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KIRSI NIIRANEN

Consequences of Spermine Synthase or Spermidine/Spermine N^1-Acetyltransferase Deficiency in Polyamine Metabolism

Studies with Gene-Disrupted Mouse Embryonic Stem Cells and Mice

Doctoral dissertation

To be presented by permission of the Faculty of Natural and Environmental Sciences of the University of Kuopio for public examination in Auditorium L22, Snellmania building, University of Kuopio, on Saturday 23rd September 2006, at 12 noon

Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio

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A.I. Virtanen Institute for Molecular Sciences

Authors's address:
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio
P.O. Box 1627
FI-70211 KUOPIO
FINLAND
Tel. +358 17 162 093
Fax +358 17 163 025
E-mail: Kirsi.Niiranen@uku.fi

Supervisors:
Professor Juhani Jänne, M.D., Ph.D.
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio

Professor Leena Alhonen, Ph.D.
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio

Rewiewers:
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University of Oulu

Dosent Pauli Seppänen, Ph.D.
Director of Research and Development
Savonia University of Applied Sciences
Kuopio

Opponent:
Professor Matti Poutanen, Ph.D.
Department of Physiology
Institute of Biomedicine
University of Turku

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ABSTRACT

A vast number of studies have linked polyamines to numerous cellular functions but less is known about the possible physiological functions of the individual polyamines. The specific gene targeting technique has provided a new potential way to resolve this mystery. We generated two separate embryonic stem (ES) cell lines where either spermine synthase gene (SPMSy) or spermidine/spermine N^1-acetyltransferase gene (SSAT) was disrupted. The SSAT-deficient ES cells enabled the further creation of SSAT knockout mice.

We wanted to study the actual role of spermine in mammalian cells as this polyamine is lacking from bacterial cells and also to gain more information of SSAT, which is considered to be the key enzyme in polyamine catabolism. Gene-disrupted models showed only slightly changed polyamine homeostasis at the single polyamine level and the total polyamine pools remained practically unchanged. Spermine-deficient cells compensated for the lack of spermine by significantly increasing the amount of spermidine. SPMSy knockout cells had normal growth rates compared with control cells but showed enhanced sensitivity to antiproliferative drugs. However, spermine may be essential during mouse embryogenesis as the generation of SPMSy-deficient mice failed.

SSAT knockout cells proliferated also normally and were predictably more resistant than wild-type cells to the cytotoxicity evoked by the polyamine analogue N^1, N^{11}-diethylnorspermine (DENSPM). Polyamine interconversion studies showed that SSAT activity was absolutely necessary for spermidine catabolism, as indicated by the increased spermidine levels in SSAT-deficient cells. Interestingly, SSAT seemed to have only marginal importance in spermine catabolism because SSAT knockout cells were able to backconvert spermine to spermidine even more efficiently than control cells.

Since SSAT is regarded to be crucial in maintaining normal polyamine pools and therefore preventing the accumulation of toxic levels of higher polyamines, it was somewhat surprising that a lack of a functional SSAT gene would have no effect on the viability, fertility or phenotype of the mice. The indispensability of SSAT in spermidine back-conversion was also detected *in vivo* as the molar ratio of spermidine to spermine was elevated in most of SSAT knockout tissues. DENSPM showed divergent results *in vitro* and *in vivo*, hence SSAT-deficient mice were unexpectedly more sensitive to the drug than their wild-type littermates. DENSPM is considered to exert cytotoxic effects through SSAT induction and thus depletion of higher polyamine pools, but the toxicity of this drug appears to be mediated by other mechanisms in the SSAT-deficient mice. Furthermore, the results with the SSAT knockout mice supported the role of polyamine catabolism in the regulation of glucose and energy metabolism, as these mice were insulin resistant at old age.

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Medical Subject Headings: polyamines/metabolism; spermine synthase; acetyltransferases; stem cells; gene targeting; animals, genetically modified; mice, knockout; energy metabolism; glucose/metabolism; insulin resistance

"Ihmeellistä", sanoi orava hämmästyneenä. "Tuota pahvilaatikkoa ei ollut täällä aikaisemmin. Tässä on varmasti jotakin vinossa. Tai sitten tämä on kokonaan väärä luola. Tai minä olen ehkä väärä orava, mutta sitä en oikein uskoisi."

Tove Jansson, Taikatalvi

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Kuopio, September 2006

Kirsi Niiranen

ABBREVIATIONS

AdoMet	S-adenosylmethionine
AdoMetDC	S-adenosylmethionine decarboxylase
AZ	antizyme
AZI	antizyme inhibitor
CAG	chicken β-actin promoter
CHO	Chinese hamster ovary
dcAdoMet	decarboxylated S-adenosylmethionine
DENSPM	N^1, N^{11}-diethylnorspermine
DFMO	α-difluoromethylornithine
DMEM	Dulbecco's modified Eagle's medium
EDTA	ethylenediaminetetraacetic acid
ES cell	embryonic stem cell
Gy	Gyro
HPLC	high performance liquid chromatography
Hyp	hypophosphatemic
KI	knock-in
KO	knockout
MDL 72527	N^1, N^4-bis(2,3-butadienyl)-1,4-butanediamine
MGBG	methylglyoxal bis(guanylhydrazone)
MHC	α-myosin heavy chain
MT	metallothionein
N^1-Ac-SPD	N^1-acetylspermidine
N^1-Ac-SPM	N^1-acetylspermine
NEO	neomycin phosphotransferase
ODC	ornithine decarboxylase
PAO	polyamine oxidase
PHEX	phosphate regulating gene with homologies to endopeptidases on the X-chromosome
PUT	putrescine
RNAi	RNA interference
siRNA	small interfering RNA
SMO	spermine oxidase
SPD	spermidine
SPDSy	spermidine synthase
SPM	spermine
SPM-KO	spermine synthase knockout
SPMSy	spermine synthase
SSAT	spermidine/spermine N^1-acetyltransferase
SSAT-KO	spermidine/spermine N^1-acetyltransferase knockout
WT	wild-type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

I Korhonen V. P., Niiranen K., Halmekytö M., Pietilä M., Diegelman P., Parkkinen J. J., Eloranta T., Porter C. W., Alhonen L., and Jänne J. (2001). Spermine deficiency resulting from targeted disruption of the spermine synthase gene in embryonic stem cells leads to enhanced sensitivity to antiproliferative drugs. Mol Pharmacol *59(2)*, 231-238.

II Niiranen K., Pietilä M., Pirttilä T. J., Järvinen A., Halmekytö M., Korhonen V. P., Keinänen T. A., Alhonen L., and Jänne J. (2002). Targeted disruption of spermidine/spermine N^1-acetyltransferase gene in mouse embryonic stem cells. Effects on polyamine homeostasis and sensitivity to polyamine analogues. J Biol Chem *277(28)*, 25323-25328.

III Niiranen K., Keinänen T. A., Pirinen E., Heikkinen S., Tusa M., Fatrai S., Suppola S., Pietilä M., Uimari A., Laakso M., Alhonen L., and Jänne J. (2006). Mice with targeted disruption of spermidine/spermine N^1-acetyltransferase gene maintain nearly normal tissue polyamine homeostasis but show signs of insulin resistance upon aging. JCMM, in press.

CONTENTS

1 INTRODUCTION

The polyamines spermidine (SPD) and spermine (SPM) and their diamine precursor putrescine (PUT) are ubiquitous cellular cations that have many important biological roles in cells. Polyamines play an essential role in cell growth and in the synthesis of proteins and nucleic acids (Tabor and Tabor, 1984). They are also involved in signaling processes and have specific interactions with particular ion channels (Williams, 1997). The polyamine content is generally increased in rapidly growing cells and their intracellular levels are strictly regulated by a variety of mechanisms affecting their biosynthesis, degradation, uptake and excretion. Earlier studies of regulation of polyamine content were concentrated on the biosynthetic reactions, especially on the role of the key regulatory enzyme, ornithine decarboxylase (ODC). Recently the importance of the catabolic processes, particularly the highly regulated acetylation step by spermidine/spermine N^1-acetyltransferase (SSAT), has become apparent. SSAT has been considered to be the rate-controlling enzyme in polyamine catabolism by N^1-acetylation of spermidine or spermine. Currently its role in spermine degradation seems to less unequivocal since spermine can be also oxidized directly to spermidine in a reaction catalyzed by a recently discovered enzyme, spermine oxidase (SMO) (Bellelli et al., 2004; Vujcic et al., 2002; Wang et al., 2003).

The specific cellular functions of polyamines are still largely unknown. In order to study the actual role of the individual polyamines, the generation of specific gene knockouts has proved to be very important. We have generated two separate mouse embryonic stem (ES) cell lines with targeted disruption of spermine synthase (SPMSy) gene or SSAT gene and furthermore the metabolic consequences of absence of SSAT was studied in SSAT gene-disrupted mice. In polyamine biosynthesis, spermine synthase catalyzes the formation of spermine. Bacterial cells are unable to synthesize spermine and thus, its specific role has to be elucidated in mammalian cells. SPMSy gene-disrupted cells were totally devoid of the polyamine spermine but instead their spermidine content was doubled. SPMSy-deficient cells grew normally in comparison with the parental cells and did not exhibit any morphological changes, but the cells were more sensitive to antiproliferative drugs. The generation of SPMSy knockout (SPM-KO) mice was not successful, as chimeric mice were not able to transmit the disrupted gene to their offspring. However, it has been possible to study spermine synthase deficiency *in vivo* with Gyro (Gy) mice, which have a chromosomal deletion in the Xp22.1 chromosome region, containing the

spermine synthase gene and one of the phosphate metabolism regulating genes. In addition to hypophosphatemia, male Gy mice show also multiple neurological abnormalities, sterility and have a very short lifespan apparently as consequences of SPMSy gene disruption (Grieff et al., 1997; Lorenz et al., 1998; Meyer et al., 1998).

SSAT is considered as the key enzyme in polyamine back-conversion. Since there are no specific SSAT inhibitors, attempts have been made to clarify the cellular functions of the enzyme with the aid of polyamine analogues which induce SSAT. The SSAT knockout (SSAT-KO) ES cells did not contain any inducible SSAT activity or SSAT protein. The growth rate and polyamine pools were similar as those found in wild-type (WT) ES cells except that they had constantly elevated cellular levels of spermidine. SSAT-KO cells were predictably more resistant than wild-type cells to the growth inhibitory effect of one of the most potent inducers of SSAT, N^1, N^{11}-diethylnorspermine (DENSPM), but this was not due to a more rapid depletion of the higher polyamines in parental cells in response to the SSAT induction, as spermine and spermidine decreased similarly in both cell lines. The role of SSAT in spermine back-conversion seemed to be only marginal, as targeted cells converted radiolabeled spermine to spermidine even more effectively than the wild-type cells. However, SSAT activity was indispensable for the back-conversion of spermidine to putrescine via N^1-acetylspermidine, since SSAT-deficient cells did not catabolize any detectable amounts of radioactive putrescine from [^{14}C]spermidine. The generation of SSAT knockout mice was successful and the mice were viable, fertile and phenotypically indistinguishable from their wild-type littermates. A more detailed characterization revealed slightly affected polyamine pools as the spermidine/spermine ratio was increased in most of the studied tissues. Surprisingly, in contrast to the SSAT-deficient ES cells, SSAT-KO animals were significantly more sensitive towards the polyamine analogue DENSPM than their wild-type littermates. Depletion of cellular polyamines or accumulation of the analogue was not the reason for this observed toxicity, as the polyamine pools remained almost unchanged in comparison with non-treated animals and the uptake of the drug was either the same or decreased in different tissues as compared with controls. Interestingly, older SSAT-deficient mice showed an opposite phenotype compared to SSAT overexpressing mice with respect to insulin resistance and impaired glucose tolerance (Pirinen unpublished). This is support for a role for polyamine catabolism in the regulation of glucose metabolism.

2 REVIEW OF THE LITERATURE

2.1. Properties and functions of polyamines

The polyamines putrescine, spermidine and spermine are low molecular weight aliphatic amines found in all living cells. Polyamines are essential for normal cell growth and their intracellular levels are regulated by a balance between biosynthesis, catabolism and transport. The amino groups of the polyamines are all protonated at physiological pH values (Tabor and Tabor, 1984). Therefore, these polycations can interact electrostatically with a variety of negatively charged structures such as DNA, RNA, proteins and membrane phospholipids, although in some of the reactions their presence can be substituted by inorganic cations such as Mg^{2+} or Ca^{2+}. However, polyamines form stronger and more specific interactions than the inorganic cations due to the widely distributed charge along the length of the aliphatic carbon chain. It has been shown that binding energy decreases from the most charged spermine to spermidine, with putrescine being the least effective in several biological processes (Seiler, 1990).

Polyamines are linked to several biochemical roles and their importance in cell proliferation is well established. Eukaryotic initiation factor 5A needs spermidine for one essential process, hypusination modification, and if hypusine synthesis is inhibited, cells cease to grow (Park et al., 1994). It has been shown that polyamine depletion increases the expression of the nuclear phosphoprotein p53 gene and also stabilizes its mRNA in intestinal crypt cells (Li et al., 1999; Li et al., 2001). Thus polyamine depletion-induced expression of the p53 gene may play an important role in growth inhibition. In addition to cell proliferation, polyamines have been also linked to apoptosis which can be activated by depletion of higher polyamine pools or oxidative stress caused by accumulation of H_2O_2 during increased catabolism of the higher polyamines (Seiler and Raul, 2005). Polyamines have been reported to stabilize DNA (Flink and Pettijohn, 1975) and to promote the transition of B-DNA to Z-DNA (Howell et al., 1996). Polyamines can also stimulate gene transcription and translation (Coffino, 2000). They undergo other interactions with nucleic acids for example polyamine-stimulated translational frameshifting is required to decode the antizyme mRNA (Matsufuji et al., 1995). Endogenous polyamines have also specific interactions with various types of ion channels. There are polyamine binding sites on glutamate receptors, inwardly rectifying potassium channels and voltage-dependent Ca^{2+} channels (Williams, 1997). In particular, spermine and spermidine have dual effects on the N-methyl-D-

aspartate receptor, both increasing and decreasing receptor activation. Polyamines can also increase membrane rigidity by binding to proteins and acidic phospholipids in membranes (Schuber, 1989). In addition, for many years it has been known that polyamines possess anti-oxidant and free radical scavenging properties and thus may protect against lipid peroxidation as an example (Tadolini, 1988).

2.2. Polyamine biosynthesis in mammalian cells

Most living species are capable of synthesizing polyamines. The biosynthetic enzymes are ornithine decarboxylase, S-adenosylmethionine decarboxylase (AdoMetDC), spermidine synthase (SPDSy) and spermine synthase. Polyamine biosynthesis in mammalian cells begins with the production of putrescine by direct decarboxylation of L-ornithine (Figure 1). This rate-controlling step in polyamine biosynthesis is catalyzed by ODC. Another key step is the decarboxylation of S-adenosylmethionine to decarboxylated S-adenosylmethionine (dcAdoMet) through the action of AdoMetDC. DcAdoMet serves as an aminopropyl donor in the synthesis of higher polyamines. The addition of an aminopropyl group to putrescine leads to the synthesis of spermidine and the addition of a second aminopropyl moiety to spermidine leads to the formation of spermine. These reactions are catalyzed by two constitutively expressed and stable enzymes SPDSy and SPMSy, respectively.

Although *de novo*-biosynthesis is the major route in the production of polyamines in mammalian cells, their transport in and out of the cell also plays an important role in maintaining cellular polyamine homeostasis. In general, factors that increase polyamine formation increase also their uptake from the environment, whereas high intracellular polyamine levels enhance their export out of cells. Rapidly proliferating cells have a high polyamine demand and exogenous polyamines are transported to cells when the cells' own synthetic production cannot meet the demand. Exogenous polyamines may be obtained from diet (Bardocz, 1993), through synthesis by intestinal microorganisms (Hessels et al., 1989) or by the release from other cells. Mammalian cells have an active polyamine transport system which is a carrier-mediated, energy-requiring process capable of transporting polyamines against a significant concentration gradient. However, the mammalian polyamine transporter has not yet been cloned and its exact mechanism remains to be clarified in detail (Seiler and Dezeure, 1990).

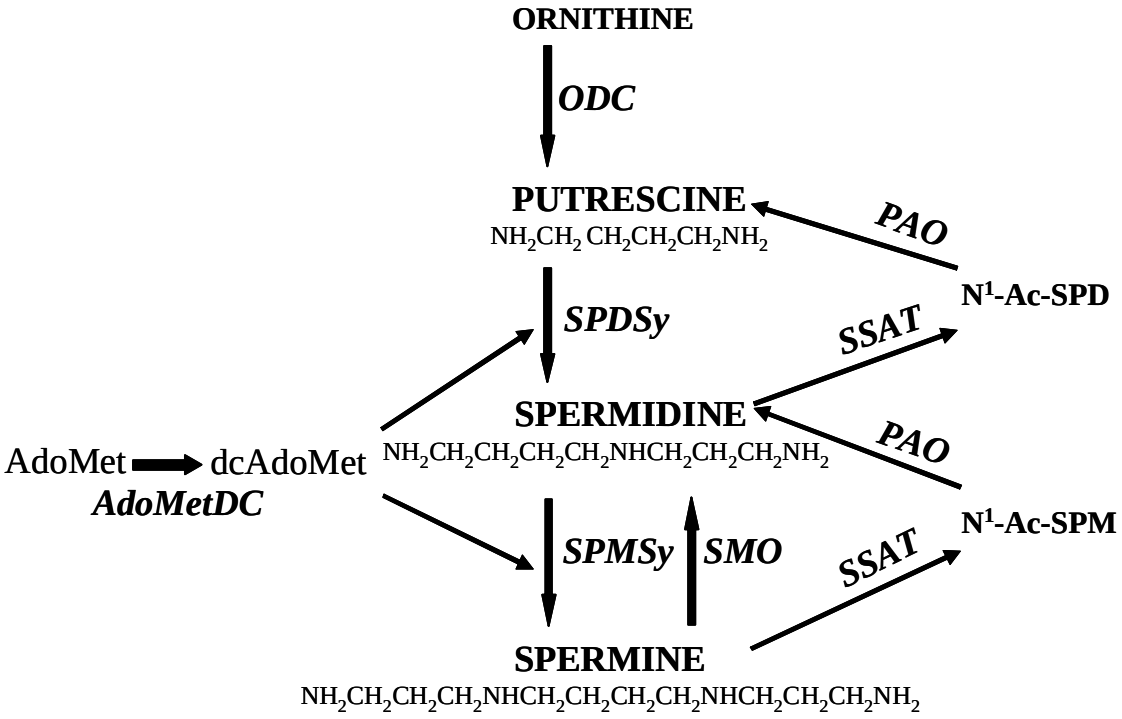

Figure 1. The polyamine metabolic pathway

ODC, ornithine decarboxylase; AdoMet, S-adenosylmethionine; dcAdoMet, decarboxylated S-adenosylmethionine; AdoMetDC, S-adenosylmethionine decarboxylase, SPDSy, spermidine synthase; SPMSy, spermine synthase; SMO, spermine oxidase; SSAT, spermidine/spermine N^1-acetyltransferase; PAO, polyamine oxidase; N^1-Ac-SPM, N^1-acetylspermine; N^1-Ac-SPD, N^1-acetylspermidine

2.2.1. Ornithine decarboxylase

Ornithine decarboxylase (EC 4.1.1.17) catalyzes the first step in the mammalian polyamine biosynthetic pathway, forming putrescine from the amino acid ornithine. Eukaryotic ODC is a homodimer with two shared active sites between the monomers (Coleman et al., 1994) and the human ODC gene is located on the short arm of chromosome 2 (Winqvist et al., 1986). Like most decarboxylases, ODC requires pyridoxal-5'-phosphate as a cofactor (Poulin et al., 1992). ODC is strictly regulated at the transcriptional and translational levels as well as by its RNA and protein half-life (Pegg, 2006). ODC enzyme activity exhibits induction during late G_1 and at G_2/M phases of the cell cycle and is increased in a number of preneoplastic conditions. The gene

encoding ODC has been implicated as a proto-oncogene in multiple tumor types when expressed at very high levels (Auvinen et al., 1992; Pegg et al., 1995a). Thus, ODC inhibitors have been tested as chemopreventive agents with the most potent being based on the best known compound α-difluoromethylornithine (DFMO) (Poulin et al., 1992).

ODC has an extremely short turnover rate with a biological half-life of 10-30 minutes and the enzyme is highly regulated at many levels (Davis et al., 1992; Heby and Persson, 1990; Shantz and Pegg, 1999). Post-transcriptionally, ODC is negatively regulated by a unique degradation mechanism mediated by the antizyme (AZ) family which functions as a regulator of polyamine homeostasis. AZs are small regulatory proteins, whose expression is regulated by a ribosomal frameshift mechanism. AZ was first identified in mammals and several isoforms have been reported; ubiquitously expressed antizymes 1 and 2 and the tissue-specific antizyme 3 and the less well characterized antizyme 4. Polyamines induce AZ expression by promoting +1 ribosomal frameshifting during decoding of the AZ mRNA (Matsufuji et al., 1995). AZ1 limits polyamine accumulation by binding noncovalently to the ODC monomer, forming the ODC/AZ heterodimer and thus blocking the formation of the active ODC homodimer (Li and Coffino, 1992; Mitchell and Chen, 1990) and targeting this complex to the 26S proteosome for rapid degradation in an ubiquitin-independent manner (Elias et al., 1995; Murakami et al., 1992). ODC is degraded to peptides of 5-11 amino acids, whereas AZ is released and recycled to destabilize more ODC monomers. AZ1 also inactivates polyamine uptake at the cell membrane and can bind to an antizyme inhibitor (AZI) with higher affinity than to the ODC monomer, releasing ODC from the ODC-AZ complex. AZI also seems to stabilize ODC by reducing the amount of AZ1 available to ODC destabilization (Murakami et al., 1989). AZ2 also binds to ODC and inhibits polyamine transport but in contrast to AZ1, binding of AZ2 does not result in degradation of ODC (Zhu et al., 1999). AZ3 is testis-specific and its expression is restricted to certain stages of spermatogenesis (Ivanov et al., 2000). AZ4 has not yet been characterized in detail but it is capable of binding to ODC and inhibiting its activity in a similar manner as the other antizymes (Mangold and Leberer, 2005).

Polyamines normally promote cell growth, but they can also have an opposite effect, inducing apoptosis at high concentrations. The consequences of massive putrescine accumulation *in vivo* were firstly studied in detail with ODC transgenic mouse line using the human ODC gene under its own promoter. The transgene was overexpressed nearly in all studied tissues but the most

extensively increased ODC activity and thus the highest putrescine pools were detected in testis and brain. Extensive polyamine homeostatic control was observed since the overexpression of ODC had only minimal effects on tissue levels of higher polyamines, except for testis (Halmekytö et al., 1991a). Histological analysis of ODC transgenic mice showed abnormalities only in testis and clarified the cause for male infertility; this was attributable to the greatly reduced germinal epithelium and to the absence of ongoing spermatogenesis (Halmekytö et al., 1991b). In addition, despite the claimed oncoprotein-like nature of ODC, transgenic mice did not show enhanced spontaneous tumorigenesis as compared to wild-type animals at the age of two years (Alhonen et al., 1995).

ODC overexpression has been also targeted to keratinocytes (Megosh et al., 1995; O'Brien et al., 1997) and to heart (Shantz et al., 2001) using tissue-specific promoters. Target tissues showed the expected elevations in the content of putrescine and spermidine. The skin-targeted ODC transgenic mice were hairless and had increased skin wrinkling and nail growth, and furthermore, the mice were more sensitive to a variety of carcinogenic stimuli. The mice with heart-targeted ODC showed a slight cardiac hypertrophy and an increased hypertrophic response to isoproterenol (Shantz et al., 2001). The mouse metallothionein (MT) I promoter has been used to control inducible overexpression of ODC (Alhonen et al., 1996) but increased ODC activity was detected in heart, testis, brain, and especially in liver of the transgenic animals even in the absence of exposure to heavy metals. The highly elevated putrescine levels after zinc-treatment led to only transient increases in spermine and spermidine levels.

Mutants lacking the active ODC gene

Several strains of *Saccharomyces cerevisiae* have been isolated which contained mutations at a single chromosomal ornithine decarboxylase gene. One of these strains showed no detectable ODC activity and completely lacked putrescine, spermidine and spermine. This mutant strain was totally dependent on supplementation with exogenous polyamines for growth. The mutated strain appeared normal when supplemented with higher polyamines and thus it seems probable that the most important function of putrescine in yeast is to serve as a precursor for the biosynthesis of spermidine and spermine (Cohn et al., 1980). A cessation of growth in polyamine-free medium was also seen in haploid *S. cerevisiae* when the ODC gene was disrupted (Schwartz et al., 1995). A naturally occurring ODC mutant has been identified in the nematode, *Caenorhabditis elegans*,

which despite its inability to synthesize polyamines showed no apparent defects except for slightly reduced brood size (Macrae et al., 1995). When these worms were transferred to polyamine-free medium, developmental disturbances were detected, suggesting that ODC activity is most critically required during oogenesis and embryogenesis (MacRae et al., 1998). Also deletion of ODC was lethal in *Leishmania Donovani* unless supplemented with exogenous putrescine or polyamines in diet (Jiang et al., 1999).

The role of ODC in mammalian cells has been studied with Chinese hamster ovary (CHO) cells. Putrescine auxotroph CHO mutant cells, which have a severe reduction in the amount of ODC activity, did not manage to grow in polyamine-free medium and their growth rate began to decline after 48 h, after 5-6 days the cells began to die. In addition to putrescine, the cells were able to maintain normal growth rate with spermidine and spermine as their polyamine source and also a very high ornithine concentration supported growth (Steglich and Scheffler, 1982). Arginase-deficient CHO cells were first described by Pohjanpelto and coworkers (1981) and from that strain, a mutant strain without a catalytically active ODC enzyme was isolated (Pohjanpelto et al., 1985). The mutant cells were not able to synthesize putrescine and their proliferation was retarded and ceased in 6-10 days if they were not provided with an additional supply of polyamines in their culture medium. During polyamine starvation, putrescine and spermidine levels decreased to non-detectable levels while spermine was reduced to 20 % of that found in control cells.

In vertebrates, the developmental role of ODC has been studied with the aid of enzyme inhibitors. For example, Fozard and coworkers (1980) showed clearly the polyamine requirement for mouse embryogenesis, as embryonic development was arrested in pregnant mice after treatment with DFMO during days 5-8 of pregnancy. The major disadvantage of the use of inhibitors in developmental studies was the inability to distinguish whether this arrest was due to maternal ODC or embryonic ODC. Subsequently when more sophisticated strategies became available, the targeted disruption of mouse ODC gene proved the essential role of ODC in murine embryogenesis and showed that ODC null embryos developed normally to the blastocyst stage but died just after implantation showing substantial chromosomal DNA breakage associated with apoptosis (Pendeville et al., 2001). ODC-heterozygous mice were viable, normal and fertile.

2.2.2. S-adenosylmethionine decarboxylase

S-adenosylmethionine decarboxylase (EC 4.1.1.50) catalyzes the formation of decarboxylated *S*-adenosylmethionine (dcAdoMet), which serves as an aminopropyl donor in the biosynthesis of spermidine and spermine. AdoMetDC regulates the cellular homeostasis of polyamines together with ODC. All mammalian AdoMetDC sequences are highly homologous and they all are synthesized as proenzymes which undergo an autocatalytic cleavage reaction forming a tetramer with two pairs of α- and β-subunits with a pyruvate as the prosthetic group (Pegg et al., 1998). The enzyme is inducible, and strictly regulated at the levels of transcription, translation, proenzyme processing and degradation (Pegg et al., 1998). Spermidine has been shown to repress the transcription of AdoMetDC gene and spermine inhibits its mRNA translation (Shantz et al., 1992). In turn, putrescine activates the enzyme directly and increases the rate of formation of the mature enzyme as well as stimulating its catalytic activity (Pegg et al., 1988a; Stanley et al., 1994). The half-life of enzyme is inversely related to cellular concentrations of higher polyamines and varies between 1-3 h in most cell lines and tissues (Berntsson et al., 1999; Kramer et al., 1988; Morris et al., 1981; Stjernborg and Persson, 1993). The exact mechanism for AdoMetDC degradation is not yet resolved, but it has been suggested that the enzyme is ubiquitinated and degraded by the 26 S proteasome without any obvious interaction with antizyme (Yerlikaya and Stanley, 2004).

There are a few cell lines where increased AdoMetDC levels have been reported (Kramer et al., 1995; Manni et al., 2001; Suzuki et al., 1993). As an example, the overexpression of human AdoMetDC cDNA induced transformation of rodent fibroblasts, and inoculation of these cells into nude mice was tumorigenic (Paasinen-Sohns et al., 2000). A moderate increase in AdoMetDC activity, 2-4 fold, was seen in transgenic mice carrying the rat AdoMetDC transgene in their genome. These mice showed no significant changes in their spermidine and spermine levels (Heljasvaara et al., 1997). AdoMetDC transgenic mice were further bred with ODC transgenic mice and these hybrids were still able to maintain their higher polyamine homeostasis despite the fact that both enzymes have been considered as rate-controlling in polyamine biosynthesis. Primary fibroblasts from AdoMetDC mice showed that the rate of polyamine synthesis was faster in transgenic fibroblasts than in syngenic cells (Heljasvaara et al., 1997). However, transgenic cells showed increased acetylation and export of spermidine and spermine

out of cells which seemed to operate as a compensatory mechanism, preventing the toxic accumulation of the higher polyamines.

Transgenic mice overexpressing AdoMetDC have also been created using the α-myosin heavy chain (MHC) promoter which targets the expression in the heart. The MHC/AdoMetDC mice had an over 100-fold increase in enzyme activity in the heart and a significant alteration in the polyamine content leading to a two-fold decrease in putrescine and spermidine, and a two-fold increase in spermine during the first week of age. Subsequently the levels of spermine decreased to near to control levels. Transgenic mice showed normal growth, fertility and life-span. In addition transgenic mice did not display an overt cardiac phenotype though they were sensitive to cardiac hypertrophy after β-adrenergic stimulation. MHC/AdoMetDC mice were crossed with MHC/ODC, and this hybrid line showed over 1000-fold increase in cardiac ornithine decarboxylase activity. However, the combination of overexpression of the two main biosynthetic enzymes overburdened the cardiac polyamine homeostasis mechanism and hybrid mice showed embryonic lethality, presumably due to the fact that the increased spermine levels were toxic (Nisenberg et al., 2006).

AdoMetDC-deficient mice

The mouse genome contains two catalytically active S-adenosylmethionine decarboxylase genes, AMD1 and an intronless pseudogene AMD2. AMD1 gene is mapped to chromosome 10 in mouse and it has eight exons and seven introns (Nishimura et al., 1999). The AMD1 gene has been disrupted in mouse embryonic stem cells in order to study the role of spermidine and spermine in embryonic development (Nishimura et al., 2002). Homozygous AMD1 gene-disrupted embryos developed normally to the blastocyst stage but died shortly thereafter showing no observable DNA fragmentation as seen in ODC knockout embryos. Cultured AdoMetDC-deficient blastocysts revealed a requirement for spermidine in order to grow *in vitro*. Heterozygous AMD1 +/- mice were normal, viable and fertile and showed decreased AdoMetDC activity in various tissues leading to slightly elevated concentrations of putrescine and spermidine and correspondingly to decreased spermine levels as compared with wild-type littermates. The ODC activity remained rather similar in heterozygous AMD1 +/- and wild-type mice.

2.2.3. Spermidine synthase

Spermidine synthase (EC 2.5.1.16) catalyzes the transfer of the aminopropyl group from decarboxylated S-adenosylmethionine to putrescine in the biosynthesis of spermidine. SPDSy is a mainly constitutively expressed enzyme with a half-life of several days (Seiler, 1990). The enzyme has been purified from a number of species, and active human SPDSy contains two identical subunits (Kajander et al., 1989) and is localized on chromosome 1 (Myöhänen et al., 1991). SPDSy activity is mainly regulated by the availability of its substrate dcAdoMet (Jänne et al., 1978).

Constitutive overexpression of human SPDSy gene in transgenic mice displayed SPDSy activity that was 2-6 times higher as compared with syngenic mice (Kauppinen et al., 1993). The increased enzyme activity had no effect on tissue polyamine pools. SPDSy transgenic mice were also crossed with mice overexpressing the human ODC gene. Hybrid mice revealed no increased accumulation of polyamines and the molar ratio of spermidine to spermine was only slightly increased, supporting the critical role of AdoMetDC in regulating tissue higher polyamine levels.

Mutants lacking the active SPDSy gene

In *S. cerevisiae* spermidine and spermine have shown interchangeable phenotypic effects with SPDSy null mutant. This phenotypically altered mutant strain exhibited no spermidine synthase activity, lacked spermidine and spermine and had an absolute requirement for small amounts of spermidine or higher levels of spermine for the growth (Hamasaki-Katagiri et al., 1997). However, this study was further continued in order to find out whether there is specific requirement for spermidine (Chattopadhyay et al., 2003). *S. cerevisiae* mutant cells that lacked both SPDSy and the *FMS1*-encoded amine oxidase that oxidizes spermine to spermidine showed an absolute requirement for spermidine for growth. Spermine itself was not effective in the absence of the *FMS1* gene because it must be first oxidized to spermidine.

The SPDSy deficiency has been also studied in *L. donovani* (Roberts et al., 2001). The knockout line could not synthesize spermidine and showed a rapid depletion in the intracellular spermidine level with a concomitant elevation of the putrescine pool when incubated in polyamine-free medium. Polyamine auxotrophy was circumvented by supplementing with exogenous spermidine

but not by putrescine or spermine, and hence it was concluded that SPDSy was an essential enzyme in *L. donovani* promastigotes.

2.2.4. Spermine synthase

Spermine is ubiquitously present at a significant level in eukaryotic cells and is the final product of the biosynthetic pathway for polyamines. Spermine synthase (EC 2.5.1.22) catalyzes the synthesis of spermine by transferring the aminopropyl group from dcAdoMet to spermidine, forming spermine. SPMSy has similar characteristics than SPDSy being constitutively expressed, being feedback-inhibited by its product (Pegg, 1986) and consisting of two identical subunits (Kajander et al., 1989). The human and mouse SPMSy genes are located on chromosome X (Lorenz et al., 1998). Although SPMSy catalyses almost identical reactions as SPDSy, their primary structures are very different (Korhonen et al., 1995). Attempts have been made to elucidate the actual role of spermine in mammalian cells by using SPMSy specific enzyme inhibitors but unfortunately in most cases the use of an inhibitor has led only to a weak inhibition of cell growth, since depleted spermine pools were compensated with elevated spermidine concentrations (Pegg and Coward, 1993; Pegg et al., 1989; Shirahata et al., 1993).

Four separate transgenic founder mice were created carrying human SPMSy cDNA under the CMV-IE enhancer/chicken β-actin promoter (CAG/SPMSy) (Ikeguchi et al., 2004). These mice were normal, fertile and SPMSy activities varied widely between founders and studied tissues. The overexpression of SPMSy led to only a modest decrease in spermidine and a slight increase in spermine levels, without influencing the SPDSy activity. SPMSy transgenic mice were crossed with MHC-AdoMetDC and MHC-ODC mice. The former combination proved to be lethal while CAG/SPMSy x MHC/ODC hybrid mice showed improved vitality compared to single MHC/ODC mice. Although the ODC transgene provided an increased supply of putrescine and spermidine, CAG/SPMSy x MHC/ODC mice had only a 20 % increased level of spermine. On the other hand, spermidine was decreased by 44 % and thus this normalization may account for the better survival of the hybrid mice.

SPMSy-deficient models

Several studies have demonstrated that mammalian cells can be grown in culture in the presence of inhibitors of spermine synthase (Chu et al., 1995; Huber and Poulin, 1995; Pegg et al., 1995b). SPMSy-deleted *S. cerevisiae* cells showed neither SPMSy activity nor spermine but the spermidine concentration was increased (Hamasaki-Katagiri et al., 1998). This strain exhibited a normal morphology and grew normally as compared with the wild-type strain in the absence of exogenous polyamines.

The importance of spermine in higher eukaryotes was studied with Gy mice that carry an X-linked dominant mutation in their genome. Gy (Lyon et al., 1986) and Hyp (Eicher et al., 1976) mice have mutations in the Phex gene and are used as models for the genetic bone disease, X-linked hypophosphatemia (Meyer et al., 1995). In Gy mice, deletion extends also upstream from the Phex gene and covers the neighboring SPMSy gene (Grieff et al., 1997; Lorenz et al., 1998; Meyer et al., 1998). Gy males had no significant SPMSy activity and thus no spermine, but the tissue spermidine content was elevated partly due to the inability of the mice to convert spermidine further to spermine but also due to compensatorily increased activities of polyamine biosynthetic enzymes. Increased spermidine levels may also retard active polyamine transport and therefore Gy mice are not able to maintain tissue spermine levels by uptake of exogenous polyamines. Gy and Hyp mutant strains suffer from renal tubular reabsorption of phosphate, hypophosphatemia, rickets, and osteomalasia. In addition, Gy males exhibit additional symptoms including sterility in males, smaller size, inner-ear abnormalities, deafness, hyperactivity, circling behavior and reduced lifespan. In order to verify whether the lack of spermine can cause these more severe symptoms, Gy carrier female were bred with spermine overexpressing mice (Wang et al., 2004). The hybrid mice expressed high levels of SPMSy and also contained spermine in the tissues studied. Their lifespan and fertility were normal and the mice had also a normal growth rate with the exception of a reduction in body weight, this being probably due to the hypophosphatemia. It is also noteworthy that Gy mice can survive only on the B6C3H background (Meyer et al., 1995) and attempts to transfer the gene deletion to other strains have not been successful.

Fibroblast cell lines have been established from Gy males and SPMSy deficiency does not have any significant effect on the growth of these cells. Spermine is strongly bound to DNA and may

impart protection from oxidative and radiative damage against DNA. Ha and coworkers (1998) suggested that spermine is a major natural intracellular compound capable of protecting DNA from free radical attack but the primary cultures of skin fibroblasts from hemizygous Gy/Y mice surprisingly proved to be more resistant towards oxidative stress induced by H_2O_2 than the parental cells (Nilsson et al., 2000). Thus, the increased concentration of spermidine in Gy/Y cells indicates that it has role as a potent free-radical scavenger *in vitro*. UV radiation is known to cause genetic damage by breaking the bonds in the DNA. UV-radiation and oxidative stressors both produce free-radicals but exert their action via different signal transduction pathways as seen in Gy/Y cells which were, in turn, more sensitive than controls to UV-exposure, evidence of a protective role for spermine against chromatin damage. In addition, the lack of any interaction between spermine and DNA predisposed Gy-derived immortalized fibroblast to chemically induced DNA damage by the alkylating agent 1,3-bis-(2-chloroethyl)-*N*-nitrosourea (Mackintosh and Pegg, 2000). Gy derived fibroblasts have also been exposed to a variety of other anti-proliferative agents, such as polyamine analogues. The effects of spermine on the development of cytotoxicity were dependent on the drug employed and both protective actions and sensitization were detected (Ikeguchi et al., 2003).

Recently, the first clinical polyamine deficiency syndrome, Snyder-Robinson syndrome has been reported, which is caused by a defect in SPMSy, reducing the enzyme activity to 5% of controls (Cason et al., 2003). The affected males have mild to moderate mental retardation, childhood hypotonia, facial asymmetry, thin habitus and bone abnormalities. Carrier females are clinically normal.

2.3. Polyamine catabolism in mammalian cells

Higher polyamines can be interconverted to putrescine by the action of catabolic enzymes. Thus, catabolism regulates polyamine homeostasis in conjunction with polyamine transport. Polyamines may also be terminally oxidized by amine oxidases which release aldehydic compounds and reactive oxygen species with a high potential to produce cellular damage (Seiler, 1990). The extent to which the interconversion occurs in cells is not known but it is induced by increased levels of higher polyamines as well as their analogues (Wallace et al., 2003).

2.3.1. Spermidine/spermine N^1-acetyltransferase

In polyamine back-conversion, SSAT catalyzes the transfer of an acetyl group from acetyl-CoA to an aminopropyl group of spermine or spermidine, forming N^1-acetylspermine or N^1-acetylspermidine, respectively. SSAT can either monoacetylate or diacetylate spermine, and putrescine is formed from diacetylated spermine directly via N^1-acetylspermidine without spermidine being formed as an intermediate product (Vujcic et al., 2000). N^1-acetylated compounds are suitable for oxidation by polyamine oxidase (PAO) and the aminopropyl residues originated from dcAdoMet are systemically removed, ultimately yielding spermidine from N^1-acetylspermine and putrescine from N^1-acetylspermidine. The products of these reactions, spermidine and putrescine, can be used again in *de novo* synthesis. The oxidation reaction yields also hydrogen peroxide and 3-acetamidopropanal which is converted to β-alanine (Casero and Pegg, 1993; Seiler, 1987). Recently, a new back-conversion route was discovered, converting spermine directly to spermidine with the aid of SMO without prior acetylation (Vujcic et al., 2002; Wang et al., 2003).

Spermidine/spermine N^1-acetyltransferase (EC 2.3.1.57) is regarded as the key enzyme in polyamine back-conversion. Acetylation marks polyamines for their reutilization or export out of cells and thus SSAT catalyzes the reaction that reduces cellular polyamine levels. SSAT is a homodimer of a subunit containing 171 amino acids (Casero et al., 1991) and the cloned human SSAT gene has been localized to the Xp22.1 region (Xiao et al., 1992). SSAT activity is normally very low under basal cellular conditions and it has a rapid turnover rate (<30 min), therefore its regulation under cellular conditions has been difficult to study (Matsui and Pegg, 1981). However, this cytosolic enzyme is induced rapidly in response to a number of factors including various toxic agents, hormones, growth factors, polyamines and polyamine analogues (Casero and Pegg, 1993; Thomas and Thomas, 2001). Polyamines and their analogues regulate SSAT expression at many levels including increased transcription (Fogel-Petrovic et al., 1993; Xiao and Casero, 1996) and mRNA translation (Fogel-Petrovic et al., 1996; Parry et al., 1995a). Polyamine analogues have also been shown to stabilize SSAT protein (Parry et al., 1995a), probably through inhibition of enzyme ubiquitination and thus preventing its targeting to proteosomal degradation (Coleman and Pegg, 2001). One of the most potent inducers of SSAT is the polyamine analogue, DENSPM, which evokes a rapid depletion of higher polyamines in cells and is currently undergoing phase II clinical trials (Casero and Woster, 2001).

The overexpression of SSAT was first studied in *Escherichia coli*, where inducible expression of human SSAT reduced the bacterial growth rate due to depletion of cellular spermidine levels. Spermidine was converted to N^1-acetylspermidine which to a large extent was excreted out of cells (Parry et al., 1995b). SSAT overexpressing cells were also more sensitive to the growth inhibitory action of the antitumor agent N^1, N^{12}-bis(ethyl)spermine.

SSAT gene has been transiently expressed in CHO cells resulting in an enhanced accumulation of putrescine, the appearance of N^1-acetylspermidine and a decrease in the spermidine and spermine pools (Vargiu and Persson, 1994). The SSAT-induced changes in cellular polyamine content resulted in a compensatory increase in the activities of the biosynthetic enzymes (Vargiu and Persson, 1994). Activation of polyamine catabolism did not have any effects on cell growth, probably due to the short-term nature of the experiment. When SSAT was stably expressed in CHO cells using SSAT cDNA under the control of the cytomegalovirus promoter, the continuous disturbances in polyamine homeostasis led to a reduction in the rate of growth (McCloskey et al., 1999).

Transgenic mouse lines overexpressing SSAT under an endogenous promoter have been widely studied for nearly a decade. These mice showed markedly distorted tissue polyamine pools, including a massive accumulation of putrescine, the appearance of N^1-acetylspermidine, and a decrease in the spermidine and/or spermine pools (Pietilä et al., 1997; Suppola et al., 1999). Probably the most intensively examined SSAT transgenic mice (line UKU165b) carry more than 20 SSAT gene copies in their genome. The most striking phenotypic change was permanent hair loss at the age of 3 to 4 weeks and the mice remained hairless thereafter due to over-accumulation of putrescine which interfered with proper hair development. Histological analysis showed large follicular cysts in the dermis. Other observed phenotypic changes were skin wrinkling, female infertility and loss of subcutaneous fat. Despite the reduced whole body white adipose tissue mass, these mice showed no insulin resistance or fat accumulation in non-adipose tissues (Pirinen unpublished) in contrast to other lipoatrophic mouse models (Moitra et al., 1998; Shimomura et al., 1998). In addition to high insulin sensitivity and glucose tolerance, SSAT overexpressing mice displayed also an enhanced basal metabolic rate, a low tissue accumulation of triglycerides and an increased expression of oxidative phosphorylation genes in white adipose tissue (Pirinen unpublished). The antiproliferative action of polyamine analogues is generally considered to result from induced SSAT activity and thus depletion of the higher polyamines and as expected, SSAT transgenic mice were more sensitive towards toxicity of DENSPM (Alhonen et al., 1999).

The neuroprotective role of putrescine was supported by the findings that SSAT overexpression appears to protect the brain from kainate-induced toxicity (Kaasinen et al., 2000) as well as from pentylenetetrazol-induced neuron loss in the hippocampus (Kaasinen et al., 2003). Two-stage skin tumorigenesis revealed that SSAT transgenic mice developed significantly fewer papillomas than their syngenic littermates (Pietilä et al., 2001). Coleman and coworkers (2002) reported opposite results with a different mouse line where SSAT cDNA was targeted to skin using the bovine keratin 6 promoter. These animals were phenotypically indistinguishable from their syngenic littermates and showed normal hair cycle. The inconsistency between these two studies may be related to the different levels of SSAT expression, hairlessness or genetic background.

In order to elucidate whether activated polyamine catabolism can be overrode by the enhanced polyamine biosynthesis, the hybrid transgenic mice overexpressing both SSAT and ODC under MT promoter were generated (Suppola et al., 2001). Surprisingly, even more striking signs of activated hepatic polyamine catabolism were detected in the hybrid mice than seen in singly MT-SSAT transgenic animals, indicating that tissue polyamine levels are mainly regulated by catabolism. In addition, these doubly transgenic mice exhibited also more severe skin histology showing a significantly larger size of dermal cysts (Pietilä et al., 2001).

Transgenic rats overexpressing SSAT under the control of mouse MT I promoter showed markedly elevated pancreatic SSAT activity after zinc induction which led to extensive depletion of higher polyamines as well as overaccumulation of putrescine and appearance of N^1-acetylspermidine in transgenic animals (Alhonen et al., 2000). A sufficient supply of spermidine and spermine seemed to be essential for normal pancreatic integrity, since the greatly activated polyamine catabolism caused acute pancreatitis in the transgenic rats. The importance of the higher polyamines became evident also when MT-SSAT rats were subjected to partial hepatectomy. In transgenic rats, the initiation of liver regeneration was delayed compared to syngenic littermates and started at day 3 after the operation, when the hepatic spermidine pool reverted back to the normal level (Alhonen et al., 2002).

In humans, the SSAT gene has been related to a rare X-linked syndrome, keratosis follicularis spinulosa decalvans, which is characterized by follicular hyperkeratosis and which affects the skin and eyes. The symptoms are believed to be due to duplication of the region on the X-chromosome that contains the SSAT gene and the patients exhibit similar changes in their polyamine pools as the SSAT overexpressing mice (Gimelli et al., 2002). Recently SSAT has

been identified as a candidate gene mediating a risk for suicide and severe depression (Sequeira et al., 2006).

SSAT silencing using RNA interference

In the absence of specific SSAT inhibitors, the role of SSAT has been studied using small interfering RNA (siRNA) technology which selectively silences the gene post-transcriptionally (Elbashir et al., 2001a; Elbashir et al., 2001b). Chen and coworkers (2003) examined the role of SSAT induction in mediating polyamine pool depletion and apoptosis with RNA interference. Basal levels of SSAT mRNA were reduced by >80% in human embryo kidney (HEK-293) cells and human melanoma (SK-MEL-28) cells, and DENSPM-induced SSAT mRNA was decreased by 95% in SK-MEL-28 cells. Polyamine pools remained virtually unaltered in SK-MEL-28 cells when they were transfected with siSSAT and the analogue treatment caused no spermine and spermidine depletion. siSSAT also prevented DENSPM-induced cytochrome *c* release and caspase-3 cleavage which are a part of the intrinsic apoptotic cascade (Chen et al., 2001). Thus, the induction of SSAT leading to spermine pool depletion is essential for initiating events that lead to polyamine analogue-induced apoptosis. Another example using siRNA technology revealed that knockdown of endogenous SSAT specifically inhibited cell migration mediated by the α9 subunit cytoplasmic domain in mouse embryonic fibroblasts (Chen et al., 2004). Pledgie and coworkers (2005) have also reported that a stable knockdown of SSAT reduced the sensitivity of breast cancer cells to the polyamine analogue DENSPM, suggesting that SSAT plays a major role in mediating the cellular response of breast tumor cells to DENSPM.

2.3.2. Polyamine oxidase

Polyamine oxidase (EC 1.5.3.11) strongly prefers acetylated polyamines as substrates and backconverts selectively N^1-acetylspermine to spermidine and N^1-acetylspermidine to putrescine (Seiler, 1987). The enzyme can also poorly use spermine as a natural substrate (Bolkenius and Seiler, 1981). PAO is flavin adenine dinucleotide-dependent amine oxidase and is present in virtually all vertebrate tissues (Seiler, 1995). The rat liver PAO has a molecular mass of 62 kDa and has been localized both in the peroxisomes and in the cytoplasm (Hölttä, 1977). PAO is relatively constitutively expressed, its half-life is several days and its activity seems to be higher in differentiated cells (Quash et al., 1987). PAO-catalyzed reactions are limited by the availability of N^1-acetylated substrates (Seiler, 1995). The other products of the SSAT/PAO pathway, namely

H_2O_2 and 3-acetamidopropanal, are considered to be responsible for the cytotoxic response of several tumor types to specific polyamine analogues (Ha et al., 1997; McCloskey et al., 1996).

2.3.3. Spermine oxidase

Recently an additional enzyme was discovered in the mammalian polyamine catabolic pathway. Spermine oxidase is a flavoenzyme which converts spermine directly to spermidine without prior acetylation. PAO and SMO share 39% sequence similarity (Vujcic et al., 2003) and have same molecular weights (Bellelli et al., 2004). PAO preferentially oxidizes N^1-acetylated spermidine and spermine, while SMO can use only spermine as its substrate and is unable to oxidize other free or acetylated polyamines (Bellelli et al., 2004). Both enzymes liberate H_2O_2, but SMO produces 3-aminopropanal as a by-product (Vujcic et al., 2002), whereas PAO generates 3-acetamidopropanal (Murray-Stewart et al., 2002). SMO is a cytosolic protein and, similarly to SSAT, is induced by many polyamine analogues (Devereux et al., 2003). Unlike SSAT, which undergoes complex regulation in response to polyamines and polyamine analogues, SMO is regulated mainly at the level of its mRNA in response to polyamine analogues (Wang et al., 2005). Pledgie and coworkers (2005) suggested that the source of the cytotoxic H_2O_2 in polyamine analogue-treated human breast cancer cells is actually derived from SMO, not from PAO. Human SMO (PAOh1) codes for at least three additional splice variants which have different biochemical characteristics and substrate specificities depending on the cell types (Murray-Stewart et al., 2002). The mouse SMO gene is reported also to code for a number of splice variants with the major α-isoform being localized in the cytoplasm while another biochemically identical μ-isoform is found in both nuclear and cytoplasmic compartments (Cervelli et al., 2004).

2.4. Inhibitors of polyamine metabolism and polyamine analogues

Intracellular polyamines have an important role in the proliferation of normal and malignant cells and therefore polyamine metabolism has been employed as a target for cancer chemotherapy and chemoprevention strategies. Polyamines accumulate in cancerous tissues and their concentration is elevated in body fluids of cancer patients and thus the polyamine levels can be used as a diagnostic tool for monitoring the success of therapy. Patients with various types of cancer excreted high levels of polyamines in their urine and this polyamine output was decreased back to normal levels when patients were in remission (Russell et al., 1971). Polyamines can also be monitored from blood (Moulinoux et al., 1991) and cerebrospinal fluid (Marton et al., 1979).

Several polyamine biosynthesis inhibitors have been developed for the treatment of cancer. The combinations of these inhibitors have demonstrated better results than can be obtained with single agents in several studies. The most potent inhibitors are described here briefly. The best known is DFMO, which is an enzyme-activated, suicide inhibitor of ODC (Metcalf et al., 1978). Inhibition of ODC caused cell cycle arrest predominantly in G_1 (Ray et al., 2001) due to decreased putrescine and spermidine levels while the spermine pool often remained unaltered or were somewhat elevated (Gerner et al., 1986). However, clinical studies with DFMO proved disappointing. DFMO showed cytostatic rather than cytotoxic effects *in vivo* because of the compensatory up-regulation of polyamine metabolism and transport. In addition, DFMO was also found to cause hearing loss at high doses (Meyskens and Gerner, 1999). However, recently DFMO has been studied as a chemopreventative agent and has been shown to inhibit carcinogen-induced cancer development in a number of rodent models (Carbone et al., 1998; Kadmon, 1992; Meyskens and Gerner, 1999; Nigro et al., 1987). DFMO has also been used successfully as a highly efficient antiparasitic agent (Bacchi et al., 1980; Giffin et al., 1986).

Methylglyoxal bis(guanylhydrazone) (MGBG) is frequently used as a potent competitive inhibitor of AdoMetDC despite its relatively low specificity. MGBG is a structural analogue of spermidine and it is transported into the cells by the polyamine transport system (Seppänen, 1981). MGBG accumulation inhibits cell growth as well as decreasing the cellular spermidine and spermine content while significantly increasing the putrescine content (Porter et al., 1980). In addition to inhibiting AdoMetDC, MGBG stabilizes the enzyme against proteolytic degradation, inhibits diamine oxidase and induces SSAT (Pegg et al., 1973; Pegg et al., 1985; Pegg and McCann, 1992; Pegg and Williams-Ashman, 1968; Williams-Ashman and Seidenfeld, 1986). A major side-effect of MGBG-treated cells has been mitochondriotoxicity. The swelling of the mitochondria, with a subsequent decrease in ATP production, is evidence of non-specific effects of this inhibitor (Pleshkewych et al., 1980; Williams-Ashman and Seidenfeld, 1986). The clinical use of MGBG was limited by its high toxicity which could not be prevented by using a variety of treatment and dosage regimens (Warrell and Burchenal, 1983). More specific inhibitors of AdoMetDC have been developed, which are analogues of AdoMet or dcAdoMet but their use has been limited by their poor cellular uptake or their instability (Kramer et al., 1989; Pegg et al., 1988b; Stjernborg et al., 1993; Wu and Woster, 1995).

N^1, N^4-bis(2,3-butadienyl)-1,4-butanediamine (MDL 72527) is an enzyme-activated irreversible inhibitor of PAO (Bolkenius et al., 1985). It inactivates PAO in cells and tissues of experimental

animals in a time-dependent manner at micromolar concentrations. It interferes with polyamine pool homeostasis by preventing back-conversion and excretion and thus may reduce the availability of putrescine and spermidine for cell growth and survival. Recently, it has been reported that PAO is not the only enzyme which is inhibited by MDL 72527. The inhibitor also acts as a competitive inhibitor of mouse SMO and thus blocks intracellular back-conversion of spermine to spermidine by both routes (Bellelli et al., 2004). MDL 72527 has been considered as rather non-toxic, since long-term enzyme inactivation caused no apparent toxic effects or behavioral changes in healthy animals (Bolkenius et al., 1985; Sarhan et al., 1991a). However, chronic administration of this compound led to development of lethal effects due to prevention of spermine degradation (Sarhan et al., 1991b).

As the direct single enzyme inhibition strategy has limitations due the wide-ranging regulation of polyamine homeostasis, the specifically developed polyamine analogues have provided a novel and potentially effective class of compounds which target the regulation of polyamine metabolism rather than directly inhibiting the metabolic enzymes in tumor cells. The analogues enter cells through the selective polyamine transport system. The compounds can be either polyamine antimetabolites that deplete the intracellular polyamines or polyamine mimetics that displace the natural polyamines from binding sites, but do not substitute for them in terms of growth-promoting functions. In addition, analogues enhance polyamine catabolism by inducing SSAT and polyamine export out of cells (Wallace and Fraser, 2004; Wallace et al., 2003). The synthesis of the first generation of symmetrically substituted bis(alkyl)polyamine analogues was based on the theory that polyamines may utilize feedback mechanisms to auto-regulate their synthesis. The best studied compounds are DENSPM, N^1, N^{14}-bis(ethyl)-homospermine and N^1, N^{12}-bis(ethyl)spermine, which deplete the intracellular polyamine pool and exert cytotoxic effects in several cell lines (Bergeron et al., 1988; Casero et al., 1989; Chang et al., 1992). DENSPM has undergone Phase II clinical trials but has shown very little evidence of clinical activity. There are also reports of toxicity exerted by this analogue (Bernacki et al., 1995; Kanter et al., 1994). Later non-symmetrically substituted bis(alkyl)polyamine analogues were developed (Wu et al., 1996). These compounds display structure-dependent and cell type-specific cellular effects in the regulation of polyamine metabolism. More recently, a novel class of analogues has been synthesized by the SLIL Biomedical Corporation, which includes conformationally restricted, cyclic and long-chain oligoamine analogues. One of these third generation analogues, SL-11093, has proven to be effective as a chemotherapeutic agent against a human prostate tumor xenograft grown in nude mice (Frydman et al., 2003).

2.5. Techniques for studying gene function

Traditional transgenic animals have provided a powerful tool for the analysis of gene action in the whole organism. Transgenic animals have been used as models of human genetic diseases either indirectly for investigating the biology of the disease, or directly for testing potential treatments. However, one serious limitation of this technique is that the transgene integration site in the genome is random. Since a gene's location in the genome is important for its expression pattern, mouse lines carrying the same transgene may well display wildly varying phenotypes. In addition, one line might have hundreds of copies of the transgene integrated into its genome, while another line under the same experimental conditions might have only a single copy. Also retroviral vectors have been used in the generation of transgenic mice. Concentrated viral preparations carrying the gene of interested have been injected into the perivitelline space of zygotes and transferred to recipient mothers (Barquinero et al., 2004). Recently, major advances have been made using lentiviral vectors which can transduce nondividing cells (Buchschacher and Wong-Staal, 2000). Nonetheless, much information can be learned about gene function by the elimination of a gene or by the deletion of a functional domain of the protein. This can be achieved through random mutation using chemical mutagenesis, a gene trap approach or through gene targeting to generate a knockout mouse.

2.5.1. Production of gene-disrupted mice

The mouse has a long history as an invaluable model in biological studies. Their short life-span and large litter size as well as their similarities to the human genome make them suitable for genetic studies intended to investigate the mechanisms of human diseases. Developments in molecular biology and stem cell biology have allowed researchers to create custom-made mice through gene targeting in mouse embryonic stem cells. Knocking out the activity of a certain gene is one way to study the role of the gene in developing and adult animals. Currently there are hundreds of knockout mouse strains, and examples of knockout mouse models that have been developed to study human diseases vary from single gene disorders to more complex diseases, like cancer.

ES cells are derived from pluripotent, uncommitted cells of the inner cell mass of pre-implantation blastocysts. In the early 80`s, two independent groups reported for the first time on

the isolation and culture of pluripotent ES cells (Evans and Kaufman, 1981; Martin, 1981). ES cells are capable of giving rise to both somatic and germline cells (Bradley et al., 1984). Genetically manipulated ES cells are able to integrate fully into viable embryos when injected into a host blastocyst or can be aggregated with a host morula enabling further germline transmission. This capacity crucially depends on the culture conditions that keep ES cells in an undifferentiated state. To prevent differentiation, ES cells are cultured on a feeder layer such as growth inactivated mouse embryonic fibroblasts which provide both differentiation inhibiting signals as well as serving as an adherence matrix for the ES cells. Mouse ES cells can also be grown *in vitro* without feeder cells if a small cytokine, leukemia inhibitory factor, is added to the culture medium (Smith et al., 1988; Williams et al., 1988). The developments in gene targeting technology have made the production of knockout cell lines or knockout mouse strains of any desired gene possible (Figure 2) (Mansour et al., 1990; Robertson, 1991; Zimmer, 1992). In the first gene-targeted mouse, the target gene was hypoxanthine phosphoribosyl transferase (Doetschman et al., 1987; Thomas and Capecchi, 1987). The gene targeting technique achieves a homologous recombination between a genetically modified gene of interest i. e. the targeting vector, and the matching genomic sequence of the mouse embryonic stem cells. This allows the generation of mice lacking a particular gene function. The targeting vector can be introduced into cultured ES cells by several methods, including calcium-phosphate precipitation, retroviral infection, microinjection, but electroporation is most widely used technique since it is both reliable and reproducible (Thomas and Capecchi, 1987). The targeting vector contains generally 6-10 kb homologous genomic DNA and is often of the same genetic background as the ES cells to be used (te Riele et al., 1992). The desired gene is disrupted by a positive selection marker (e.g., antibiotic resistance gene). In order to minimize random integrations and to increase the efficiency of gene targeting, also a negative selection marker (e.g., herpes simplex virus thymidine kinase gene) can be cloned outside the region of homology. The majority of recombination events do not occur at the desired locus and only in a small number of the transfected cells do identical regions of targeting vector and native chromosome overlap. Thus, ES cells showing resistance to the selective agents are also screened by PCR or Southern blot to identify which clones have been correctly targeted and can be used in the production of mosaic offspring known as chimeras. The tissues of chimeric mice are comprised of a mixture of cells that are originated from both the host embryo and the ES cells. Chimeras are bred to obtain germline transmission and to produce offspring with the desired genetic modification (Thompson et al., 1989).

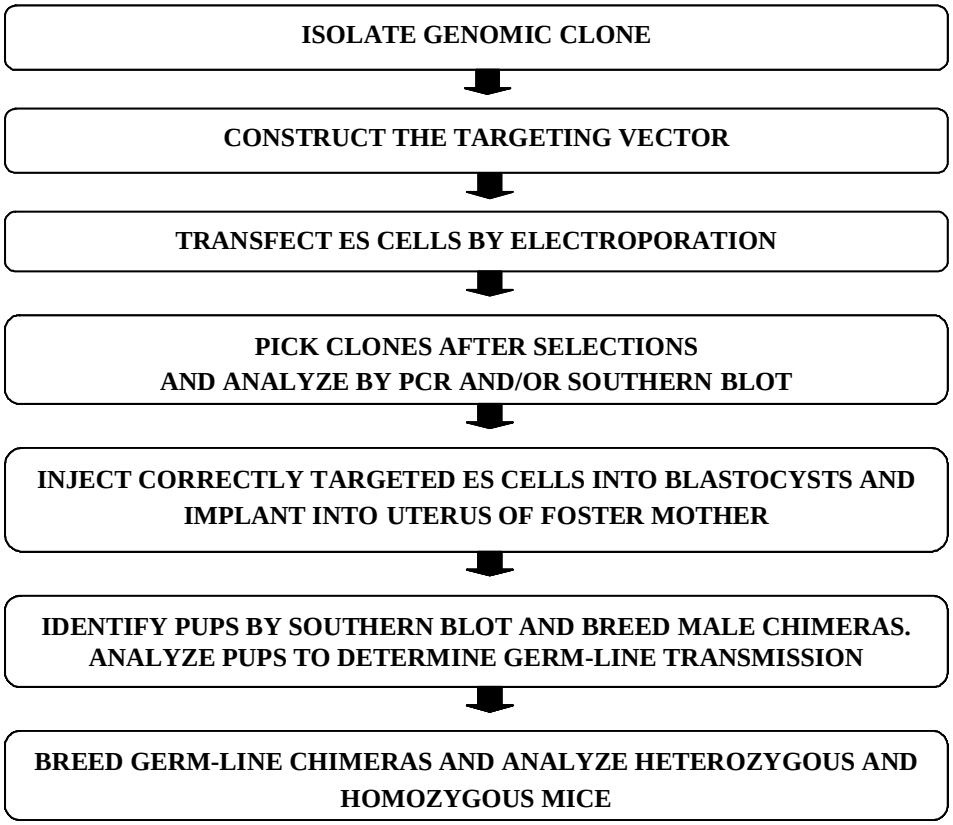

Figure 2. Schematic presentation of the generation of knockout mice

Knockout technology has been further developed during years and new types of genetic modifications have been created using the same gene targeting and ES cell methodology as for the conventional knockouts. Knock-in (KI) is the replacement of a gene by a mutated version of the same gene using homologous recombination. The knock-in vectors contain very subtle mutations such as point mutations, micro deletions or insertions and they are very useful when establishing specific disease-related models of human gene, as in Huntington's disease (Menalled, 2005). The conditional knockout technology enables the specific gene disruption in a time- and tissue-dependent manner in a particular organ, cell type, or stage of development which is particularly useful when complete gene inactivation leads to a lethal or some otherwise adverse phenotype (Lobe and Nagy, 1998). The first widely used method is the Cre-*lox*P recombinase system from bacteriophage P1. Cre recombinase catalyses a site-specific DNA recombination between two identical 34-bp *lox*P sequences (Abremski and Hoess, 1984; Sauer and Henderson,

1988) and is used to excise a gene that has been flanked by two *lox*P target sequences. To generate conditional knockout mice using the Cre/loxP system, two lines of transgenic mice are crossbred. The target mouse line has been created using the gene targeting strategy and it carries a modified allele of the gene including the *lox*P sites. Another mouse line expresses the Cre recombinase under a tissue-specific or inducible promoter (Hayashi and McMahon, 2002). Also viral delivery systems such as adenovirus (Shibata et al., 1997) or lentivirus (Pfeifer et al., 2001) can be used in timing of Cre expression. Offspring which contain both the *lox*P-flanked target locus and the *Cre* gene will produce Cre recombinase in the desired tissue type or time point depending on which promoter has been used. The resulting recombination between the *lox*P sites can result in deletions, inversions or translocations of the target locus depending on the location and orientation of the *loxP* sites. A similar system based on *S. cerevisiae* flippase recombinase has become more frequently used in mouse research (Sadowski, 1995). Tetracycline operator-based systems have been also successfully used to control the expression of numerous transgenes in cultured cells and in whole organisms, especially in mice (Zhu et al., 2002).

2.5.2. Gene trapping

Another strategy to produce knockout mice is achieved by manipulating a gene in ES cells. This is called gene trapping and this method has been applied for investigating animal gene function over a large scale (Evans et al., 1997). Gene trapping uses DNA vectors that can be introduced randomly into the genome of mouse ES cells by electroporation (Schuster-Gossler et al., 1998) or by using retroviral vectors (Friedrich and Soriano, 1991). The basic gene-trap vector contains a splice acceptor site upstream of the promoterless reporter gene and is designed to be expressed only after it is inserted into a genomic locus downstream of a functional promoter. The fusion transcripts mimics the expression of the endogenous gene and usually leads to its transcriptional disruption (Evans et al., 1997). ES cells, in which the reporter gene has been activated, are transferred to the host embryo in order to produce mutant mice. Compared to conventional knockout, single gene-trap vector can be used to mutate thousands of individual genes in mouse ES cells and this method generates mutant mice faster as there is no need to target each gene separately. However, the generation of homozygous mutant animals for useful gene knockout models requires also breeding of heterozygous lines with gene trap mutations. Many genes with unknown functions have been discovered and characterized from libraries developed by gene trapping techniques (Stanford et al., 2001). One major disadvantage of this technique is that is not

as efficient or as specific as gene targeting because the successful insertion of the gene-trap vector into a gene does not automatically lead to a loss of function. In addition, certain genes may never be trapped either due to statistics or because the gene is not active in ES cells.

2.5.3. Somatic cell nuclear transfer

Until recently, targeted modification of the animal genome was restricted to the mouse because isolation of germline competent embryonic stem cells has failed in any other mammalian species. However, Wilmut and coworkers (1997) described successful cloning of the sheep by somatic cell nuclear transfer and this opened up the possibility for an alternative route to generate animals with targeted mutations in their germline. The basic idea is to perform gene targeting in somatic cells and then to transfer the genetically modified nuclei into unfertilized enucleated oocytes (Kubota et al., 2000; Schnieke et al., 1997). The resulting embryos are transferred to a foster mother and the new individuals are genetically identical to the somatic cell donor and should contain the genetic modification in every cell of their body. So far, many species have been cloned but the technique also suffers from problems like low efficiency and a high incidence of developmental abnormalities (Hill et al., 1999; Kato et al., 1998; Kubota et al., 2000; Renard et al., 1999; Young et al., 1998). Somatic cell nuclear transfer can also be used as a source of viable stem cells and, in addition, to reproductive cloning; it offers therapeutic possibilities to create embryonic stem cells that exactly match a patient (Wakayama et al., 2001).

2.5.4. Small interfering RNAs

The generation of gene targeted mutant mice via homologous recombination in ES cells is a laborious process and also problematic if the targeted gene is essential for embryonic development. Therefore a number of antisense strategies have been developed in an attempt to silence the gene expression by targeting specific RNAs for degradation but effective gene silencing in complex mammalian systems has proven difficult. Three classes of antisense agents can be distinguished by their mode of action: single-stranded antisense oligodeoxynucleotides; catalytic active RNA/DNA such as ribozymes and small interfering RNA molecules known as siRNAs (Vidal et al., 2005).

RNA interference (RNAi) is a phenomenon in which double-stranded RNA suppresses expression of a target protein by inducing specific degradation of the target mRNA (Hannon,

2002). The main effectors of the RNAi process are the small interfering RNAs. The siRNAs are complementary to the mRNA of the target gene and are incorporated into an RNAi targeting complex with nuclease activity (RISC; RNA-induced silencing complex) inducing degradation of the target mRNA (Hammond et al., 2001; Hutvagner and Zamore, 2002; Nykänen et al., 2001; Sharp, 2001). Thus the translation of the encoded protein is prevented and this method provides a simple alternative to the creation of cell lines or transgenic animals with knockdown phenotypes i.e. gene expression levels are reduced (Elbashir et al., 2001a). RNAi has revolutionized the study of gene function, and is now being explored as a therapeutic tool (reviewed in Dorsett and Tuschl, 2004; Hannon and Rossi, 2004). Today, specific gene silencing by using siRNA has been widely applied in species ranging from *C. elegans* to non-human primates (Fire et al., 1998; Zimmermann et al., 2006).

3 AIMS OF THE STUDY

The general aim was to study the specific functions of two genes involved in polyamine metabolism by using conventional knockout techniques. The polyamine biosynthetic gene SPMSy was chosen for the disruption because bacterial cells are unable to synthesize spermine and it was intended to elucidate the actual role of this polyamine in mammalian cells. SSAT gene was the second gene of interest as its product is considered to be the key enzyme in polyamine catabolism. It has not been previously possible to study the metabolic significance of changes in SSAT activity due to the lack of specific inhibitors. In addition, as SSAT-overexpressing mice revealed striking changes in their phenotype, polyamine pools and glucose metabolism, the extent of the possible consequences in SSAT null genotype would be interesting to study in parallel research.

The specific aims of this study were to:

I Generate distinct SPMSy- and SSAT-deficient embryonic stem cell lines and knockout mouse strains

II Study the consequences of SPMSy gene disruption on polyamine homeostasis and to clarify the specific role of spermine in mammals

III Study the importance of SSAT in polyamine catabolism and homeostasis

IV Characterize SSAT null mice and to study their glucose metabolism

4 MATERIALS AND METHODS

4.1. Generation of the knockout ES cell lines

Genomic clones of the mouse SPMSy gene and SSAT gene were isolated from a 129/SvJ mouse genomic library in lambda DASH II vector (Stratagene, La Jolla, CA). SPMSy gene was isolated by PCR-based screening method (Israel, 1993) using oligonucleotides which amplified a region between exons 6 and 7 as described in (**I**). Phage inserts of isolated positive clones were subcloned and sequenced revealing a 14.3 kb fragment containing exons 3-9 of the spermine synthase gene. The SPMSy-targeting vector, pMSPMNEO, covered exons 3-6 and a positive selection marker, neomycin phosphotransferase (NEO) gene from pGT-N28 (New England Biolabs, Beverly, MA) were inserted in the opposite orientation on the exon 5. The construct also included herpes simplex virus thymidine kinase gene from pTV-O plasmid as a negative selection marker which was inserted outside the homology region into 5' end of the clone. Thirty micrograms of linearized and purified targeting vector was introduced into the mouse ES cell line RW-4 (Genome Systems Inc., St.Louis, MO) by electroporation (Gene Pulser, Biorad Life Science, Hercules, CA). Clones that survived from neomycin-ganciclovir selections were screened first by PCR and correct targeting of PCR-positive clones was confirmed by Southern blot analysis. The absence of additional random integration of construct was ensured with NEO-specific probe (**I**).

SSAT gene was screened from the 129/SvJ mouse genomic library by using a probe which covered nucleotides 137-744 of mouse SSAT cDNA. The SSAT-targeting vector contained all six exons of the SSAT gene and was inserted into the pTV-O vector. One part of exon 1 was deleted and replaced by the NEO gene from pMC1Neo (Stratagene La Jolla, CA). The targeting vector was introduced into the mouse ES cell line RW-4 by electroporation similarly to the SPMSy construct. Those clones that survived from positive-negative selection were identified first by PCR with the results being verified by Southern blot hybridization as described more detail in (**II**).

4.2. Experiments with the knockout ES cell lines

Parental RW-4, SPM-KO and SSAT-KO ES cell lines were cultured in an undifferentiated state on mitomycin-inactivated mouse fetal fibroblasts (feeder fibroblasts) in Dulbecco's modified Eagle's medium (DMEM; Life Technologies or Invitrogen) supplemented with 2 mM glutamine, 10-15 % fetal bovine serum and murine leukemia inhibitory factor (1000 units/ml) (ESGRO, Invitrogen). Later, the cells were adapted to grow without feeder fibroblasts in DMEM supplemented with 2 mM glutamine and 10 % fetal bovine serum.

Parental and SPMSy-deficient cells were seeded 24-h prior to treatments with 1 mM DFMO, 10 µM MGBG, 50 µM DENSPM or 0.1-100 µM etoposide. The effects of spermine on the growth of SPMSy knockout cells were examined without feeder fibroblasts in medium supplemented with 1 mM aminoguanidine (Sigma, St. Louis, MO, USA), an inhibitor of serum amine oxidase, in order to protect the cultured cells against serum amine oxidase-dependent polyamine toxicity (Parchment et al., 1990). The cell number in the various experiments was measured electronically by Coulter Counter (Coulter, Luton, England). DNA fragmentation was determined as described earlier by (Nomura et al., 1999).

Parental and SSAT-deficient ES cells were seeded 24 h prior to the treatments. DENSPM concentration varied depending of experiment and typically 50 µM or 100 µM DENSPM was used. Exogenous polyamines at 100 µM concentration of each were added to cell cultures. In tracer studies, cells were plated in six-well culture plates and exposed to 50 µM DENSPM for 48 h. Then the inhibitors of amine oxidases, 20 µM MDL 72527 (gift from Hoechst-Roussel), 1 mM semicarbazide (Sigma, St. Louis, MO, USA) and 1 mM aminoguanidine, were added for an additional 24 h with 50 µM DENSPM. After the growth medium was removed, the cells were washed twice with phosphate buffered saline, and DMEM without serum supplemented with either 10 µM [^{14}C]spermidine (specific radioactivity 112 mCi/mmol; Amersham Biosciences) or [^{14}C]spermine (specific radioactivity 110 mCi/mmol; Amersham Biosciences) being added for 3 h pulse labeling. After the incubation, the cells were washed with phosphate buffered saline, trypsinized, counted, and subjected to further analyses.

4.3. Generation of the SSAT knockout mice

C57BL/6J blastocysts were injected with one of the correctly targeted undifferentiated SSAT-KO ES cell clones and were transplanted into pseudopregnant females at Karolinska Institutet (Stockholm, Sweden). The chimeric animals, three females and one male, were transported to the University of Kuopio for further breeding. The chimeric male gave germline transmission, and the offspring were screened for the presence of the disrupted SSAT gene by PCR and Southern blot analysis using PCR oligonucleotides and Southern blot probes as described (**II**). The SSAT-deficient mice were backcrossed in the C57BL/6J mouse strain in order to dilute the embryonic stem cell-derived 129/SvJ genetic background. Age and sex-matched C57BL/6J mice, or wild-type littermates from breedings, were used as controls in all experiments. The animals were kept at the National Laboratory Animal Center of the University of Kuopio on a 12-h day/night cycle and were fed normal rodent chow (R3, Lactamin AB, Stockholm). All experimental protocols were approved by the Animal Care and Use Committee of the University of Kuopio.

4.4. Experiments with the SSAT knockout mice

The absence of the functional SSAT gene in knockout mice was confirmed by PCR and Southern blot analysis using the same oligonucleotides and probes as described earlier in SSAT-KO ES cell characterization (**II**). Polyamine homeostasis in SSAT-deficient mice was studied with 24 h CCl_4 treatment (0.1 ml/kg i.p. (1 % w/v in corn oil)) and with DENSPM (125 mg/kg i.p. for 3 days). In the DENSPM-survival study, mice received daily injections of the analogue (125 mg/kg/for 10 days) and the survival of animals was followed. Polyamine back-conversion was also studied with intraperitoneal injections of 2 µCi [^{14}C]spermidine (specific radioactivity 112 mCi/mmol; Amersham Biosciences) or 2 µCi [^{14}C]spermine (specific radioactivity 113 mCi/mmol; Amersham Biosciences).

In the glucose tolerance test, mice were fasted for 16 h before intraperitoneal injection of 2 mg/g D(+)-glucose (BDH, England). Blood samples were collected from a tail vein of nonanaesthesized animals at 0, 15, 30, 60, 90, and 120 min after the injection. The insulin tolerance test was carried out after 12 h fasting and mice were injected with 0.25 mU/g insulin (Actrapid, Novo Nordic, Denmark). Blood samples were taken from a tail vein at different time points (0, 20, 40, and 80 min). Plasma glucose levels were measured microfluorometrically

(Passonneau and Lowry, 1993). Plasma insulin levels were determined using rat insulin ELISA kit (Crystal Chem Inc, USA) with mouse insulin standards.

4.5. Analytical methods

Southern blot. Southern blot analyses were carried out to confirm the absences of a functional SPMSy gene, a SSAT gene or the additional random integration sites of the constructs (**I-III**). DNA was isolated from the ES cells or tissues of SSAT null mice by using the method of Blin and Stafford (1976). A total of 10 µg of DNA from selected clones or 10-15 µg from tissues were digested, electrophoresed and transferred onto nylon membranes using capillary transfer. Blots were hybridized with digoxigenin labeled probe external to the targeting vector and with NEO-probe (**I-III**) and chemiluminescent detection (Roche Applied Science) was performed (Engler-Blum et al., 1993).

Western blot. Cell lysates were prepared from feeder fibroblast free cell cultures. SSAT-KO cells and their controls were exposed to 100 µM DENSPM prior to the analysis. The samples contained 50 µg of protein and were electrophoresed in 12 % SDS-polyacrylamide gel (Laemmli, 1970) and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blots were blocked with 5 % (w/v) nonfat dried milk in Tris-buffered saline and incubated with SPMSy peptide-specific antibodies (**I**) or with the C-terminal antibody of the SSAT (**II**). An alkaline phosphatase-labeled antirabbit IgG (Zymed, San Francisco, CA) was used as the detection antibody. The blots were developed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate staining.

Enzyme activities. Cells were lysed (25 mM Tris-HCl pH 7.4, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% Triton X-100) and the tissues were homogenized in lysis buffer (25 mM Tris·HCl pH 7.4, 1 mM dithiothreitol, 0.1 mM EDTA). SPDSy and SPMSy activities (**I**) were measured from the supernatants with methyl-^{14}C-labeled dcAdoMet as the substrate (Raina et al., 1983). The activities of ODC and AdoMetDC were determined as described previously (Jänne et al., 1971; Jänne and Williams-Ashman, 1971). PAO activity was measured essentially according to Kumazawa and coworkers (1990) except for using radioactive diacetylspermine, instead of acetylspermine, as the substrate. SSAT activity (**II, III**) was assayed from centrifuged homogenates (Bernacki et al., 1995).

Polyamines and their analogues. Polyamines and their acetylated derivates were measured from cell (**I, II**) or tissue homogenates (**III**) with the aid of high performance liquid chromatography (HPLC) (Hyvönen et al., 1992). The samples from [^{14}C]spermidine and [^{14}C]spermine tracer studies were analyzed by injecting 20 µl supernatant into an HPLC and one minute fractions were collected and counted for radioactivity. The HPLC was used also in determination of MGBG (**I**) (Yarlett and Bacchi, 1988) and DENSPM **(I-III)** (Porter et al., 1985).

Metabolic assays. Triglycerides and cholesterol were determined from plasma samples of SSAT-KO and WT male mice after a 16 h fast using Microlab 200 analyzer (Merck, Germany) and commercial kits for triglycerides and cholesterol (Diasys diagnostic Systems GmbH, Germany **(III)**.

Histology. The tissue specimens for histology were fixed in 10 % phosphate-buffered formalin, processed, and embedded in paraffin, cut into four-micrometer thick sections and stained with haematoxylin and eosine using the standard procedures (**III**).

Statistical analyses. Two-way and one-way (with Dunnett's *post hoc* multiple comparison test) analysis of variance (ANOVA) or Student's two-tailed *t* test, when applicable, were used for statistical analyses with the aid of a software package, GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA). Insulin levels were logarithmically transformed for statistical analyses.

5 RESULTS

5.1. SPMSy knockout cells (I)

A large part of the functional SPMSy gene was isolated from the mouse strain 129/SvJ genomic library containing 14318 base pairs and covering the exons 3 to 9 encoding amino acids 58 to 315. The SPMSy gene was disrupted by NEO gene insertion into exon 5 and replacement of the wild-type SPMSy gene by an inactive copy on mouse ES cells. Altogether 44 clones resistant both to G418 and ganciclovir were isolated and one of them was targeted correctly and showed only the mutated allele. Since the functional mouse SPMSy gene is located on chromosome X and the used RW-4 ES cells are XY karyotype, this gene disruption led to hemizygous SPMSy deficiency. The absence of SPMSy activity decreased the spermine concentration below the detection levels. The loss of one biosynthetic enzyme and hence to an absence of spermine led to increased activities of both ODC and AdoMetDC but not SPDSy. Although the activity of SPDSy remained at control cell levels, the actual function of this enzyme was enhanced in knockout cells due to improved availability of the shared substrate, dcAdoMet, which led to decreased putrescine concentration and significantly elevated spermidine content. However, as increased spermidine levels compensated for the loss of spermine, the total polyamine pool remained virtually unaltered. Spermine depletion caused no morphological effects on cell structure as seen in transmission electron microscopy and showed only a minimal effect on the growth rate of the ES cells as compared with parental RW-4 cells. The synthesis of spermine was not essential for ES cells growth *in vitro* as this slightly slower growth rate in knockout cells was not enhanced with additional spermine supplement. Nonetheless, the spermine-deficient cells were more sensitive to the antiproliferative actions of two polyamine biosynthesis inhibitors, 1 mM DFMO and 10 µM MGBG. Inhibition of AdoMetDC led to the reduction of spermidine and spermine pools and increased the putrescine level in both cell lines but in targeted cells, the MGBG treatment caused a cytotoxic effect after 48-h exposure instead of the cytostatic effects observed on control cells. The cytotoxicity exerted by MGBG seemed to be due to increased uptake of the drug in the spermine-deficient cells as MGBG is structurally related to the higher polyamines and thus is transported into cells by the same carrier as used by the natural polyamines (Seppänen, 1981).

The spermine analogue, 50 µM DENSPM, reduced growth rates both in parental and in SPMSy knockout cell lines as compared with non-treated controls, but this sensitivity was likewise enhanced in the targeted cells and after 48-h exposure the cells started to die while parental cells only ceased to grow. When the polyamine pools of these two cell lines were compared, it was observed that the cytotoxic effect of DENSPM in spermine-deficient cells was not directly attributable to the depletion of total polyamine pools or the higher DENSPM concentration in cells. For instance, after 24-h treatment, the spermidine concentration in the targeted cells was higher than the combined spermidine and spermine pools in control cells and also the initially elevated uptake of the drug in targeted cells was normalized to the level found in the parental cells. Moreover, cells were cultured with the presence of increasing concentrations (0.1-100 µM) of etoposide, which induces DNA damage in the cells by inhibiting the nuclear enzyme topoisomerase II. This enzyme is responsible for repair of breakage and reunion reactions of DNA which are necessary for normal cellular function (Baldwin and Osheroff, 2005). Growth curves showed clearly significantly higher sensitivity of SPMSy-deficient cells to etoposide in both time- and dose-dependent manner. Also an ethidium bromide stained agarose gel revealed a typical etoposide-induced DNA fragmentation pattern from knockout cells treated with 10 or 100 µM etoposide.

Undifferentiated SPMSy-deficient ES cells were also used in morula aggregations and blastocyst injections in order to generate knockout mice line but unfortunately after several breedings and characterization of numerous pups it was evident that it was not likely that we would achieve any germline offspring originating from these chimeric animals.

5.2. SSAT knockout cells (II)

A 18-kb fragment of mouse SSAT gene was isolated from a 129/SvJ genomic library. The targeting vector was constructed by cloning a fragment of SSAT gene containing all six exons to pTV-O vector. The SSAT gene was disrupted by inserting the NEO gene into exon 1. Targeting the mouse SSAT gene in ES cells gave 16 clones that survived from selections and three of them were correctly targeted and contained no additional integration sites of the construct, as confirmed by Southern blot analysis. The SSAT activity is normally very low in cells. Therefore the absence of inducible SSAT protein in SSAT-KO cells was verified by Western blot analysis where the cells were first treated with a highly potent SSAT inducer, 100 µM DENSPM, for 24-

h. The lack of SSAT activity was further confirmed when cells were cultured with 50 µM DENSPM. The parental cells showed direct induction of SSAT activity in response to the drug while in targeted cells the activity remained at basal level during the whole five day treatment. Disruption of the SSAT gene in ES cells caused a constant elevation of the spermidine pool, but in other respects only minor changes in the polyamine pools were detected as compared with parental cells. To further study the role of SSAT in maintaining polyamine homeostasis and thus protecting cells from cytotoxic levels of polyamines, cells were grown for 24-h in a culture media supplemented with either 100 µM putrescine, 100 µM spermidine, or 100 µM spermine. The knockout cells did not accumulate exogenous polyamines above the parental cell line levels and no observed decrease in cell viability was detected in any of the cell lines. Polyamine analogues, like DENSPM, have been believed to exert their cytotoxic effects through induction of SSAT and the subsequent depletion of cellular spermidine and spermine pools. As expected, SSAT-KO cells were significantly more resistant to the antiproliferative effect of five day treatment with 50 µM DENSPM. The sensitivity of parental cells to the drug was not due to a more efficient DENSPM-uptake or to polyamine depletion, as the spermine level was depleted identically and the spermidine level decreased only slightly slower in targeted cells. It is probable that the replacement of natural polyamines from their intracellular binding sites by DENSPM had depleted the intracellular polyamine pools without any involvement of SSAT activity. However, the role of SSAT is considered to be crucial in the back-conversion of spermine to spermidine as well as spermidine to putrescine. This assumption was tested using radioactive spermidine and spermine as tracers. For the spermidine interconversion study, SSAT-deficient cells were exposed to 10 µM [^{14}C]spermidine for 3-h in the absence or presence of 50 µM DENSPM and 20 µM polyamine oxidase inhibitor, MDL 72527. DENSPM-treated parental cells revealed an accumulation of the products of spermidine back-conversion, N^1-acetylspermidine and putrescine, which in turn remained at the non-treated level in the targeted cells. PAO inhibition by MDL 72527 had no effect on SSAT-KO cell metabolism but it tripled the formation of radioactive N^1-acetylspermidine in the wild-type cells by preventing its oxidation further to putrescine. These results indicate that SSAT is essential for the back-conversion of spermidine.

In the case of spermine interconversion, SSAT-deficient cells surprisingly converted 10 µM [^{14}C]spermine to radioactive spermidine even more rapidly than parental cells without any formation of acetylated spermine. This was not due to stimulated PAO activity, as its activity was similar in parental and targeted cells with or without DENSPM. The treatment with DENSPM

did not enhance the rate of spermine interconversion in either cell line; instead it seemed to inhibit formation of spermidine from spermine or to facilitate the spermidine efflux in the targeted cells. Spermine interconversion was only partially blocked with MDL 72527 or amine oxidase inhibitors, aminoguanidine and semicarbazide. Thus SSAT-disrupted ES cells proved that spermine can be oxidized directly to spermidine without its prior acetylation by SSAT.

5.3. SSAT knockout mice (III)

Undifferentiated SSAT-KO ES cells were used in the blastocyst injection experiments and three chimeric females and one male were born. The chimeric male gave germline transmission to his female offspring and the SSAT-KO mouse line was established and further backcrossed in the C57BL/6J mouse strain. SSAT-deficient mice had a normal lifespan, were viable, fertile and phenotypically indistinguishable from their wild-type littermates. The characterization of SSAT-KO mice by PCR and by Southern blot showed the correctly targeted and appropriately sized DNA pattern. The lack of inducible SSAT activity was confirmed with CCl_4 and DENSPM treatments. After CCl_4-treatment (0.1 ml/kg i.p. 1 % w/v in corn oil) SSAT activity was increased only in livers of wild-type mice which led to markedly elevated putrescine and acetylated spermidine pools. In SSAT-KO mice, the activity remained at the low basal level but surprisingly, the hepatic spermidine and spermine pools decreased similarly as in wild-type mice.

In order to verify the absence of inducible SSAT and to study the effects of the analogue on the tissue polyamine pools, SSAT-KO and wild-type mice were subjected to a 3-day DENSPM-treatment (125 mg/kg/daily i.p.). SSAT activity was significantly increased in all studied tissues of wild-type mice whereas the SSAT knockout mice showed once again basal level enzyme activity. DENSPM significantly decreased tissue higher polyamine pools and enhanced putrescine levels in wild-type mice while no such changes were seen in knockout mice. The analogue accumulated similarly in both the wild-type and SSAT-KO mice.

Polyamine pools of wild-type and SSAT-KO mice were studied regularly during a two-year surveillance period. According to our earlier results, SSAT-deficient ES cells maintained polyamine homeostasis relatively well with the exception that they had about a 30 % elevated spermidine concentration. This phenomenon was also seen in SSAT knockout mice where we detected an elevated molar ratio of spermidine to spermine in most studied tissues.

In order to confirm the spermidine and spermine interconversion results which had been observed in ES cells, SSAT-KO and wild-type mice were tested in a similar experiment. After 2 h of 2 µCi [^{14}C]spermine injection, no acetylated spermine was detected in wild-type or SSAT-KO mice and the amount of radioactive spermidine was similar in both groups (Figure 3A, unpublished results). However, when mice were injected first with the SSAT inductor, CCl_4 (0.1 ml/kg i.p. 1 % w/v in corn oil), and subsequently with 2 µCi [^{14}C]spermidine, increased amounts of N^1-acetylspermidine and significantly elevated levels of radioactive putrescine were clearly detected in wild-type mice after 3 h (Figure 3B, unpublished results). In knockout mice, traces of acetylated [^{14}C]spermidine were also found but the amount of putrescine was only marginally elevated (0.3 ± 0.3 %) as compared with the untreated group (0 %) confirming the importance of functional SSAT activity in the back-conversion of spermidine (Figure 3B, unpublished results).

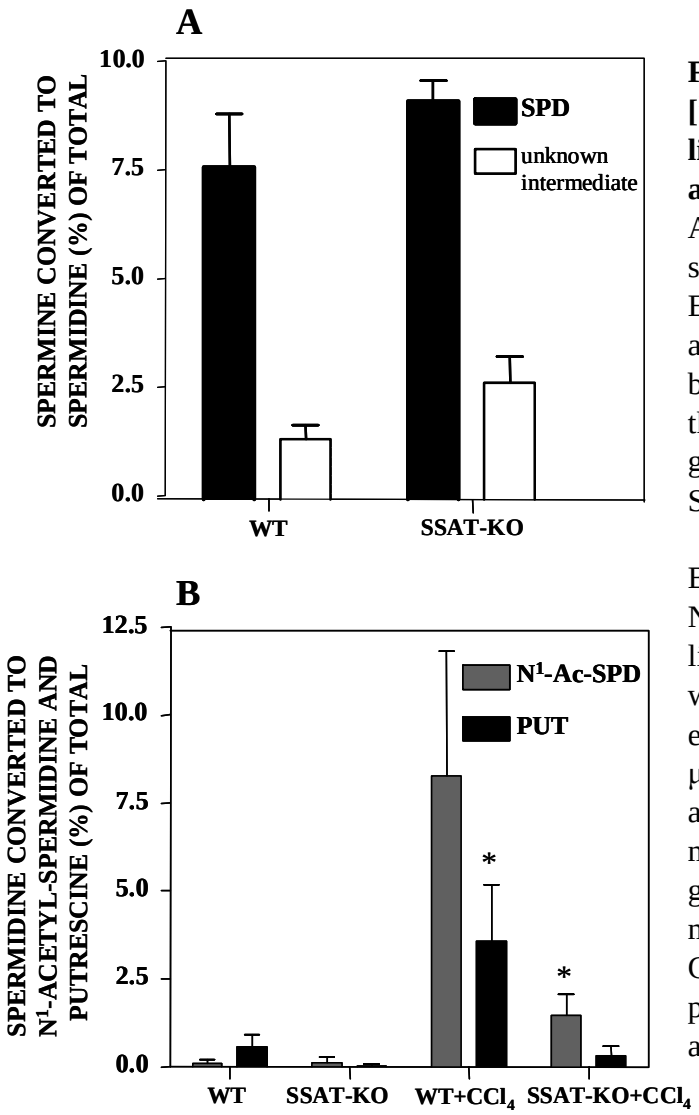

Figure 3. [^{14}C]spermine and [^{14}C]spermidine backconversion in the livers of SSAT knockout (SSAT-KO) and wild-type (WT) mice.

A. Conversion of radioactive spermine to spermidine and unknown intermediate. Each animal received 2 µCi [^{14}C]spermine as an intraperitoneal injection 2 h or 4 h before sacrifice. Each column represents the mean ± S.D., where n(2 h) = 4 in both genotypes and n(4 h) = 2 (WT), 4(KO) SPM, spermine; SPD, spermidine.

B. Conversion of radioactive spermidine to N^1-acetylspermidine and putrescine in the livers of SSAT knockout (SSAT-KO) and wild-type (WT) mice. Animals were exposed to 1 % CCl_4 for 24 h, whereafter 2 µCi [^{14}C]spermidine was injected for an additional 3 h. Each column represents the mean ± S.D., where n = 3 in both genotypes. Animals were 16 week-old male mice. *$p<0.05$, in comparison with CCl_4-treated and untreated animals. PUT, putrescine; N^1-Ac-SPD, N^1-acetylspermidine

In the DENSPM-survival study, SSAT knockout mice were surprisingly more sensitive towards DENSPM (125 mg/kg/ i.p. daily) than their wild-type littermates. The reason for this enhanced sensitivity was not the increased tissue uptake of DENSPM or greater depletion of cellular polyamines compared to wild-type mice. The analogue caused typical polyamine pool changes in wild-type animals after 72-h exposure, with tissue spermine and spermidine being significantly reduced and putrescine increased whereas almost no response was found in SSAT-KO mice. A histological analysis failed to show any explanatory changes in liver, spleen, kidney and pancreas of SSAT-KO mice as compared with controls.

In addition to the importance of SSAT in spermidine interconversion, it also seems to be a factor involved in the regulation of glucose and energy metabolism, as demonstrated in studies with SSAT overexpressing mice (Pirinen unpublished). 16-month-old SSAT-KO males showed elevated plasma glucose levels in the fasted state and the glucose tolerance test revealed glucose levels similar to WT mice but the insulin levels were significantly elevated during the experiment indicating increased insulin secretion and insulin resistance in the SSAT-KO mice. The insulin tolerance test revealed elevated plasma glucose levels at all measurement points and further confirmed insulin resistance of older SSAT-KO males. Subsequently it was defined that the actual onset of the insulin resistance of SSAT gene-disrupted mice began at the age of 12 months and fasted plasma glucose levels were then 9.0 ± 0.8 mM in WT mice as compared with the significantly increased 10.0 ± 1.0 mM in SSAT-KO mice ($p<0.05$). In addition, a histological analysis of the kidneys from 24-month-old knockout animals showed dilation of Bowman's space with atrophy of glomerulus and associated cells, dilation of the tubules in the inner medulla, inflammation and necrosis of renal papillae and inflammation of the surrounding fat. The majority of these findings are compatible with the hypothesis that the symptoms are attributable to diabetic nephropathy.

6 DISCUSSION

The knockout mice have proven that it is possible to aim the inserted gene at a precise location in the mouse genome. This technique enabled the replacement or knockout of a specific gene with a mutated or inactive allele. New mutant alleles can be passed through the germline to produce an unlimited number of mutant offspring, and different mutations can be combined with variants at other loci to study gene interactions. Currently there are several hundred different strains of knockout mice which have been beneficial in expanding our understanding of the human genome and its roles in disease. Polyamine research is no exception in this respect and new insights into the actual roles of polyamine metabolic enzymes as well individual polyamines have been obtained with specific gene disruption.

6.1. Generation of SPMSy and SSAT gene knockouts

The disruption of ODC and AdoMetDC have proven that these key polyamine biosynthetic enzymes are crucial during embryogenesis (Nishimura et al., 2002; Pendeville et al., 2001). Our aim was to generate individual SPMSy and SSAT gene-disrupted ES cell lines which would later be utilized in the possible creation of the knockout mice. As a result of this study, we created both SPMSy- and SSAT-deficient ES cell lines and SSAT knockout mice. Studies with SSAT-KO mice revealed that this enzyme is not essential for mammalian embryonic development and the disrupted gene was inherited in a Mendelian fashion.

The generation of SPMSy-deficient mice was more problematic and it failed as SPM-KO chimeric mice were not able to transmit the mutated allele to their offspring. This suggests that the absence of germline transmission is evidence that spermine is essential for mouse development, at least in the 129/SvJ background. This result was further confirmed with the SPMSy gene and Phex gene mutated Gy mice which survived only on the B6C3H background. Every attempt to transfer this gene deletion to other strains has failed and the genetic factors allowing survival in the absence of spermine in the B6C3H strain are unknown. Spermine seems to have a crucial role during embryogenesis, as SPMSy expression is highest on day 11 (Lorenz et al., 1998). However, in Gy mice, adequate supplies of spermine might be available during embryonic development due to polyamine transport. Further changes in polyamine levels may occur during postnatal life because Gy mice had a reduced viability from birth and they were prone to sudden death at adult age. Thus it can be concluded that in the more complex

environment of a multicellular organism, spermine may be required in some specialized cell type or under certain circumstances.

6.2. Polyamine homeostasis in SPMSy and SSAT knockout models

Polyamine homeostasis is complexly controlled at the level of the biosynthetic and catabolic enzymes as well at the transport stage. The extent of this regulation was clearly seen in both SPMSy and SSAT gene-disrupted ES cell lines *in vitro* as well as in SSAT knockout mice *in vivo*. All of these knockout models were able to maintain their total polyamine pools relatively well though one common feature was constantly elevated spermidine pool albeit the primary reasons for this accumulation were divergent. The almost doubled spermidine concentration in SPM-KO cells resulted from increased activities of the main biosynthetic enzymes, ODC and AdoMetDC, and the improved availability of decarboxylated AdoMet exclusively for spermidine synthase. Spermine has been reported to be more active than spermidine in the repression of AdoMetDC activity (Pegg et al., 1998) and it also seems to have a major regulatory role to ODC, as even the greatly increased SPD levels in knockout cells could not prevent these inductions. Spermidine is known to stimulate polyamine catabolism by inducing SSAT, but no increased activity was detected in SPM-KO cells (unpublished results). Similar results were also seen in Gy fibroblasts (Nilsson et al., 2000).

Even though SSAT is believed to function as an essential component in regulating polyamine homeostasis by backconverting spermine and spermidine to acetylated polyamines and thus facilitating their excretion and providing better substrates for PAO, the SSAT-KO cells and mice showed only minor disturbances in their polyamine levels. Even the exposure of SSAT-deficient cells to an excess of polyamines induced no toxicity or polyamine accumulation. Therefore, the polyamine interconversion route was subjected to a more detailed study in order to follow the catabolism of higher polyamines and to clarify the reason for the significantly elevated spermidine concentration in SSAT-deficient cells. The tracer studies revealed conclusively the importance of SSAT in spermidine back-conversion since after DENSPM-treatment only parental cells were capable of converting [^{14}C]spermidine to N^1-acetylspermidine and furthermore to putrescine. In addition, the MDL 72527 treatment proved that spermidine back-conversion in parental cells indeed proceeds via the SSAT/PAO pathway, as the acetylated spermidine level was increased to an even greater content while it was not further oxidized to radioactive

putrescine. Spermidine back-conversion was absent in the SSAT-KO cells, as the products of labeled spermidine remained at the background level under all experimental conditions.

On the other hand, interconversion of spermine opened up the possibility for the existence of another back-conversion route and an alternative route for spermine catabolism has been described by Vujcic and coworkers (2002). SMO catalyzes spermine oxidation directly to spermidine and thus spermine can be backconverted by the aid of two enzymes. Which route is more preferable depends presumably on the cell type and general conditions. The active function of SMO was detected from the SSAT-deficient cells which converted spermine to spermidine about four times more rapidly than the parental cells. Therefore knockout cells may have compensated for the loss of SSAT by increasing their SMO activity in order to prevent the accumulation of toxic levels of spermine in the cells. Parental cells were not able to compete with this enhanced rate of spermine catabolism even with simultaneous action of DENSPM-induced SSAT and SMO. The reason for this reduced conversion in parental cells was not the decreased PAO activity or the further metabolism of spermidine to N^1-acetylspermidine or putrescine. We obtained confirmation that in ES cells, SMO is the driving force for spermine catabolism and the actual role of SSAT is rather modest when we examined the effects of combined DENSPM and MDL 72527 treatment. MDL 72527 inhibited only 40-50 % of the formation of spermidine from spermine in both parental and knockout cell lines. Known originally as a PAO inhibitor this compund has also been reported to inhibit SMO but it has a much lower affinity for purified spermine oxidase than it has for purified PAO (K_i 63 µM versus 1 µM) (Bellelli et al., 2004; Wu et al., 2005) and this 60-fold difference proved to be relevant in the incomplete inhibition of spermine interconversion. DENSPM has been reported to induce SMO in several studies (Pledgie et al., 2005; Vujcic et al., 2002; Wang et al., 2001), but instead of induction, DENSPM appeared to have an inhibitory effect on spermine interconversion in the targeted cells. In summary, it is probable that the significantly higher spermidine pool in the targeted cells may originate from the blockade of spermidine catabolism together with accelerated spermine conversion to spermidine.

SSAT-disrupted mice showed only slightly altered polyamine homeostasis and exhibited an increased spermidine/spermine ratio in almost every analyzed tissue regardless of age. The SSAT inducers, CCl_4 and DENSPM, confirmed the absence of inducible SSAT in the knockout mice. Both drugs have been shown to cause higher polyamine pool depletion, the appearance of N^1-acetylspermidine and the accumulation of putrescine in response to SSAT induction.

Unexpectedly, CCl_4-treated livers of SSAT-KO mice also showed decreased spermine and spermidine pools and thus inducible SSAT seems to lack a distinct role in higher polyamine depletion in response to this treatment. The depletion of spermidine in null mice might result from enhanced excretion of spermidine out of CCl_4-damaged hepatocytes. The absence of inducible SSAT seemed to protect the tissues from DENSPM-induced polyamine pool depletion, as the drug had practically no effect on polyamine levels in knockout mice even after three days' treatment. DENSPM-induced SSAT activity and thus higher polyamine pool depletion and enhanced putrescine accumulation was detected only in the wild-type mice tissues. These results highlighted the difference between *in vivo* and *in vitro* studies, as in SSAT-KO cells the analogue depleted higher polyamine pools similarly compared to parental cells. The importance of SSAT in spermidine back-conversion *in vivo* was confirmed in an experiment using radioactively labeled spermidine. [^{14}C]spermidine combined with prior CCl_4-treatment increased SSAT activity in WT mice leading to increased levels of N^1-acetylated spermidine and putrescine in the liver. In knockout mice, the radioactive putrescine remained near to the basal level. The traces of acetylated N^1-acetylspermidine were also detected in SSAT-KO mice which may point to the presence of acetylases other than SSAT as it has been shown earlier that inducible SSAT is responsible only some proportion of the measured N^1-acetylspermidine formation under basal conditions (Persson and Pegg, 1984). The tracer studies with [^{14}C]spermine confirmed also *in vivo* that SSAT activity was not required for the back-conversion of spermine and that spermine is apparently degraded by SMO with little or no contribution of SSAT.

6.3. Treatments with polyamine metabolism inhibitors, polyamine analogues and etoposide

Both SPMSy- and SSAT-deficient ES cell lines had nearly normal growth rates compared to parental cells under standard cell culture conditions. However, when spermine-deficient cells were treated with polyamine biosynthesis inhibitors, DFMO or MGBG, significantly reduced viability was detected. An inhibition of the ODC depleted the spermidine pool less efficiently in targeted cells than in parental cells and it seems that maintenance of the spermine level is more advantageous for growth and is preferred to the maintenance of spermidine levels under these conditions. DFMO had also a stronger antiproliferative effect in Gy fibroblasts than in parental cells (Nilsson et al., 2000). The importance of spermine for cell growth was further demonstrated when cells were grown in a medium supplemented with MGBG, an inhibitor of AdoMetDC or DENSPM, a polyamine analogue. As higher polyamine pools were depleted almost equally

effectively in both cell lines, the cytotoxic effects of these drugs in SPMSy-deficient cells might be related to the lack of spermine. A higher amount of spermidine was not sufficient to protect SPM-KO cells from drug-induced cytotoxicity and thus spermine seems to have a specific role under the stress of antiproliferative agents. SPMSy knockout cells were also more sensitive to the anticancer drug, etoposide. This cytotoxic compound inhibits DNA topoisomerase II and induces single and double strand breaks in DNA. DNA fragmentation analysis has shown that spermine seems to protect DNA from the induction of strand breaks in control cells. The protective role of spermine has been also demonstrated with spermine synthase-deficient fibroblast cell lines derived from Gy mice. The studies indicate that Gy cells were more easily killed by the chloroethylating agent 1,3-bis-(2-chloroethyl)-*N*-nitrosourea and UV light than cells from control mice (Mackintosh and Pegg, 2000; Nilsson et al., 2000).

The cytotoxicity exerted by polyamine analogues is considered to be closely correlated with their ability to induce SSAT and thus to deplete higher polyamine pools. SSAT-KO deficient cells were expectedly more resistant to the cytotoxic action of the polyamine analogue, DENSPM, but the enhanced resistance was not attributable to higher spermidine and spermine levels in targeted cells as the analogue decreased the polyamine pools almost to the same extent in both cell lines. Irrespective of the actual reason, the effects of polyamine analogues are cell line-specific; one possibility to explain the increased resistance of the targeted cells towards DENSPM is that there is blockade of the accumulation H_2O_2 and potentially cytotoxic aldehydes and thus reactive oxygen species derived from the SSAT/PAO pathway. However, it has been recently reported that SMO, instead of PAO, is the primary source of cytotoxic H_2O_2 (Pledgie et al., 2005). Therefore parental cells might produce these harmful compounds both from SSAT/PAO and from SMO pathways while the targeted cells have only one active catabolism route. On the other hand, the SMO activity seems to be elevated in SSAT knockout cells and thus probably also the production of H_2O_2, but the aldehydic by-product between these oxidases is different since PAO produces 3-acetamidopropanal whereas SMO forms 3-aminopropanal. Also the increased amount of spermidine may also protect SSAT-KO cells from DENSPM-induced cytotoxicity as it has been reported to possess a potential role as a free-radical scavenger *in vitro* (Nilsson et al., 2000).

Based on the SSAT-KO ES cells results, the working hypothesis was that SSAT-deficient mice would also be more resistant to DENSPM-induced toxicity than their wild-type littermates. Surprisingly, SSAT knockout animals were significantly more sensitive to the DENSPM.

Another difference in targeted cells was that the tissue polyamine pools remained almost unchanged in response to the analogue in contrast to the significant reduction observed in the polyamine pools in the wild-type mice. As the depletion of cellular polyamines or increased tissue accumulation of the drug were not the reasons for this significant toxicity in knockout mice, it is possible that these polyamine analogues exert direct cellular toxicity not dependent on SSAT or polyamine depletion. Recent evidence has indicated that SMO can oxidize spermine directly, bypassing the SSAT/ PAO pathway and producing 3-aminopropanal. In fact, there are several reports that have demonstrated the neurotoxicity of 3-aminopropanal *in vitro* (Yu et al., 2004) and its pathological relevance *in vivo* (Ivanova et al., 2002; Ivanova et al., 1998). The cytotoxicity of this aldehyde is believed to be mediated by its cleavage to acrolein (Calingasan et al., 1999). Tomitori and coworkers (2005) also found that acrolein was produced from 3-acetamidopropanal generated via PAO catalyzed reaction in stroke patients and normal subjects but its production was low compared to that of SMO. Therefore, the cytotoxicity of 3-acetamidopropanal may be considerably different from its non-acetylated analogue. If this is the case, the SSAT/PAO system may represent a parallel pathway for the cell to generate putrescine from higher polyamines without being a risk of the consequences of 3-aminopropanal formation. Thus, two unanswered questions remain: what is the relationship between these two oxidative reactions and what is their relative importance under normal and pathological conditions. Also another possible mechanism for neurotoxicity exerted by 3-aminopropanal has been reported (Li et al., 2003). This small aldehyde has a structure of a weak lysosomotropic base which concentrates to the acidic vacuolar compartment and causes lysosomal rupture. The release of lysosomic enzymes initiates caspase activation and apoptotic cell death.

6.4. Characterization of the SSAT knockout mice

The generation of SSAT gene-disrupted mice from undifferentiated SSAT-KO ES cells was successful. SSAT-KO mice were healthy, viable, fertile and phenotypically indistinguishable from wild-type mice. In addition no macroscopic or microscopic changes were detected in the tissues of SSAT-KO mice. Our previous studies with SSAT and double transgenic (MT-ODC/MT-SSAT) mice lines have revealed that accelerated polyamine catabolism reduced life expectancy by at least 50 % (Suppola et al., 2001). As stated earlier, SSAT knockout mice had only slightly altered polyamine homeostasis and the lack of a functional SSAT gene did not affect their lifespan as determined in our long-term survival study.

The role of SSAT in tumorigenesis has been studied widely in cell cultures and in genetically modified animals using polyamine analogues. Based on these studies, SSAT seems to have a dual role in tumor development depending on the cellular environment. In the long-term aging study, there was no difference in the incidence of spontaneous tumors during aging between SSAT-KO and wild-type mice. Direct evidence of the importance of SSAT in intestinal tumorigenesis, without any consequential polyamine analogue or inhibitor treatments, was detected when SSAT-KO mice were crossed with ApcMIN/+ mice which are highly susceptible to the development of spontaneous intestinal adenomas (Tucker et al., 2005). The deletion of SSAT reduced tumorigenesis as the hybrids of ApcMIN/SSAT knockout mice developed 75% fewer adenomas in the small intestine than normal MIN mice. In addition, crossings with ApcMIN to SSAT overexpressing mice increased the development of adenomas by 3-fold in the small intestine and by 6-fold in the colon. Therefore the inhibition of SSAT expression and thus blockade of this polyamine catabolism pathway may represent a nontoxic prevention or treatment strategy for gastrointestinal cancers.

Type 2 diabetes is a common metabolic condition worldwide. The primary cause is impaired insulin secretion from pancreatic β-cells or insulin resistance of peripheral tissues or both (Kahn, 1994). The principal mechanisms responsible for the development of insulin resistance are so far unknown, although both genetic and environmental factors are involved, such as obesity and a sedentary lifestyle. The ectopic triglyceride accumulation is recognized as one of the important mechanisms leading to insulin resistance. The use of several transgenic and knockout mouse models has provided new insights into the etiology of type 2 diabetes at the molecular level (LeRoith and Gavrilova, 2006). Therefore, it is possible that the SSAT transgenic and SSAT knockout mice lines might also provide a novel perspective in exploring the mechanisms underpinning this disease. The role of polyamines in glucose metabolism has been evaluated earlier and spermidine and spermine have been shown to mimic the actions of insulin in isolated rat adipocytes (Lockwood and East, 1974). Furthermore, putrescine and spermidine have been demonstrated to be necessary for the proinsulin biosynthesis in isolated mouse pancreatic-islet cells, whereas spermine depletion decreases insulin-mRNA contents, insulin biosynthesis, islet insulin content and glucose-sensitive insulin release (Welsh and Sjöholm, 1988). Thus polyamines seemed to have either permissive or stimulatory functions at several different sites of insulin production.

Our SSAT overexpressing mice appear to exhibit a metabolically reverse phenotype to type 2 diabetic patients showing improved glucose tolerance, high insulin sensitivity, reduced amount of white adipose tissue, high basal metabolic rate, enhanced mitochondrial biogenesis and low accumulation of triglycerides in liver and skeletal muscle (Pirinen unpublished). In addition, SSAT-KO mice showed elevated fasting plasma glucose levels at the age of 12 months. A glucose tolerance test conducted in 16-month-old SSAT-KO mice revealed the signs of insulin resistance as raised fasted plasma glucose levels were accompanied by significantly elevated plasma insulin levels during the test. The insulin tolerance test confirmed the impaired action of insulin since plasma glucose levels of SSAT-KO mice were elevated at all time points. The body weight, amount of white adipose tissue or plasma triglycerides remained similar between the genotypes during aging. The histology of 24-months old SSAT-KO mice revealed microscopic changes in the kidneys, which might have been caused by constantly elevated glucose levels; the factor believed to be responsible for the elevated risk of developing diabetic nephropathy. Based on recent unpublished findings, changes in polyamine flux due to enhanced or reduced polyamine catabolism and therefore consumption of important metabolic intermediates such as ATP and acetyl-CoA may be responsible for the metabolic changes observed in SSAT overexpressing and knockout mice. Therefore our SSAT mouse models showed that it is polyamine catabolism rather than the individual polyamines themselves which play an important role in the regulation of glucose and energy metabolism. This may offer a new metabolic concept for drug development to treat obesity and type 2 diabetes.

7 SUMMARY

This study was undertaken to examine the physiological functions of spermine in mammalian cells and to elucidate the role of SSAT in polyamine interconversion and in the maintenance of polyamine homeostasis. The following results were obtained:

I Spermine was not indispensable for ES-cell growth to occur *in vitro* but it may be essential during mouse embryogenesis at least in mice of the 129/SvJ genetic background.

II SSAT-deficiency did not affect ES cell growth rate or mouse embryonic development. SSAT does not seem to play a central role in the maintenance of polyamine homeostasis as spermidine and spermine appear to have different pathways for their degradation of which only spermidine catabolism is SSAT-dependent both *in vitro* and *in vivo*.

III Experiments with SSAT-deficient ES cells and SSAT knockout mice demonstrated that under certain circumstances, the cytotoxicity of polyamine analogues may be based on mechanisms other than SSAT-induced depletion of the higher polyamines.

IV SSAT knockout mice were viable, fertile and phenotypically indistinguishable from their wild-type littermates. However, SSAT-deficient mice developed insulin resistance at old age, possibly indicating that polyamine catabolism has a role in the regulation of glucose and energy metabolism.

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ORIGINAL PUBLICATIONS

I - III

I

SPERMINE DEFICIENCY RESULTING FROM TARGETED DISRUPTION OF THE SPERMINE SYNTHASE GENE IN EMBRYONIC STEM CELLS LEADS TO ENHANCED SENSITIVITY TO ANTIPROLIFERATIVE DRUGS

Korhonen V. P., Niiranen K., Halmekytö M., Pietilä M., Diegelman P., Parkkinen J. J., Eloranta T., Porter C. W., Alhonen L. and Jänne J.

Molecular Pharmacology (2001) 59(2): 231-238

II

TARGETED DISRUPTION OF SPERMIDINE/SPERMINE N^1-ACETYLTRANSFERASE GENE IN MOUSE EMBRYONIC STEM CELLS.

EFFECTS ON POLYAMINE HOMEOSTASIS AND SENSITIVITY TO POLYAMINE ANALOGUES

Niiranen K., Pietilä M., Pirttilä T. J., Järvinen A., Halmekytö M., Korhonen V. P., Keinänen T. A., Alhonen L. and Jänne J.

Journal of Biological Chemistry (2002) 277(28): 25323-25328

III

MICE WITH TARGETED DISRUPTION OF SPERMIDINE/SPERMINE N^1-ACETYLTRANSFERASE GENE MAINTAIN NEARLY NORMAL TISSUE POLYAMINE HOMEOSTASIS BUT SHOW SIGNS OF INSULIN RESISTANCE UPON AGING

Niiranen K., Keinänen T. A., Pirinen E., Heikkinen S., Tusa M., Fatrai S., Suppola S., Pietilä M., Uimari A., Laakso M., Alhonen L. and Jänne J.

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