tetracycline removal resulted in a rapid ~10-fold increase in SSAT mRNA and a ~20-fold increase in enzyme activity (Figure 2). Consistent with this response, high levels of the SSAT products N^1-acetylspermidine, N^1-acetylspermine and N^1,N^{12}-diacetylspermine were found to accumulate both intracellularly and extracellularly (Figure 3). SSAT induction led to significant growth inhibition which in contrast to MCF-7 cells (11), was not accompanied by polyamine pool depletion (Figures 3 and 4). Rather, intracellular spermidine and spermine pools were elevated or maintained at control levels by a robust compensatory increase in biosynthesis at the levels of ODC and SAMDC activities (Figure 5). This, in turn, gave rise to a high rate of metabolic flux through both the biosynthetic and catabolic pathways. Interruption of that flux with α-difluoromethylornithine (DFMO), an inhibitor of ODC and well-known antiproliferative agent, unexpectedly prevented growth inhibition during Tet removal (Figure 6). As diagramed in Figure 7, it appears that flux-induced growth inhibition may derive from excess product accumulation (i.e. acetylated polyamines) and/or to metabolite depletion such as a ~50% reduction in *S*-adenosylmethionine (SAM) pools or a ~50% decrease in the SSAT cofactor acetyl-CoA (Figure 8). In summary, the results demonstrate that activation of polyamine catabolism by conditional SSAT overexpression leads to: (a) altered polyamine pool homeostasis (b) a compensatory increase in polyamine biosynthesis, (c) heightened metabolic flux through both the biosynthetic and catabolic pathways, (d) depletion

of metabolite pools such as the polyamine precursor SAM and the SSAT cofactor, acetylCoenzyme A (acetyl-CoA) and (e) rapid and sustained growth inhibition. Whether these responses are unique to prostate derived tumor cells remains to be demonstrated. Likewise, the metabolic perturbation responsible for cell growth inhibition is also uncertain but given the role of SAM in methylation reactions and acetyl-CoA in various aspects of intermediate metabolism, fatty acid synthesis and histone acetylation, it is reasonable to assume that under conditions of SSAT induction, substances could become limiting to cell growth. In addition to elucidating previously unrecognized metabolic consequences to SSAT overexpression, this important study shows that activation of polyamine catabolism gives rise to growth inhibition and thereby provides genetic validation of the idea that small molecule induction of SSAT may be effective as an anticancer strategy for treating prostate cancer. The critical studies involve in vivo validation of this concept as proposed in Task 3.

Task 2. To determine the generality of cellular and metabolic consequences seen in Task 1 on representative prostate cancer cell lines in which SSAT has been adenovirally transduced.

As shown in Figure 9, we successfully prepared and purified a SSAT expressing adenovirus in which human SSAT cDNA was cloned into a replication-deficient adenoviral system containing an internal ribosomal entry site (IRES) for GFP. Thus, both SSAT and GFP were under the control of a CMV promoter for maximum expression. The virus was prepared in human 293 cells which seemed to be immune to adverse effects on cell growth. The goal of these studies was to use the adenovirus to transduce SSAT into a number of prostate cancer cell lines and examine the effects on polyamine metabolism and cell growth. The expectation was that findings would be similar to those with LNCaP cells in Task 1, namely, that growth would be inhibited. An unexpected complication was that while the SSAT adenovirus could be used to transduce SSAT into 293 cells in the absence of toxicity, this was not the case with LNCaP or PC-3 cells. In these prostate carcinoma cells, the MOI for SSAT-bearing adenovirus was the same as that of the vector-bearing adenovirus. Northern blots indicated that SSAT was not effectively transduced presumably because it had aged for nearly one year after being purified. Taking these difficulties in consideration with the success of studies undertaken in Task 3, we propose to abandon Task 2 and focus on additional cross-breeding opportunities under Task 3. These include making use of newly developed SSAT knock-out mice and SMO transgenic mice.

Task 3. *To confirm that responses to SSAT over-expression seen in vitro (as determined in Tasks 1 and 2) are translatable to antitumor activity using three novel in vivo approaches. These include (a) conditional regulation of SSAT in LNCaP tumors to evaluate therapeutic potential, (b) adenoviral based SSAT therapy of human prostate tumor xenografts to test therapeutic generality and (c) cross-breeding SSAT over-expressing transgenic mice (12) with prostate carcinoma prone (TRAMP, 13) mice to evaluate chemopreventive potential.*

We showed in Task 1 that activation of polyamine catabolism via conditional overexpression of SSAT has antiproliferative consequences in LNCaP prostate carcinoma cells. Growth inhibition was found to be directly related to high metabolic flux arising from activation of polyamine catabolism and a compensatory increase in polyamine biosynthetic activity. Among other metabolic perturbations, the latter led to a depletion of SAM and acetylCoA pools in a manner that may be linked to the growth effects. In Task 2, we seek to examine the *in vivo* consequences of SSAT overexpression in a mouse model genetically predisposed to develop

prostate cancer. TRAMP (TRansgenic Adenocarcinoma of the Mouse Prostate) female C57BL/6 mice that express the SV40 early genes (T/t antigens) under an androgen-driven probasin promoter (13) were cross-bred with male C57BL/6 transgenic mice that systemically overexpress SSAT (12). Experiments were staged by small animal nuclear magnetic resonance image (MRI) tracking of tumor appearance and growth (Figure 10). At 30 weeks of age (Figure 11), the average genitourinary tract weights for for TRAMP mice were 1,417 ± 181 mg compared to 344 ± 52 mg for TRAMP/SSAT mice (Figure 12). Thus, SSAT overexpression reduces the size of the normal prostate and suppresses prostate tumors development. Since this could represent a delay in tumor development, we examined the effects at a later age. By 36 wk, the average TRAMP genitourinary tract increased by ~2-fold relative to the 30 wk data while those of TRAMP/SSAT actually decreased by 33% as opposed to growing larger. Since the differential effect increased between groups, we conclude that the tumor suppressive effect was not temporary but rather progressive. The average total histopathological grade for 30 wk TRAMP prostates was 5.0 ± 0.2 as compared to 4.2 ± 1.0 for TRAMP/SSAT prostates (Table 1) indicating that tumor growth but not tumor progression was suppressed. Immunohistochemistry revealed that SV40 large T-antigen expression in the prostate epithelium was similar in TRAMP versus TRAMP/SSAT mice indicating that the driving oncogene was intact and unaffected (Figure 13). Consistent with the 18-fold increase in SSAT activity in the TRAMP/SSAT transgenics, intracellular N^1-acetylspermidine and putrescine pools were markedly increased (i.e. 35-fold and 16-fold, respectively) relative to the TRAMP mice while spermidine and spermine pools were largely unaffected due to a compensatory 5- to 7-fold increase in the biosynthetic enzyme activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase (Table 2). This increase in biosynthesis is presumed to lead to heightened metabolic flux as was detected in LNCaP cells. This in turn gave rise to a marked decrease in prostate levels of the SSAT cofactor acetyl-CoA which 71% lower in TRAMP/SSAT than in TRAMP mice. By contrast, liver levels of SSAT in these same mice were relatively unaffected (Figure 14). While it is known from earlier studies (12) that most SSAT mouse tissues display a compensatory increase in biosynthetic enzyme activities, it is not known whether acetyl-CoA levels are decreased as in the prostate or remain the same as in the liver. If the latter prevails, unique metabolic dependence of the prostate on acetyl-CoA may account for the observed tissue selectivity of the SSAT antitumor and antiprostate effects. These findings are consistent with observations described above for LNCaP cells and provide important and previously unrecognzied insights into metabolic linkages between polyamine catabolism and acetylCoA metabolism that should be taken account whenever SSAT is induced or overexpressed. In addition to these mechanistic connections between SSAT, acetyl-CoA and antitumor effects, the present study definitively validates the utility of activated polyamine catabolism as an antiproliferative strategy. Given the fact that SSAT overexpression affected normal prostates (SSAT) as well as neoplastic prostates (TRAMP/SSAT), the approach may find usefulness in treating prostatic hyperplasia.

Unscheduled Findings.

Findings under Tasks 1 and 2 provide genetic evidence for the discovery and development of small molecule inducers of SSAT as potential anticancer agents. Although this process has not yet begun, we have hit upon an relevant lead involving a widely used anticancer agent (14). The finding could have important implications in understanding drug action and in designing new anticancer drug combinations. The lead derives from an Affymetrix Oligonucleotide array analysis of >10,000 genes of A2780 ovarian carcinoma cells exposed to oxaliplatin or cisplatin. Unexpectedly, SSAT was among the top 20 up-regulated genes. We went on to confirm by

Northern blot that SSAT gene expression increased in a dose-dependent manner, with oxaliplatin being twice as effective as cisplatin. The 15-fold increase in oxaliplatin-induced SSAT mRNA was accompanied by only a 2-fold increase in enzyme activity. In cells treated 2 h with oxaliplatin and then exposed for 24 h to the polyamine analogue, N^1,N^{11}-diethylnorspermine (DENSPM), a synergistic >200-fold increase in SSAT activity was observed together with markedly reduced polyamine pools. Cytotoxicity analysis of oxaliplatin treatment followed by low dose DENSPM suggests a distinctly greater than additive drug interaction. Although these studies were conducted under conditions selected for microarray analysis (i.e. short exposures/high dose) we have since deployed oxaliplatin at pharmacologically attainable (i.e. 5 μM for 20 h) and found an even greater increase in SSAT mRNA which during cotreatment with DENSPM, increased synergistically to even greater SSAT levels than described above. These results reveal a mechanistic synergy whereby oxaliplatin potently induces SSAT mRNA and DENSPM facilitates its translation to enzymatically active protein. With appropriate *in vitro* and *in vivo* optimization, these findings could lead to an effective therapeutic strategy. While oxaliplatin is not usually used in treating prostatic cancer, there have been some reports that it is quite active against this disease.

D. Key Research Accomplishments:

- Conditional expression of SSAT in LNCaP prostate carcinoma cells leads to disturbances in polyamine homeostasis and a compensatory increase in metabolic flux through both the biosynthetic and catabolic arms of the polyamine pathway. Inhibition of ODC prevents growth inhbition, and thereby provides a direct linkage between flux and growth inhibition.

- We showed for the first time that overexpression of SSAT impacts on other aspects of cellular metabolism including SAM pools and acetyl-CoA pools. The former may affect methylation and the latter can affect such processes as fatty acid synthesis, intermediate metabolism and histone acetylation. It is not clear how ~50% reduction in SAM and acetyl-CoA pools contributes to cell growth inhibition.

- Cross-breeding SSAT transgenic mice with TRAMP mice that are genetically predisposed to prostate cancer, markedly suppresses tumor development and this effect correlates closely with massive reductions in prostatic acetylCoA pools. The extent of the effect is greater than seen with any previously reported cross-breeding of TRAMP mice.

- Taken together, the data provide in vitro and in vivo genetic validation of the concept that pharmacological induction of SSAT could represent an effective therapeutic strategy for prostate cancer. Recent recognition that oxaliplatin and cisplatin drugs potently induce SSAT may be relevant to this idea as well.

Related findings under different Porter Programs.

- We have genomically identified and biochemically characterized three new polyamine catabolic enzymes--polyamine oxidase (PAO), spermine oxidase (SMO) and a second SSAT (SSAT-2, . We have developed antibodies to all thre which are useable in Western blots and in immunohistochemistry (IHC). IHC indicates that PAO, but not SMO, preferentially stains the prostate epithelium and that this staining decreases in prostate cancer (unpublished). We

are now using these antibodies to screen various tissue microarrays including prostate and cell line microarrays obtained from the NCI.

- Our collaborators (Dr. Juhani Janne of Kuopio, Finaland) have developed a SSAT knock-out mouse and found that it lacks an apparent phenotype (XX and unpublished data). In collaboration with Dr. Frank Berger, we have cross-bred the SSAT ko mice with APC^{min} mice that are genetically programmed to develop intestinal cancer and found a substantial decrease in adenomatous polyp formation. As indicated below, we have begun to cross-breed the SSAT knock-out mice with the TRAMP mice to examine whether in this model, it might increase tumorigenesis.

- In collaboration with Dr. Leena Alhonen of Kuopio Finland, we have developed a SMO transgenic mouse which after being characterized, will be cross-bred with the TRAMP mouse. SMO catalyzes the direct back-conversion of spermine to spermidine without going through the acetylated spermine step required by PAO. It is an entirely new enzyme that was not previously recognized until our characterization of it (16)

E. Reportable Outcomes: **Copies will be provided when In Press or Published**

- Hector, S., Porter, C.W., Kramer, D.L., Clark, K., Chen, Y. and Pendyala, L., Polyamine Catabolism in platinum drug action: interactions between oxaliplatin and the polyamine analogue N^1, N^{11}-diethylnorspermine at the level of spermidine/spermine N^1-acetyltransferase. Molecular Cancer Therapeutics (Acceptance pending revision).

- Kee, K., Vujcic, S., Kisiel, N., Diegelman, P., Kramer, D.L., and Porter, C.W. Activation of polyamine catabolism as a novel strategy for treating and/or preventing prostate cancer. Proc. Am. Assoc. Cancer Res. 44:1277, 2003.

- Kee, K., Vujcic, S., Merali, S., Diegelman, P., Kisiel, N., Powell, C.T., Kramer, D.L., and Porter, C.W., Metabolic and antiproliferative consequences of activated polyamine catabolism in LNCaP prostate carcinoma cells, (Submitted).

- Kee, K, Foster, B.A., Merali, S., Vujcic, S., Mazurchuk, R.V., Kramer, D.L. and Porter, C.W., Activated polyamine catabolism depletes acetyl-coA pools and suppresses prostate tumor growth in TRAMP mice. (To be submitted).

F. Conclusions & Future Directions:

These finding provide genetic evidence for the concept that selective small molecule induction of SSAT may be effective in treating prostate hyperplasia and cancer. The findings further defines the downstream metabolic consequences of this strategy include marked reduction of acetyl-CoA pools in a manner that correlates very closely with suppression of tumor growth. Since this seems to occur selectively in both the normal and neoplastic prostate and since the phenotypic consequences of sustained transgenic overexpression of SSAT include only hairloss and female infertility, this strategy appears to be selectively directed at prostate disease. Future directions under this funding source will include cross breeding SSAT knockout mice with

TRAMP mice, characterizing SMO transgenic mice and cross breeding them with TRAMP mice and further studies into the role of acetyl-CoA in SSAT-induced growth inhibition of LNCaP cells as well as in DENSPM induced growth inhibition (which includes potent SSAT induction). In studies beyond this funding source, resources and expertise will be recruited for high throughput search for a selective small molecule inducer of SSAT followed by lead optimization chemistry.

G. References:

1. Mi, Z., Kramer, D. L., Miller, J. T., Bergeron, R. J., Bernacki, R., and Porter, C. W. Human prostatic carcinoma cell lines display altered regulation of polyamine transport in response to polyamine analogs and inhibitors. Prostate, *34:* 51-60, 1998.
2. Heston, W. D. Prostatic polyamines and polyamine targeting as a new approach to therapy of prostatic cancer. Cancer Surveys, *11:* 217-238, 1991.
3. Porter, C. W., Regenass, U., and Bergeron, R. J. Polyamine inhibitors and analogs as potential anticancer agents. *In:* R. H. Dowling, U. R. Folsch, and C. Loser (eds.), Falk Symposium on Polyamines in the Gastrointestinal Tract, pp. 301-322. Dordrecht, Netherlands: Kluwer Academic Publishers Group, 1992.
4. Casero, R. A., Jr., Celano, P., Ervin, S. J., Porter, C. W., Bergeron, R. J., and Libby, P. R. Differential induction of spermidine/spermine N^1-acetyltransferase in human lung cancer cells by the bis(ethyl)polyamine analogues. Cancer Res, *49:* 3829-3833, 1989.
5. Shappell, N. W., Miller, J. T., Bergeron, R. J., and Porter, C. W. Differential effects of the spermine analog, N^1,N^{12}-bis(ethyl)-spermine, on polyamine metabolism and cell growth in human melanoma cell lines and melanocytes. Anticancer Res, *12:* 1083-1089, 1992.
6. Pegg, A. E., Wechter, R., Pakala, R., and Bergeron, R. J. Effect of N^1,N^{12}-bis(ethyl)spermine and related compounds on growth and polyamine acetylation, content, and excretion in human colon tumor cells. J Biol Chem, *264:* 11744-11749, 1989.
7. Porter, C. W., Ganis, B., Libby, P. R., and Bergeron, R. J. Correlations between polyamine analogue-induced increases in spermidine/spermine N^1-acetyltransferase activity, polyamine pool depletion, and growth inhibition in human melanoma cell lines. Cancer Res, *51:* 3715-3720, 1991.
8. McCloskey, D. E. and Pegg, A. E. Altered spermidine/spermine N^1-acetyltransferase activity as a mechanism of cellular resistance to bis(ethyl)polyamine analogues. J Biol Chem, *275:* 28708-28714, 2000.
9. Chen, Y., Kramer, D. L., Li, F., and Porter, C. W. Loss of inhibitor of apoptosis proteins as a determinant of polyamine analog-induced apoptosis in human melanoma cells. Oncogene, *22:* 4964-4972, 2003.
10. Chen, Y., Kramer, D. L., Jell, J., Vujcic, S., and Porter, C. W. Small interfering RNA suppression of polyamine analog-induced spermidine/spermine N^1-acetyltransferase. Mol Pharmacol, *64:* 1153-1159, 2003.
11. Vujcic, S., Halmekyto, M., Diegelman, P., Gan, G., Kramer, D. L., Janne, J., and Porter, C. W. Effects of conditional overexpression of spermidine/spermine N^1-acetyltransferase on polyamine pool dynamics, cell growth, and sensitivity to polyamine analogs. J Biol Chem, *275:* 38319-38328, 2000.
12. Pietilä, M., Alhonen, L., Halmekytö, M., Kanter, P., Jänne, J. and Porter, C.W., Activation of polyamine catabolism profoundly alters tissue polyamine pools and affects hair growth and female fertility in transgenic mice overexpressing spermidine/spermine N^1-acetyltransferase. J. Biol. Chem. 272:18746-18751, 1997.

13. Greenberg, N.M., DeMayo, F., Finegold, M.J., Medina, D., Tilley, W.D., Aspinal, J.O., Cunha, G.R., Donjacour, A.A., Matusik, R.J., Rosen, J.M., Proc. Nat'l. Acad. Sci. USA 92:3439-43, 1995.
14. Hector, Suzanne, Porter, C. W., Kramer, D. L., Clark, K, Chen, Y, and Pendyala, L., Polyamine catabolism in platinum drug action: interactions between oxaliplatin and the polyamine analogue N^{I}, N^{II}-diethylnorspermine at the level of spermidine/spermine n^{I}-acetyltransferase. Molecular Cancer Therapeutics (Accepted pending minor revisions).
15. Vujcic, S., Liang, P., Diegelman, P., Kramer, D.L. and Porter, C.W. Genomic identification and biochemical characterization of the mammalian polyamine oxidase involved in polyamine back-conversion. Biochem J. 370:19-28, 2003.
16. Vujcic, S., Diegelman, P., Bacchi, C.J., Kramer, D.L., and Porter, C.W., Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin. Biochem. J. 367:665-675, 2002.
17. Chen, Y., Vujcic, S., Liang, P., Diegelman, P., Kramer, D., and Porter, C.W. Genomic identification and biochemical characterization of a second spermidine/spermine N^{I}-acetyltransferase. Biochem J. (Accelerated Pub) 373:661-667, 2003.
18. Niiranen, K., Pietila, M., Pirttila, T.J., Jarvinen, A., Halmekyto, M., Korhonen, V.P., Alhonen, L., Janne, J. Targeted disruption of spermidine/spermine N1-acetyltrasferase gene in mouse embryonic stem cells. J. Biol. Chem. 277:25323-8, 2002.

APPENDIX 1

TABLES AND FIGURE

DAMD17-03-1-0024

Table 1. Genitourinary Disease Index of TRAMP/SSAT Mice

Mouse Genotype	Age (wk)	# of Animals	Average Prostate Grade			Average GU Weight (g)	GU Disease Index	*P*-value
			Overall	Worst	Total			
TRAMP	30	15	2.1 ± 0.1	2.9 ± 0.1	5.0 ± 0.2	1.44 ± 0.18	7.44 ± 1.12	<0.0001
TRAMPxSSAT	30	10	2.0 ± 0.2	2.2 ± 0.2	4.2 ± 1.0	0.36 ± 0.06	1.51 ± 0.27	
TRAMP	36	8	2.8 ± 0.4	3.5 ± 0.4	6.3 ± 0.7	2.92 ± 0.81	22.57 ± 9.75	<0.06
TRAMPxSSAT	36	6	2.5 ± 0.5	3.2 ± 0.5	5.6 ± 1.0	0.24 ± 0.12	2.08 ± 1.33	

GU Disease Index = (average overall grade + average highest grade) x average GU weight, as determined per animal.
Statistical significance (*P*-value) was determined by analysis of variance (ANOVA) with Fisher's PLSD test.

Table 2. Tissue Polyamine Metabolism in TRAMP x SSAT Littermates

Mouse Genotype	Tissue	Polyamine Enzymes			Polyamine Pools			
		SSAT	ODC	SAMDC	AcSpd	Put	Spd	Spm
		(pmol/min/mg protein)	(pmol/h/mg protein)		(pmol/mg protein)			
Wild-type	Prostate	5 ± 1	<20	60 ± 33	<40	140 ± 12	3,710 ± 402	7,990 ± 739
SSAT	Prostate	90 ± 20	190 ± 23	560 ± 81	2,440 ± 613	3,730 ± 805	7,350 ± 423	7,030 ± 458
TRAMP	Prostate	5 ± 1	<20	50 ± 19	<40	214 ± 34	4,830 ± 443	7,960 ± 881
TRAMP/SSAT	Prostate	90 ± 28	100 ± 21	370 ± 88	1,390 ± 187	3,520 ± 529	4,780 ± 858	5,280 ± 662
Wild-type	Liver	15 ± 4	<20	120 ± 45	<40	<40	7,900 ± 368	8,510 ± 225
SSAT	Liver	50 ± 12	180 ± 32	940 ± 134	790 ± 210	4,260 ± 805	13,990 ± 794	5,060 ± 146
TRAMP	Liver	15 ± 2	<20	110 ± 75	<40	<40	9,510 ± 1389	10,840 ± 1,507
TRAMP/SSAT	Liver	30 ± 4	210 ± 15	550 ± 45	710 ± 241	4,520 ± 1,235	15,010 ± 2,376	5,770 ± 935

Data are mean value ± standard error, where n is 3.

Figure 1

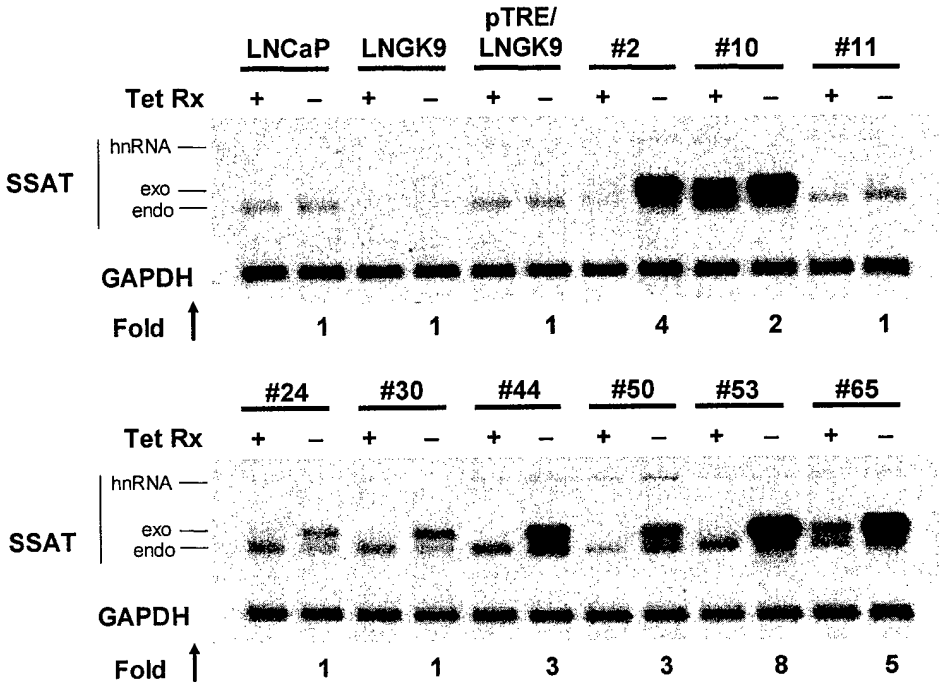

Figure 1. Conditional overexpression of SSAT mRNA in LNCaP clones transfected with Tet-regulatable human SSAT cDNA. Representative examples from over 100 selected clones were cultured for 48 h in the presence (+) or absence (–) of 1 μg/ml Tet. Note the endogenous (*endo*) and exogenous (*exo, i.e.* plasmid transcript) mature SSAT mRNA can be distinguished on the basis of size (i.e. ~1.3 versus ~1.5 kb, respectively). For quantitation, exogenous SSAT mRNA bands were scanned fluorometrically, normalized to the glyceraldehyde-3'-phosphate dehydrogenase (GAPDH) signal, and expressed as fold increase (Fold ↑) in –Tet relative to +Tet.

Figure 2

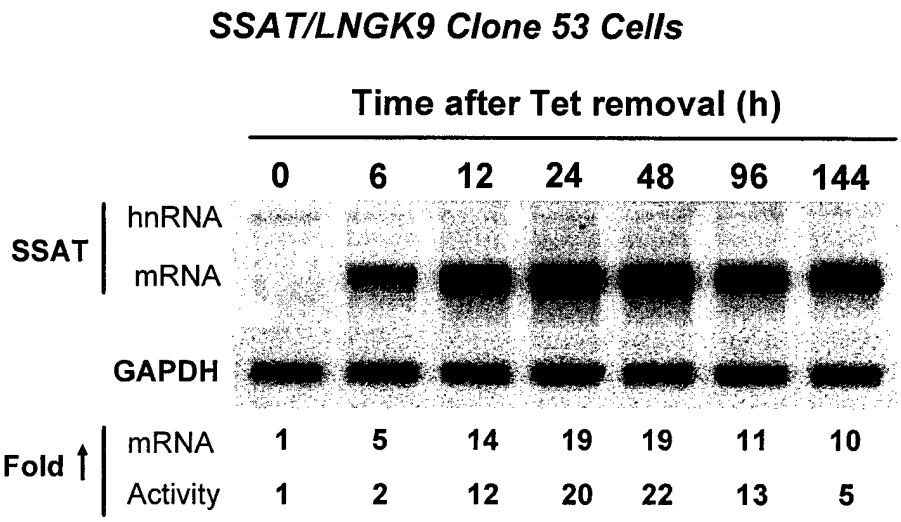

Figure 2. Time-dependent increases in SSAT mRNA and activity in SSAT/LNCaP clone 53 following Tet removal. Following removal of 1 μg/ml Tet, SSAT mRNA increases rapidly before reaching a plateau between 24 and 48 h. For quantitation, SSAT mRNA bands were scanned fluorometrically, normalized to the GAPDH signal, and expressed as fold increase (Fold ↑) relative to +Tet at 0 h (lane 1). SSAT activity increased in parallel to SSAT mRNA both expressed as fold increase.

Figure 3

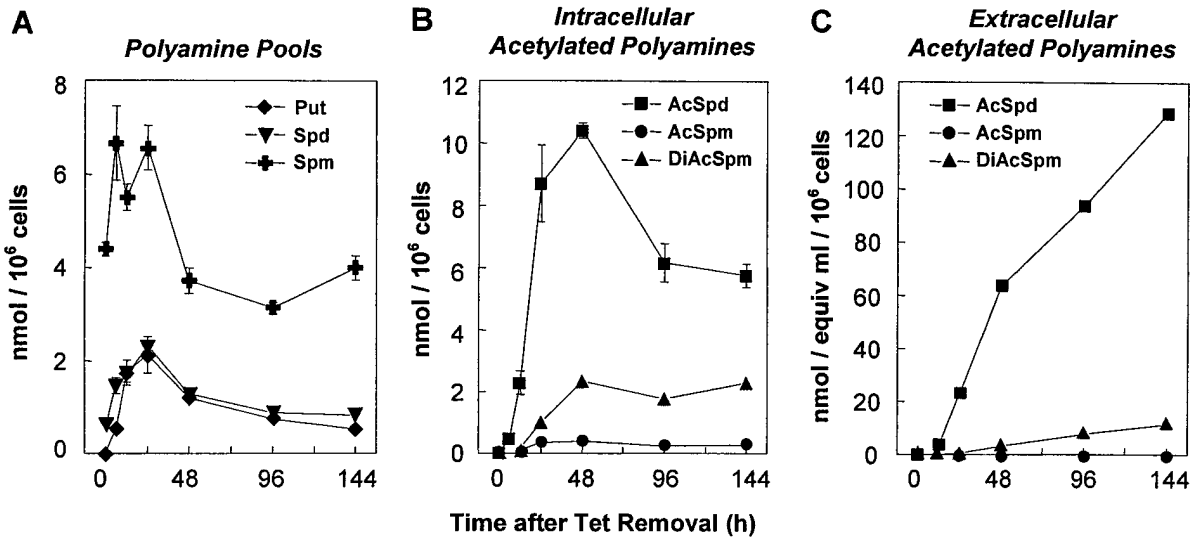

Figure 4. Time-dependent effects of conditional SSAT overexpression on intracellular and extracelluar polyamines. Intracellular polyamines pools increased transiently for 24 h following Tet removal and then decreased steadily. Note that, even at 144 h, Put, Spd and Spm pools remained similar to or slightly above basal levels (0 h) (Panel A). Intracellular AcSpd and AcSpm increased markedly following Tet removal (Panel B). At the same time, huge amounts AcSpd accumulated in the media (Panel C) indicating that acetylated products are readily exported. Significant levels of DiAcSpm were detected intracellularly and extracellularly (Panels B and C). (AcSpd, N^1-acetylspermidine; AcSpm, N^1-acetylspermine; DiAcSpm, N^1, N^{12}-diacetylspermine; Put, putrescine; Spd, spermidine; Spm, spermine). Data represents mean ± standard error, where n is 3.

Figure 4

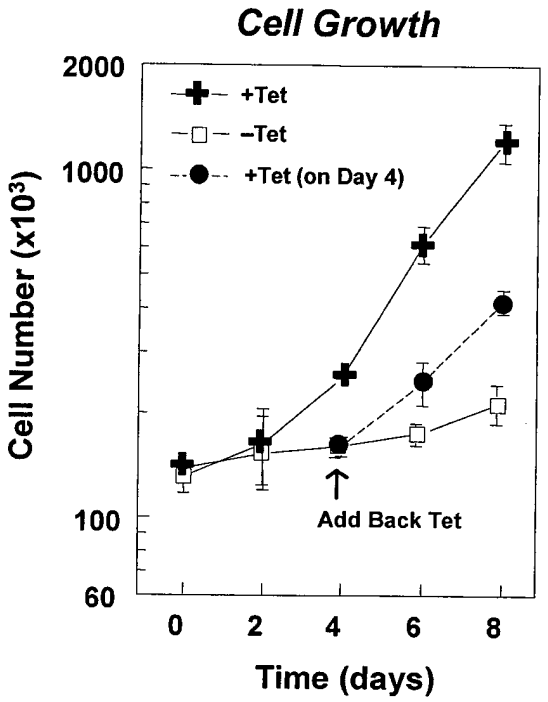

Figure 4. Effects of conditional SSAT overexpression on growth kinetics of LNCaP prostate carcinoma cells. Removal of Tet (□) resulted in an immediate inhibition of cell growth. Note that restoration of Tet at 96 h (●) leads to a rapid resumption of cell growth. Data represents mean ± standard error, where n is 3.

Figure 5

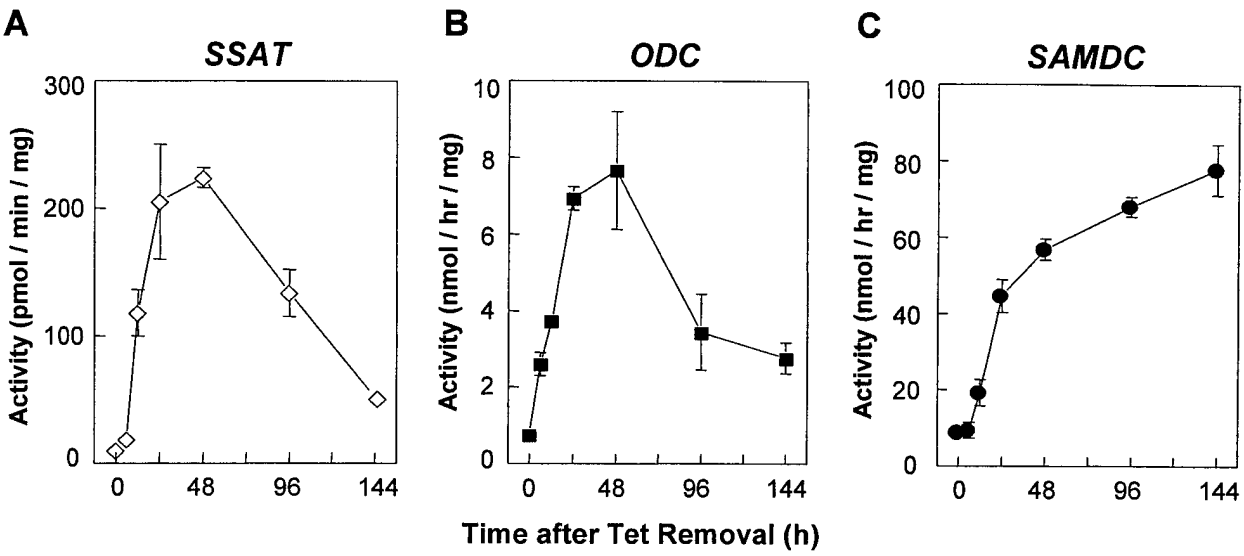

Figure 5. Time-dependent effects of conditional SSAT overexpression on polyamine biosynthetic enzyme activities. Following Tet removal, SSAT activity (◇) increased sharply to a maximum of ~20-fold at 48 h before undergoing a steady decline. Similarly, ODC activity (■) increased to a maximum of ~10-fold at 48 h before declining steadily (3B). By contrast, SAMDC activity (●) increased steadily over the course of 144 h to a maximum of ~18-fold that of basal levels (3C). Data represents mean values ± standard error, where n is 3.

Figure 6

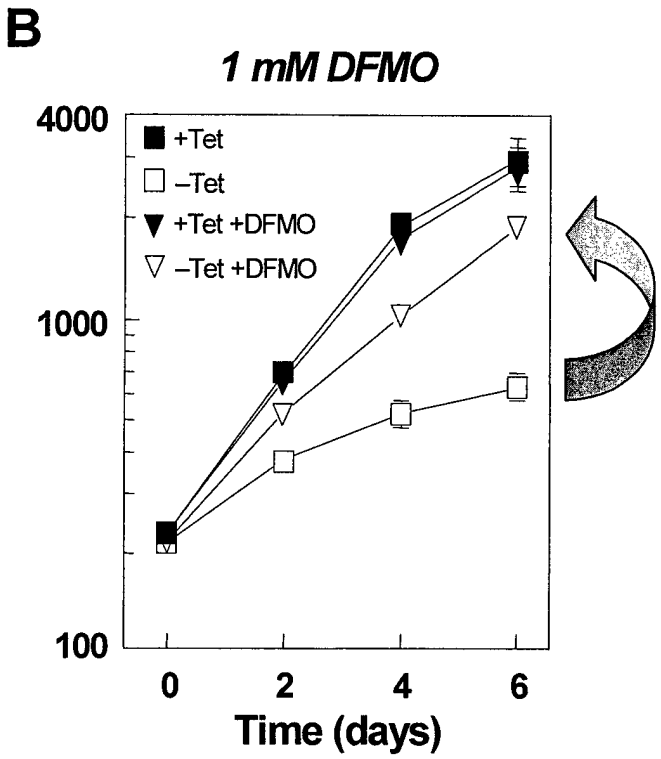

Figure 7. Effects of ODC inhibition on SSAT-induced cell growth inhibition. When included during Tet removal, the ODC inhibitor DFMO (1 mM) was very effective in preventing SSAT-induced growth inhibition (7B). Data represents mean ± standard error, where n is 3.

Figure 7

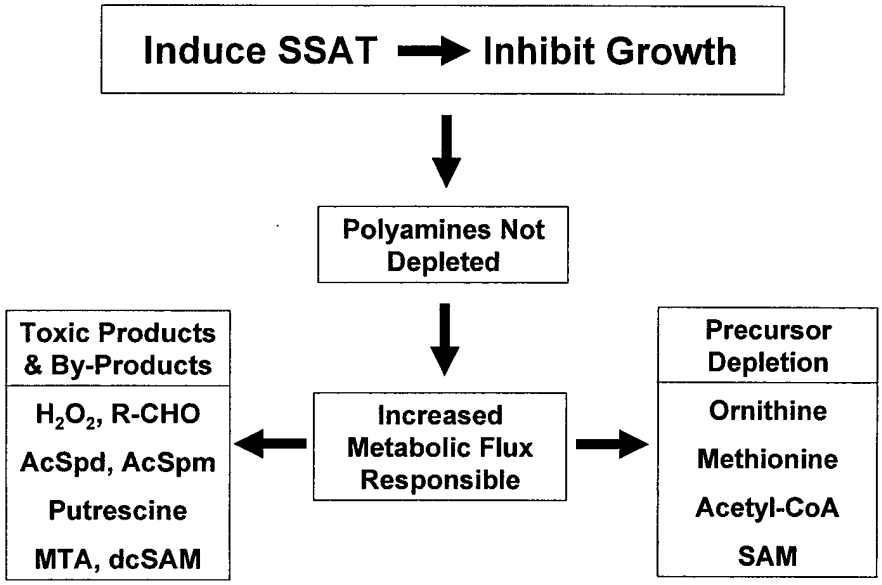

Figure 7. Diagrammatic representation of the possible causes of growth inhibition in SSAT overexpressing LNCaP cells. While not leading to polyamine pool depletion, SSAT overexpression causes a high rate of metabolic flux which when inhibited by DFMO, prevents growth inhibition. The possible causes of growth inhibition via this metabolic flux are presented as product or by-product excess (left box) or as precursor depletion (right box). Of the product/by-product possibilities, hydrogen peroxide (H_2O_2) and reactive aldehydes (R-CHO; such as acetamidopropanal), dcSAM and acetylated polyamines have been experimentally excluded. Of the possible precursor possibilities, ornithine and methionine have been experimentally excluded.

Figure 8

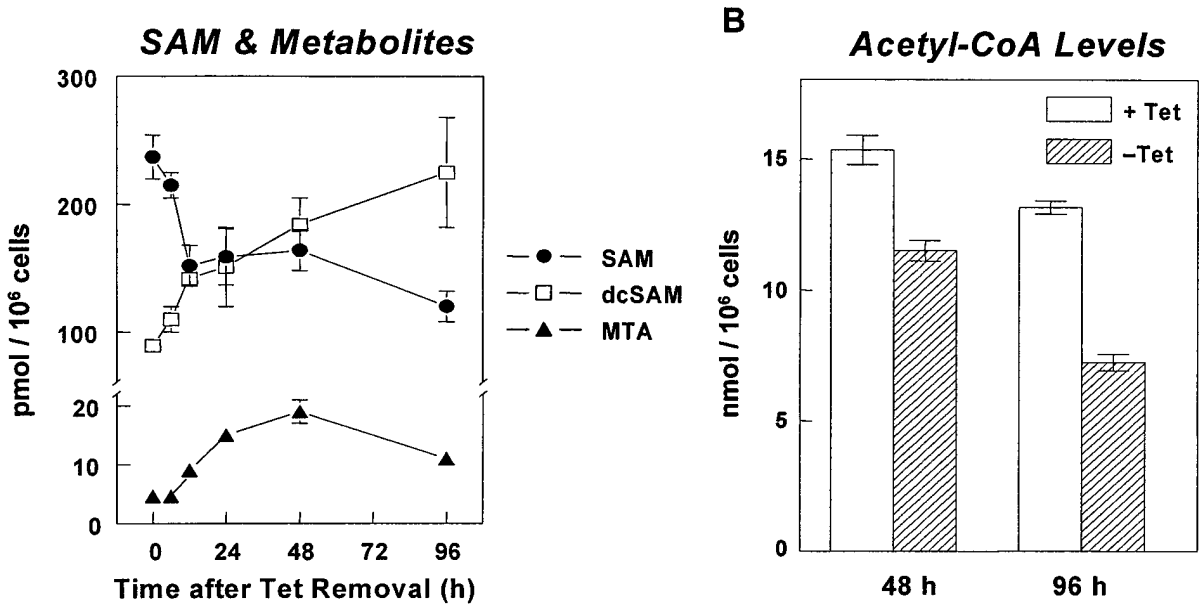

Figure 8. Left Panel: Effects of SSAT overexpression on SAM, dcSAM and MTA pools. Upon Tet removal (–Tet) intracellular levels of the SAMDC substrate SAM (●) declined steadily to ~50% at 96 h while the SAMDC product, dcSAM (□) increased steadily to a maximum of 250% at 96 h. At the same time, the by-product of dcSAM metabolism via spermidine and spermine synthases, MTA (▲), increased to a maximum of ~400% at 48 h. The limit of detection for MTA is <5 pmol/10^6 cells. Data represents mean ± standard error, where n is 3.

Figure 8. Right Panel. Effects of SSAT overexpression on acetyl-CoA levels. Graph represents an electropherogram from capillary electrophoresis showing levels of acetyl-CoA and an exogenous internal standard, isobutyryl-CoA in the presence (+Tet) and absence (–Tet) of Tet at 96 h. Data represents mean ± standard error, where n is 3.

Figure 9

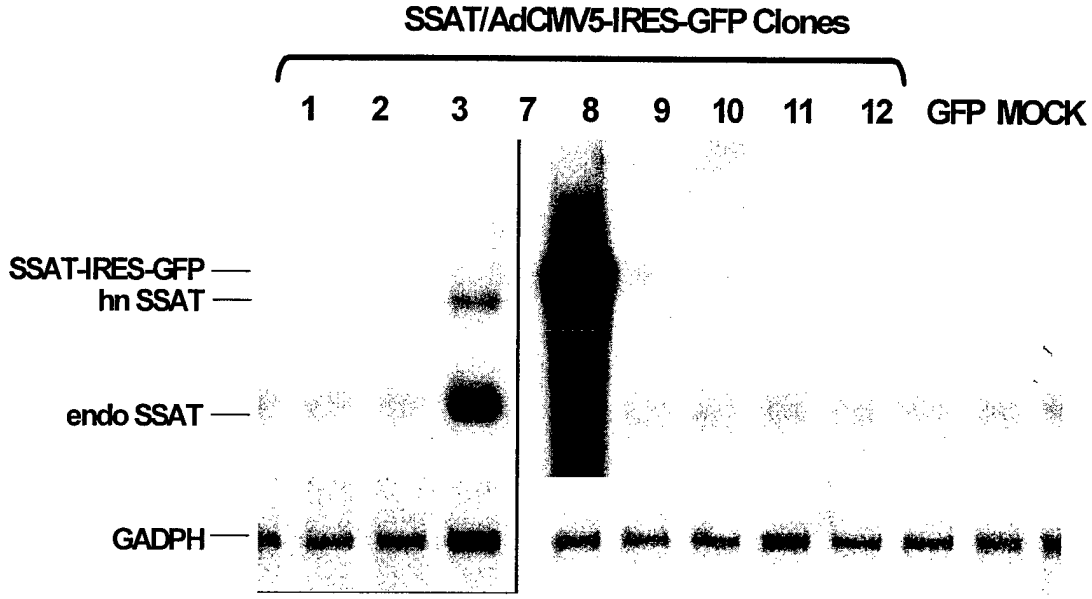

Figure 9. SSAT mRNA expression in adenovirally transduced 293 cells. The highest expressing clone (lane 1) was selected for further amplification and purification. The finding also indicates that the SSAT adenovirus can be raised in human cells.

Figure 10

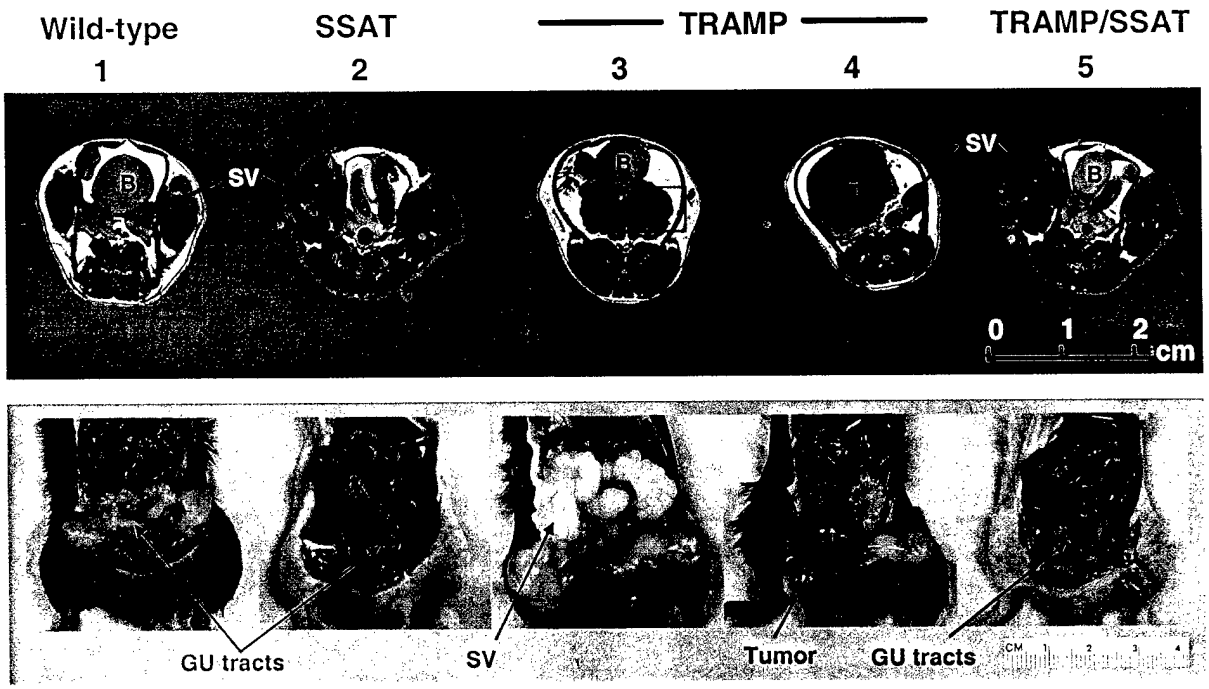

Figure 10. Magnetic resonance imaging correlates in situ observation of mice from TRAMP/SSAT cross. Representative high resolution transaxial magnetic resonance images of mice at 30-weeks showing bladder (B), seminal vesicles (SV) and prostate tumor (T) (upper panel). The MRI correlated with the *in situ* observation of mouse genitourinary tracts at postmortem necropsy (lower panel). Note that the TRAMP animals in C57BL/6 background results in 2 distinct phenotypes: (from the left) seminal vesicle involvement (mouse 3) or prostate involvement (mouse 4).

Figure 11

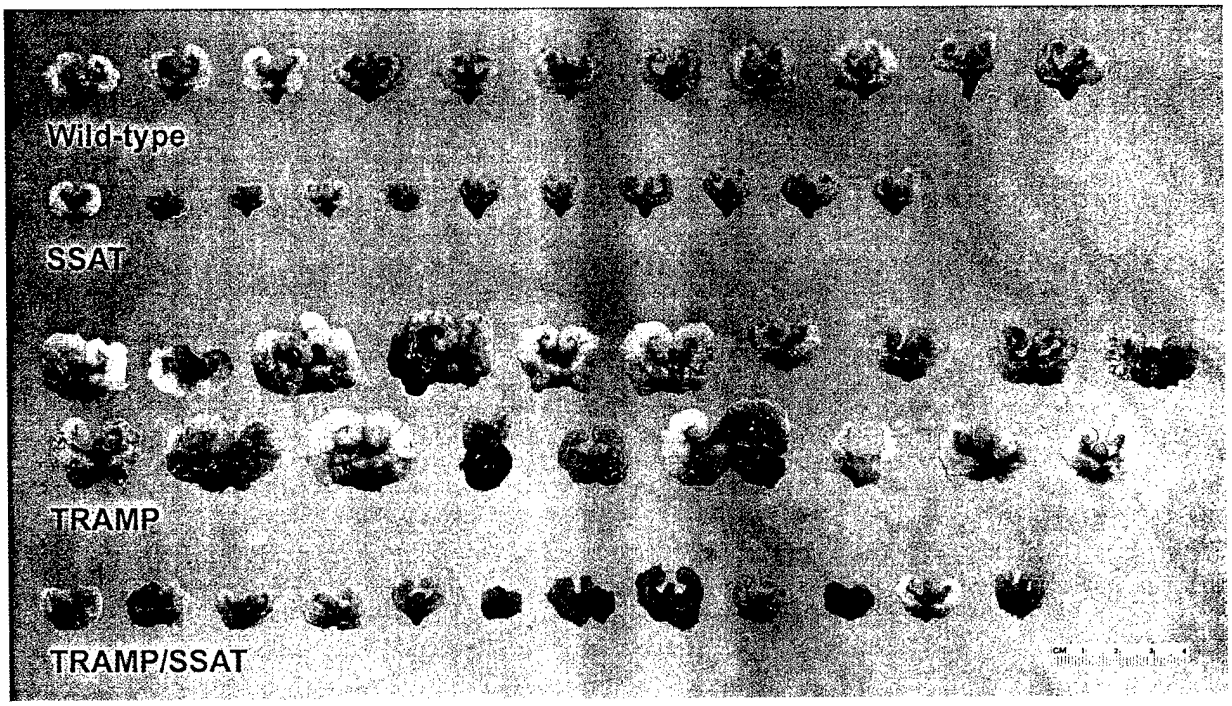

Figure 11. Comparison at 30-wks of genitourinary (GU) tracts of four genetic cohorts deriving from the TRAMP x SSAT cross. GU tracts for mice from each of four genetic cohorts were removed by microdissection at 30 wks, weighed and individually photographed. Note that the GU tracts of TRAMP/SSAT mice were much smaller and less variable than those of TRAMP mice.

Figure 12

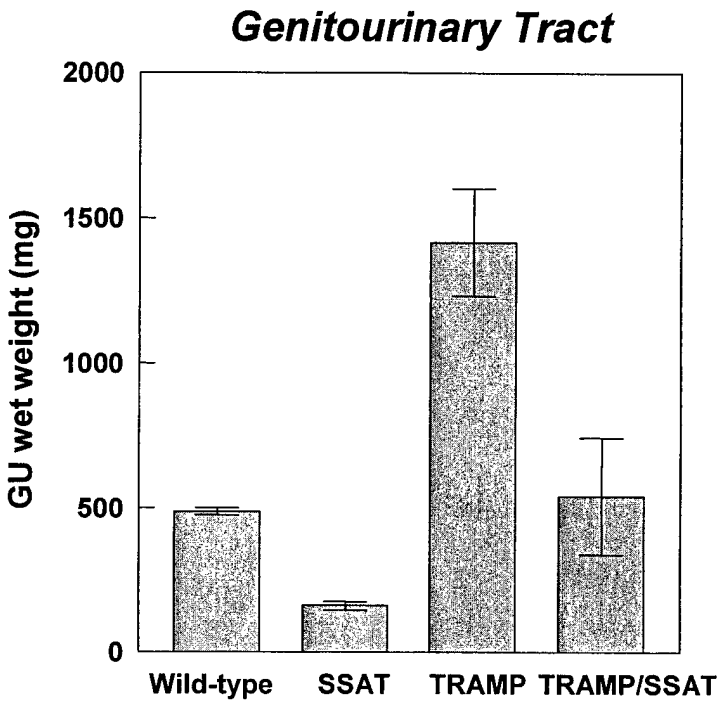

Figure 12. Disease index at 30 wks of GU tracts of tracts of four genetic cohorts deriving from the TRAMP/SSAT cross. The disease index for each GU tract was calculated by multiplying the average total histopathology grade score of all the prostate lobes times the weight of each GU tract [i.e. GU Disease Index = (average overall grade + average highest grade) x GU weight]. Raw data is presented in Table **1**. Note that the GU tract disease index of TRAMP mice was more variable and higher than that of TRAMP/SSAT mice.

Figure 13

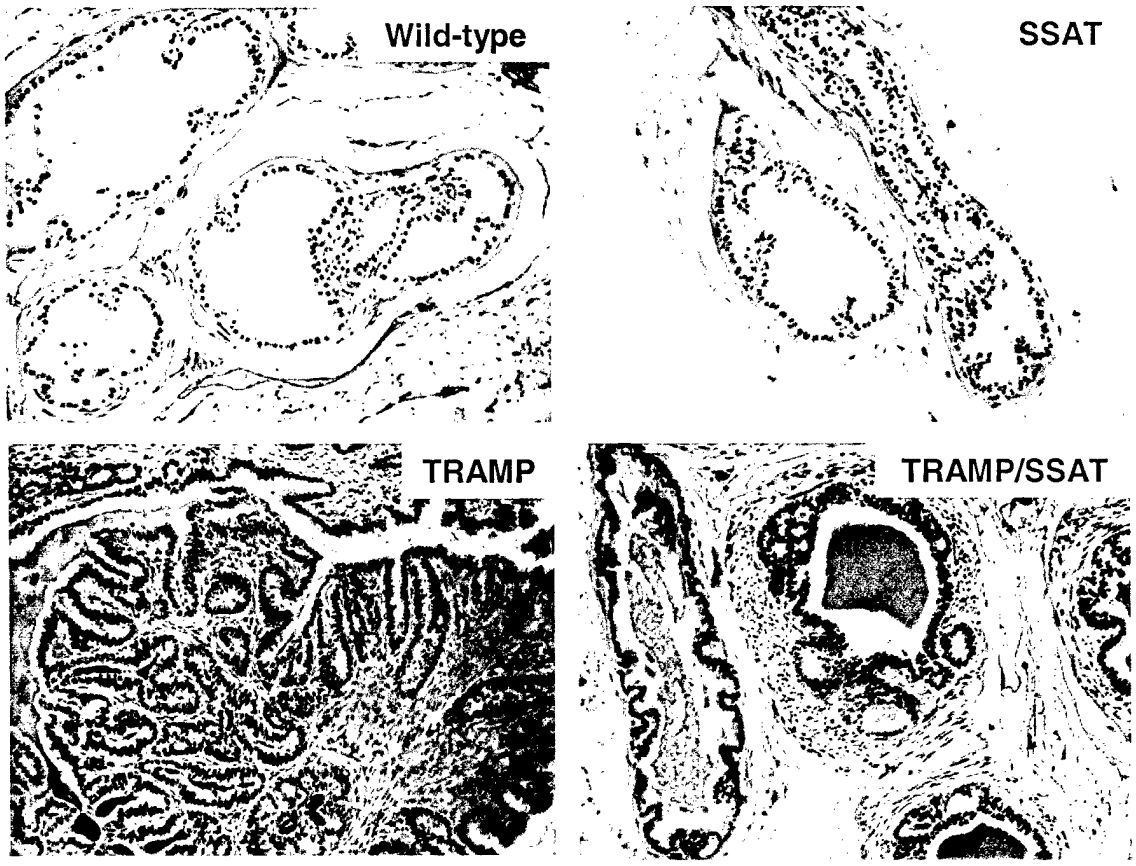

Figure 13. Immunohistochemistry of SV40 large T-antigen expression in the dorsal lobe of the mouse prostate. SSAT overexpression in transgenic mice did not affect SV40 large T-antigen expression in the prostate epithelium of TRAMP transgenic animals.

Figure 14

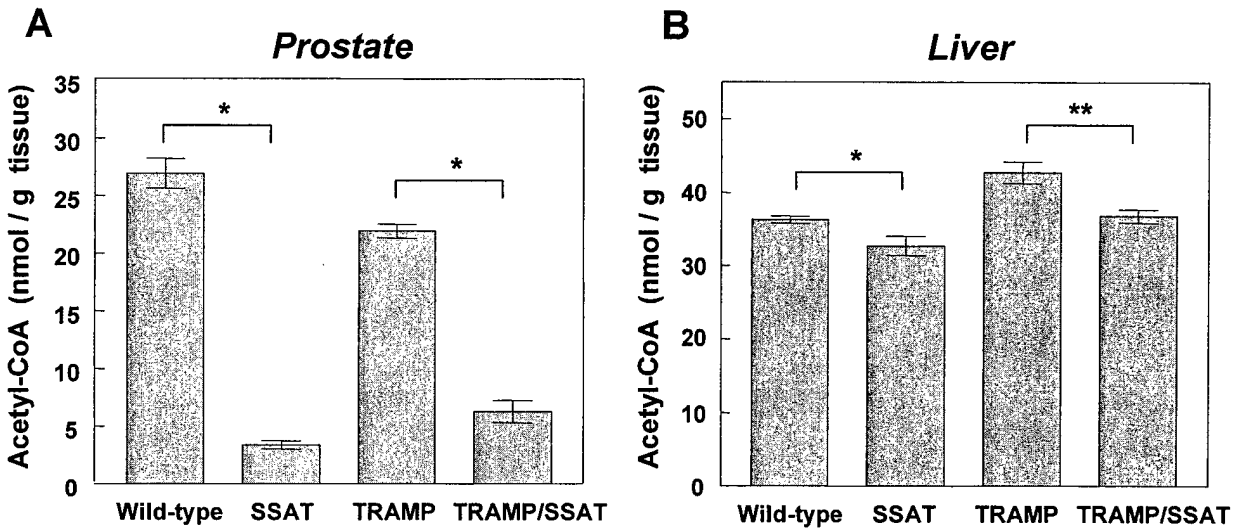

Figure 14. Relative acetyl-CoA pools in prostates and livers of various TRAMP x SSAT genetic cohorts. Note that acetyl-CoA is markedly reduced in the prostates of SSAT overexpressing mice but relatively much less affected in the liver.