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Marc Cerrada-Gimenez

Development and Use of Tools to Study Metabolic Consequences of Altered Polyamine Catabolism

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A.I.VIRTANEN INSTITUTE

MARC CERRADA-GIMENEZ

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ABSTRACT

Polyamines are a group of naturally occurring molecules that contain two or more amino groups. In eukaryotes, polyamine metabolism is tightly controlled at both the synthetic and catabolic pathways. The rate limiting enzymes for the polyamine metabolism are ornithine decarboxylase (ODC) in the synthetic pathway and spermidine/spermine N^1-acetyltransferase (SSAT) in catabolism. Polyamines are involved in a wide range of cellular functions, and due to their positive charge, they can interact with many negatively charged cellular components, such as proteins, phospholipids or DNA. This wide range of polyamines functions means that alterations in their normal levels have been linked with many different diseases, such as obesity and diabetes, cancer, and pancreatitis.

The use of polyamine analogues to investigate the polyamine metabolism is common practice. However, *in vivo* studies require larger amounts of the α-methylated-polyamine analogues than the current production methods can supply. Therefore, an improved synthetic method for α-methylated-polyamines was developed, allowing for increased production yields and the *in vivo* testing of these analogues.

In mice, the overexpression of the SSAT gene when it is placed under the control of a metallothionein I heavy metal inducible promoter (MT-SSAT) produces bizarre phenotypic changes. The acute SSAT activation offers a model to investigate physiological changes produced by altering the polyamine catabolism, and could produce new information regarding the polyamine metabolism.

The proteomic profiling of the MT-SSAT livers demonstrated that most of the altered protein expressions were directly related to the activation of polyamine catabolism. Some of the altered proteins were regulated by peroxisome proliferator-activated receptor γ co-activator 1α (PGC 1α). Moreover, the MT-SSAT mice exhibited improved insulin sensitivity and glucose tolerance when compared with their non-transgenic littermates. ATP levels were much lower in the transgenic mice due to the constant activation of the polyamine cycle, which consumes four ATP equivalents per turn. The decreased energy levels activated PGC 1α. Moreover, the continuous activation of polyamine catabolism, such as seen in the MT-SSAT mice, was accompanied by increased oxidative stress. Consequently, the transcription factor p53 appeared activated in the transgenic mice. The acute activation of p53 was responsible for the accelerated aging of the MT-SSAT mice.

National Library of Medicine Classification: QU 61, QU 135, QY 60.R6

Medical Subject Headings (MeSH): Acetyltransferases; Chromatography, Liquid; Energy Metabolism; Enzymes; Liver/metabolism; Mice; Mice, Transgenic; Oxidative Stress; Polyamines/metabolism; Proteomics; Tandem Mass Spectrometry

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TIIVISTELMÄ

Polyamiinit ovat ryhmä luontaisesti esiintyviä molekyylejä, joissa on yksi tai useampi aminoryhmä. Eukaryooteissa polyamiiniaineenvaihdunnan reitit ovat tiukasti säädeltyjä sekä synteesin että katabolian osalta. Polyamiinien synteesireitin rajoittava entsyymi on ornitiinidekarboksylaasi (ODC) ja hajotusreitin puolestaan spermidiini/spermiini N^1-asetyylitransferaasi (SSAT). Polyamiinit osallistuvat lukuisiin erilaisiin solun toimintoihin, ja positiivisen varauksensa vuoksi ne ovat vuorovaikutuksessa monien negatiivisesti varautuneiden solun rakenneosien, kuten proteiinien, fosfolipidien ja DNA:n kanssa. Koska polyamiinit osallistuvat niin moninaisiin solun toimintoihin, niiden pitoisuuksissa tapahtuvien muutosten on todettu olevan yhteydessä monenlaisiin tauteihin, kuten ylipainoon ja diabetekseen, syöpään ja haimatulehdukseen.

Polyamiinianalogeja käytetään yleisesti polyamiiniaineenvaihdunnan tutkimuksessa. Kuitenkin in vivo-tutkimukset vaativat suurempia määriä α-metyloituja polyamiinianalogeja kuin nykyisillä menetelmillä saadaan tuotettua. Sen vuoksi kehitimme entistä paremman menetelmän α-metyloitujen polyamiinianalogien tuottamiseen, mikä mahdollistaa niiden riittävän saannon in vivo-kokeita varten.

Siirtogeenisissä hiirissä SSAT-geenin ilmentyminen raskasmetalleilla aktivoituvan metallotioneiini I-promoottorin alaisuudessa (MT-SSAT) tuottaa erityislaatuisia muutoksia hiiren ilmiasuun. Akuutti SSAT-aktivaatio tarjoaa mallin, jolla voidaan tutkia, millaisia fysiologisia muutoksia polyamiinien hajotusreitin muutokset aiheuttavat, ja tarjoaa uutta tietoa polyamiiniaineenvaihdunnan merkityksestä.

MT-SSAT-hiirten proteominen profilointi osoitti, että useimmat muutokset proteiinien ilmentymisessä olivat suoraan yhteydessä polyamiinikatabolian aktivaatioon. Osa proteiineista oli sellaisia, joita säätelee peroksisomiproliferaattori-aktivoituvan reseptori gamman ko-aktivaattori 1α (PGC 1α). Lisäksi MT-SSAT-hiiret olivat insuliiniherkempiä ja niillä oli lisääntynyt glukoosinsietokyky ei-siirtogeenisiin verrokkihiiriin nähden. Siirtogeenisillä hiirillä oli myös kudoksissa alentuneet ATP-pitoisuudet, mikä johtuu jatkuvasti aktivoituneesta polyamiinien hajotussyklistä, joka kuluttaa energiaa neljä ATP-ekvivalenttia kierrosta kohti. Vähentynyt energiamäärä soluissa aktivoi PGC 1α:a. Lisäksi MT-SSAT -hiirten jatkuva polyamiinikatabolian aktivaatio johti niissä lisääntyneeseen oksidatiiviseen stressiin. Tämän seurauksena transkriptiofaktori p53 oli siirtogeenisissä hiirissä aktivoitunut. Akuutti p53:n aktivaatio MT-SSAT hiirissä johti nopeutuneeseen vanhenemiseen.

Yleinen suomalainen asiasanasto: aineenvaihdunta, entsyymit, polyamiinit, hiiret, solufysiologia, vanheminen

Per la Laia i l'Erik

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"A long time ago, in a *country* far far away …"

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Kuopio, October 2010

List of original publications

This thesis is based on the following publications that are referred to in the text by their corresponding Roman numerals:

I Järvinen A, Cerrada-Gimenez M, Grigorenko N, Khomutov A, Vepsäläinen J, Sinervirta R, Keinänen TA, Alhonen L, and Jänne J. α-Methyl polyamines: efficient synthesis and tolerance studies in vivo and in vitro. First evidence for dormant stereospecificity of polyamine oxidase. *J Med Chem* 2006; 49 (1): 399-406

II Cerrada-Gimenez M, Häyrinen J, Juutinen S, Reponen T, Jänne J and Alhonen L. Proteomic analysis of livers from a transgenic mouse line with activated polyamine catabolism. *Amino Acids* 2009; 38: 613-622

III Cerrada-Gimenez M, Pietilä M, Loimas S, Pirinen E, Hyvönen M, Keinänen T, Jänne J and Alhonen L. Continuous oxidative stress due to activation of polyamine catabolism accelerates aging and protects against hepatotoxic insults. *Transgenic Res* 2010*;* In press

IV Cerrada-Gimenez M, Häkkinen M, Vepsälainen J, Reponen T, Auriola S, Alhonen L and Keinänen T. Analyzing polyamine flux by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Submitted; 2010

Unpublished results are also presented.

Contents

Abbreviations

2D-PAGE	2-dimensional polyacrylamide gel electrophoresis
ADC	arginine decarboxylase
Acetyl-CoA	acetyl coenzyme A
AdoHcy	*S*-adenosyl-L-homocysteine
AdoMet	*S*-adenosyl-L-methionine
AdoMetDC	*S*-adenosyl-L-methionine decarboxylase
ALDH	aldehyde dehydrogenase
APAO	acetylated polyamine oxidase
APC	adenomatosis polyposis coli
AUH	agmatinase
AZI	antizyme inhibitor
BMI	body mass index
CAT	catalase
CYP450 2E1	cytochrome P450 2E1
dcAdoMet	decarboxylated *S*-adenosyl-L-methionine
DFMO	DL-α-difluoromethyl ornithine
Foxa2	forkhead box A2, previously known as hepatocyte nuclear factor 3β
Gck	glucokinase
Glut2	glucose transporter 2
GST	glutathione S-transferase
GSTpi	glutathione S-transferase pi
HNF1α	hepatocyte nuclear factor 1α
HNF4α	hepatocyte nuclear factor 4α
HPLC	high-performance liquid chromatography
Ins1	insulin 1
Ins2	insulin 2
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
MAT1	hepatic methionine adenosyltransferase
MAT2	extrahepatic methionine adenosyltransferase
MT	metallothionein
MTA	5´-methylthioadenosine
OAZ	antizyme
ODC	ornithine decarboxylase
PGC 1α	peroxisome proliferator-activated receptor γ co-activator 1α
PPAR	peroxisome proliferator-activated receptor
ROS	reactive oxygen species
QR1	NAD(P)H quinone oxidoreductase enzyme

RT-PCR	real-time polymerase chain reaction
SOD	superoxide dismutase
SMO	spermine oxidase
SPDSY	spermidine synthase
SPSY	spermine synthase
SSAT	spermidine/spermine N^1-acetyltransferase
T2D	type 2 diabetes
WAT	white adipose tissue
WHO	World Health Organization

1 Introduction

Polyamines are a family of organic compounds whose chemical structure is based on a carbon backbone chain with two or more nitrogen heteroatoms, thus the name polyamine. Polyamines are encountered in all living organisms, from prokaryotes to eukaryotes, although one exception does exist, two orders of *Archaea*, the *Methanobacteriales* and the *Halobacteriales*, do not contain polyamines in their cells (Hamana et al., 1992). In the higher eukaryotes, the term polyamine is commonly used to refer to the diamine putrescine (1,4-diaminobuthane (*Figure 1 compound (1)*), the triamine spermidine (*N*-(3-aminopropyl)butane-1,4-diamine (*Figure 1 compound (2)*), the tetraamine spermine (*N,N*'-bis(3-aminopropyl)butane-1,4-diamine (*Figure 1 compound (3)*), and their acetylated derivates. Eukaryotic cells only synthesize linear polyamines. However, lower organisms such as *Eubacteria*, *Archaebacteria*, or *Cyanobacteria*, produce polyamines with more complicated structures, such as branched polyamines (Hamana et al., 1992).

In the year 1678, "The Father of Microbiology", Antonie P. van Leeuwenhoek, after isolating crystals of spermine phosphate from human semen, described for the first time a polyamine (van Leeuwenhoek 1678). It was two hundred years later before Brieger identified putrescine (1885). The common names of the different polyamines are derived from the original source used for their isolation. Human semen was the original source for spermine and spermidine, whereas putrescine was isolated from putrefying flesh.

More than 330 years after polyamines were first isolated (van Leeuwenhoek 1678), and more than 80 years after their modern re-discovery by Dudley et al (1924), their exact functions still remain a mystery. The presence of the amino groups in the polyamine structure makes them positively charged at physiological pH, and this particular characteristic allows polyamines to substitute for inorganic cations, such as magnesium or calcium in cellular physiology. Moreover, the fact that the positive charges are not equally spaced along the carbon backbone extends the versatility of these molecules over that of simple inorganic cations (Jänne et al., 2004). The combination of these features, positive and differentially spaced charges, means that polyamines can interact with negatively charged bio-molecules, such as phospholipids, DNA and RNA (Jänne et al., 2004). This interaction between the polyamines and the various biomolecules is probably the basis of their biological functions. The intracellular concentrations of polyamines are at the millimolar level. However, most of the polyamines are expected to be bound by ionic interactions to

different cell components, and therefore, the amount of free polyamines is anticipated to be much lower (Jänne et al., 2004).

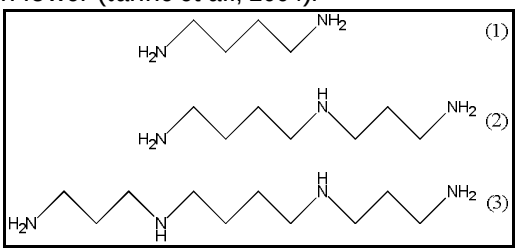

Figure 1. Chemical structures of: (1) putrescine (1,4-diaminobuthane), (2) spermidine (N-(3-aminopropyl)butane-1,4-diamine), and (3) spermine (N,N'-bis(3-aminopropyl)butane-1,4-diamine).

A useful approach for elucidating polyamine metabolism and obtain knowledge of their functions is to study transgenic animals either overexpressing or deficient in the polyamine metabolizing enzymes (Jänne et al., 2004; Jänne et al., 2005). Accordingly, the present study has taken advantage of different genetically modified mouse lines to expand the roles of polyamines in physiology and pathology. Most of the work was undertaken using a transgenic mouse line that expresses the SSAT genes under the control of a heavy metal inducible promoter (MT-SSAT (UKU 181F)) (Suppola et al., 1999), but also a mouse line overexpressing SSAT under its natural promoter (SSAT (UKU 165F) (Pietilä et al., 1997), and a mouse line with disrupted SSAT expression (SSATKO (KI1)) (Niiranen et al., 2006) were used.

The present study can be divided into three differentiated sections:

- One, the study of the effects of alpha-methylated polyamine analogues on the MT-SSAT transgenic mice. These mice are a useful model for the study of how rationally developed polyamine analogues affect polyamine metabolism and functionality. The activated polyamine catabolism in the MT-SSAT mice, which depleted the natural polyamines, made it possible to analyze whether the analogues were able to substitute the depleted polyamines and whether these analogues could be metabolized
- Two, application of new techniques in polyamine research, such as LC-MS/MS polyamine analysis. The recent recognition of polyamine flux as the cause of some metabolic disorders (Pirinen et al., 2007; Jell et al., 2007) has resurrected the interest in these compounds. These newly designed protocols for the study of polyamines allowed overcoming some of the limitations of previous methodologies
- Three, detailed characterization of the metabolic effects of activated polyamine flux in the MT-SSAT transgenic mice. The analysis of the SSAT transgenic mice has been performed in previous studies (Pirinen et al., 2007;

Pirinen et al., 2009). These studies demonstrated that activation of polyamine flux could affect many other metabolic pathways, such as insulin secretion, cholesterol and bile acid synthesis. Thus, the effects on the liver of activated polyamine catabolism, the causes of accelerated aging, and the altered insulin secretion were studied in detail

2 Review of the literature

2.1 POLYAMINES AND THEIR METABOLISM

Polyamines are actively produced in all mammalian cells, but are present at especially high levels in the liver, pancreas, and testes (Rosenthal and Tabor, 1956). One basic difference between eukaryotic and prokaryotic cells, according to their polyamine metabolism, is the absence of the spermine synthase gene and, thus, spermine can only by synthesized in the latter group only (Heby, 1981). Cellular polyamine levels are tightly controlled by the combined regulation of their metabolism (Figure 2) and transport (uptake and excretion).

2.2 Polyamine biosynthesis

In the higher eukaryotes, polyamines originate from two amino acids, L-arginine and L-methionine. During the urea cycle, L-arginine is converted into urea by arginase I in the liver (ARGI1) or arginase II in extrahepatic tissues (ARGI2). L-Ornithine is formed as a by-product of the arginase reaction. L-Ornithine can then either be cycled back into the urea cycle as citrulline, or used in polyamine metabolism. During polyamine metabolism, L-ornithine is decarboxylated by ornithine decarboxylase (ODC), a reaction that produces putrescine. This reaction is considered as the first rate-limiting step in polyamine biosynthesis.

In non-mammalian organisms, putrescine may be synthesized via an alternative pathway that involves the production of agmatine through the decarboxylation of L-arginine by arginine decarboxylase (ADC). Agmatine is then further hydrolyzed by agmatinase (AUH) to yield putrescine. Some recent evidence pointed to the presence of an agmatinase gene in the mouse and human genomes (*Agmat* and AGMAT) (Iyer et al., 2002). However, Coleman et al (2004) were unsuccessful in reproducing the original experiments, which had claimed to identify the existence of a mammalian ADC (Lortie et al., 1996; Regunathan et al., 2000). Thus, evidence for a mammalian ADC enzyme is still inconclusive. However, even though there is no proven evidence for a mammalian ADC ortholog, this pathway should not be totally eliminated as a possible source of putrescine because agmatine, which is an essential bacterial

and plant component, could be obtained from the diet.

The second amino acid precursor in polyamine biosynthesis is the essential amino acid L-methionine. Before L-methionine can be utilized in the polyamine biosynthesis, it needs to be converted into decarboxylated S-adenosyl-L-methionine (dcAdoMet) by a two-step reaction. In the first reaction, the enzyme methionine adenosyltransferase (MAT1) transforms L-methionine into S-adenosyl-L-methionine (AdoMet). The second reaction involves the decarboxylation of AdoMet into dcAdoMet by S-adenosyl-L-methionine decarboxylase (AdoMetDC). AdoMet is a common substrate for many different methylation reactions, in fact, only about 5% of the total available AdoMet is decarboxylated and committed for polyamine biosynthesis (Mudd and Poole, 1975). The enzymatic reaction producing dcAdoMet is regarded as the second rate-limiting step of the polyamine biosynthesis.

The higher polyamines, spermidine and spermine, are formed by the combination of putrescine and dcAdoMet. Spermidine synthase (SPDSY) catalyzes the combination of the diamine putrescine and an aminopropyl group from dcAdoMet. The triamine, spermidine, is then used for the production of spermine by the addition of a second aminopropyl group also provided by dcAdoMet. The spermine synthesis is catalyzed by spermine synthase (SPSY). The syntheses of spermidine and spermine produce the highly toxic 5´-methylthioadenosine (MTA) as a by-product that is salvaged back into L-methionine and adenosine (Ávila et al., 2004).

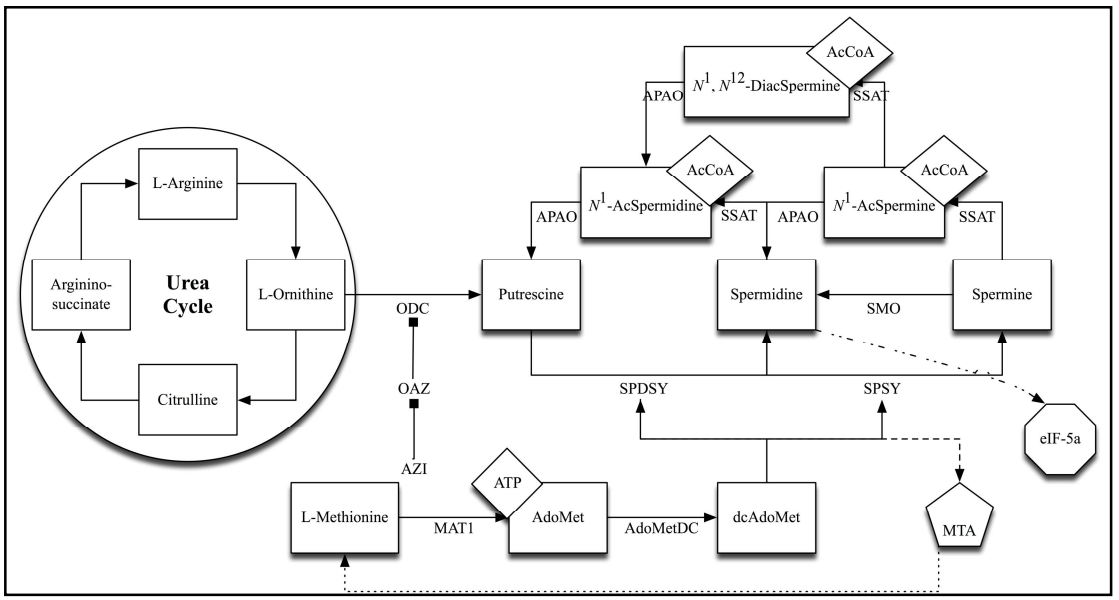

Figure 2. Schematic overview of polyamine metabolism in higher eukaryotes. The polyamine precursors are the amino acids L-ornithine, produced in the urea cycle, and L-methionine. L-ornithine is decarboxylated by ornithine decarboxylase (ODC) to produce the diamine, putrescine. L -methionine is first transformed into S-adenosyl-L-methionine (AdoMet) by methionine adenosyltransferase (MAT1), and it is further decarboxylated by S-adenosyl-L-methionine decarboxylase (AdoMetDC) yielding decarboxylated S-adenosyl-L-methionine (dcAdoMet). dcAdoMet is used by spermidine synthase (SPDSY) and spermine synthase (SPSY) to produce spermidine and spermine. Spermidine and spermine catabolism (upper scheme) is a two-step reaction in which spermine or spermidine is first acetylated by spermidine/spermine N^1-acetyltransferase (SSAT) and then oxidized by polyamine oxidase (APAO) to produce spermidine or putrescine, respectively. Spermine can also be directly backconverted into spermidine by spermine oxidase (SMO) omitting the acetylation step. Lines with arrows indicate the reaction direction (—▶), lines with squares indicate repression (—■). OAZ, antizyme; AZI, antizyme inhibitor; MTA, 5'-methylthioadenosine; eIF-5a, eukaryotic initiation factor 5a.

Table 1. List of all proteins related with the polyamine metabolism.

Protein name	Abbreviation	EC	Organism	Gene	GeneID	Uniprot
Agmatinase	AUH	3.5.3.11	H. sapiens	AGMAT	79814	Q9BSE5
			M. musculus	Agmat	75986	A2AS89
Aldehyde dehydrogenase	ALDH	1.2.1.5	H. sapiens	ALDH	216	P00352
			M. musculus	Aldh	11668	P24549
Antizyme	OAZ	ND	H. sapiens	OAZ1	4946	P54368
			M. musculus	Oaz1	18245	P54369
Antizyme inhibitor	AZI	ND	H. sapiens	AZIN1	51582	O14977
			M. musculus	Azin1	54375	O35484
Arginase I	ARGI1	3.5.3.1	H. sapiens	ARG1	383	P05089
			M. musculus	Arg1	11846	Q61176
Arginase II	ARGI2	3.5.3.1	H. sapiens	ARG2	384	P78540
			M. musculus	Arg2	11847	O08691
Arginine decarboxylase	ADC	4.1.1.19	H. sapiens	ADC	113451	Q96A70
			M. musculus	Adc	242669	B2RSR5
Catalase	CAT	1.11.1.6	H. sapiens	CAT	847	P04040
			M. musculus	Cat	12359	P24270
Deoxyhypusine hydroxylase	DOHH	1.14.99.29	H. sapiens	DOHH	83475	Q9BU89
			M. musculus	Dohh	102115	Q99LN9
Deoxyhypusine synthase	DHS	2.5.1.46	H. sapiens	DHPS	1725	P49366
			M. musculus	Dhps	330817	Q3TXU5
Eukaryotic initiation factor 5a	eIF-5a	ND	H. sapiens	EIF5A	1984	P63241
			M. musculus	Eif5a	276770	P63242
Ornithine decarboxylase	ODC	4.1.1.17	H. sapiens	ODC1	4953	P11926
			M. musculus	Odc1	18263	P00860
Polyamine oxidase	APAO	1.5.3.11	H. sapiens	PAOX	196743	Q6QHF9
			M. musculus	Paox	212503	Q8C0L6
Methionine adenosyltransferase	MAT1	2.5.1.6	H. sapiens	MAT1A	4143	Q00266
			M. musculus	Mat1a	11720	Q91X83
NAD(P)H quinone oxidoreductase	QR1	1.6.5.2	H. sapiens	NQO1	1728	P15559
			M. musculus	Nqo1	18104	Q64669
S-adenosyl-L-methionine decarboxylase	AdoMetDC	4.1.1.50	H. sapiens	AMD1	262	P17707
			M. musculus	Amd1	11702	P31154
Spermidine/spermine N^1-acetyltransferase	SSAT	2.3.1.57	H. sapiens	SAT1	6303	P21673
			M. musculus	Sat1	20229	P48026
Spermidine synthase	SPDSY	2.5.1.16	H. sapiens	SMS	6611	P52788
			M. musculus	Sms	20810	Q64674
Spermine oxidase	SMO	1.5.3.16	H. sapiens	SMOX	54498	Q9NWM0
			M. musculus	Smox	228608	Q99K82
Spermine synthase	SPSY	2.5.1.22	H. sapiens	SRM	6723	P19623
			M. musculus	Srm	20603	P97355

The column abbreviation corresponds with the abbreviation used in the text.

The E.C. column displays the enzyme classification code for the given protein. ND, not disponible.

The gene column shows the name of the gene that encodes the protein.

The GeneID column shows the correspondent code for the given gene in the Entrez Gene database, while the Uniprot column shows the code for the protein in the Uniprot database.

2.2.1 Regulation of polyamine biosynthesis

There are six different reactions in polyamine biosynthesis pathway but two of them have been considered as rate-limiting. The two rate-limiting reactions are the ones catalyzed by ODC (decarboxylation of L-ornithine to putrescine), and AdoMetDC (production of dcAdoMet).

2.2.1.1 ODC regulation

The murine ornithine decarboxylase gene spans a total of 3453 bp, and contains a total of ten exons that transcribe into a 1386 bp long mRNA, the end product of which is a 461 amino acid long protein with a mass of 51.1 kDa.

ODC is controlled at all phases of its synthesis, from the transcription to posttranslational stages. At the transcription level, the human *ODC* gene contains multiple regulatory elements, including GC-boxes, cAMP response elements (CRE), AP-1 and AP-2 motifs, a TATA box, a CAAT site, a putative insulin response element, and several Sp1 binding sites (Qin et al., 2004; Shantz and Pegg, 1999; Manzella et al., 1991). Moreover, the *Odc* promoter region contains two E boxes containing the canonical sequence for Myc/Max transcription factor binding (CACGTG) (Packham and Cleveland, 1997). The *Odc* gene is also regulated at the translational level. *Odc* mRNA contains a long 5'-UTR with a predicted complex secondary structure hindering its efficient translation. Thus, translation initiation factors, such as eukaryotic initiation factor 4E (eIF-4E), can improve the translation efficiency (Shantz and Pegg, 1994).

The ODC enzyme functions as a pyridoxal 5´-phosphate dependent decarboxylase. The active form of ODC is a homodimer containing two active sites at the interface of the two monomers (Coleman et al., 1994). Under normal conditions, ODC has a half-life of less than one hour (Shantz and Pegg, 1999). The enzyme is tightly regulated at the posttranscriptional level through a protein that shares a high similarity with ODC and named antizyme (OAZ) (Heller et al., 1976; Murakami et al., 1992). OAZ is a conserved protein among all eukaryotes, from yeast to man. There are three different OAZ isoforms. OAZ1 and OAZ2 are expressed in most vertebrate tissues, with OAZ2 being expressed at a much lower level than the other forms (Coffino 2001). OAZ3 expression is limited to the testis during the late sperm morphogenesis (Ivanov et al., 2000). OAZ plays a dual role in reducing the intracellular polyamine content. First, OAZ acts by binding to the ODC monomer, with higher affinity than ODC itself. The formation of the heterodimer, ODC-OAZ, has two consequences, first inactivation of ODC and second, OAZ targets ODC for degradation for the 26S proteasome in an

ubiquitin-independent manner (Kahana 2007). The interaction between ODC and OAZ produces a change of conformation in the ODC protein exposing the last 37 amino acids from the C-terminus that are used as a signal by the 26S proteasome (Li et al., 1993). Once ODC is presented into the 26S proteasome, OAZ is released and able to recruit new ODC monomers (Gandre et al., 2002). OAZ1 and OAZ2 differ in the way that they regulate ODC. OAZ2 is not able to induce ODC proteasomal degradation, instead it sequesters the ODC monomers, thus inhibiting the ODC enzymatic activity (Zhu et al., 1999). ODC inactivation by OAZ reduces its enzymatic activity as well as the uptake of extracellular polyamines.

OAZ itself possesses a unique translational regulation mechanism that is polyamine mediated. When cellular polyamine levels are elevated and polyamine biosynthesis needs to be inhibited, polyamines induce a translational +1 ORF frameshifting in the *Oaz* mRNA. The ribosomal frameshift allows for translation of the *Oaz* mRNA into its functional form (Matsufuji et al., 1995).

The third player in ODC regulation is a protein named antizyme inhibitor (AZI) (Fujita et al., 1982). There are two different forms of the AZI protein. AZI2 is an ODC paralogue (Pitkänen et al., 2001). AZI1 is ubiquitously expressed, whereas AZI2 expression is limited to the testis, brain and mast cells (Pitkänen et al., 2001; López-Contreras et al., 2006; Kanerva et al., 2009). AZI structures are very similar to ODC, but they lack any decarboxylase activity (Murakami et al., 1996). AZIs have a higher affinity for OAZ than does ODC itself. Therefore, the heterodimers formed between AZI and OAZ are stronger than those between ODC and OAZ (Fujita et al., 1982). AZIs stabilize the ODC homodimers prolonging their half-life (Mangold, 2005). Both, AZIs (Bercovich et al., 2004) and OAZ are degraded in an ubiquitin-dependent manner (Gandre et al., 2002). Interestingly, the interaction between OAZ and AZIs promotes the stabilization of both proteins, the opposite of the situation with the heterodimer ODC-OAZ. Recently, an alternative pathway of ODC degradation has been described (Asher et al., 2005). In this secondary pathway, ODC degradation occurs through the 20S proteasome, and is regulated by the enzyme NAD(P)H quinone oxidoreductase (QR1).

2.2.1.2 AdoMetDC regulation

The mouse AdoMetDC gene spans a total of 12530 bp, and contains eight exons that are transcribed into a 1005 bp long mRNA. The mRNA is translated into a 334 amino acid long proenzyme with a mass of 38.3 kDa. The proenzyme is autocatalytically cleaved to form the α (30.7 kDa) and β subunits (7.7 kDa), and the pyruvoyl cofactor (Stanley et al., 1989).

Similarly to ODC, AdoMetDC presents a long 5'-UTR with a predicted complex secondary structure. Included in the AdoMetDC 5'-UTR there is a 15

nucleotides-long small upstream ORF (uORF) (Shantz et al., 1994) that is believed to be responsible for the translational regulation by polyamines (Hill and Morris, 1992; Ruan et al., 1996). Putrescine enhances the conversion of the proenzyme to the active form (Pegg et al., 1988), and also produces a conformational change in the active protein lowering its K_m for the substrate (Pegg and Williams-Ashman, 1969). Moreover, MTA, a by-product produced during spermine and spermidine biosynthesis, negatively regulates AdoMetDC activity. However, since 5´-methylthioadenosine (MTA) is rapidly recycled into methionine and adenosine, it has been suggested that this control mechanism is not very relevant (Bachrach et al., 1989). AdoMetDC is degraded in an ubiquitin-dependent manner (Yerlikaya and Stanley, 2004). In humans, it has been estimated that the majority of AdoMet serves as a methyl donor in diverse methylation reactions, and only 5 % of the total is decarboxylated and destined to be used as an aminopropyl donor in polyamine biosynthesis (Mudd and Poole, 1975).

2.3 POLYAMINE CATABOLISM

Polyamine biosynthesis and catabolism are directly interconnected, meaning that all polyamines are interconversible. There are two pathways involved in polyamine catabolism. The main catabolic pathway is the acetylation pathway that involves a two-step reaction. In the first step, spermine or spermidine are acetylated by spermidine/spermine N^1-acetyltransferase (SSAT), acetyl-CoA acts as the donor of the acetyl group in this reaction (Hölttä et al., 1973). In the second step, polyamine oxidase (APAO) removes the aminpropyl group from the *N*-acetylated spermine or *N*-acetylated spermidine, producing hydrogen peroxide and an aldehyde, 3-acetaminopropanal, and transforming the original input polyamines into spermidine or putrescine, respectively (Bolkenius and Seiler, 1981). From the two by-products, hydrogen peroxide is converted into water and oxygen by catalase (CAT). The second by-product, 3-acetaminopropanal can be either detoxified by aldehyde dehydrogenase (ALDH) into *N*-acetyl-β-alanine or spontaneously converted into the highly reactive acrolein by beta elimination. *N*-acetyl-β-alanine is further metabolized by a coupled reaction of deacetylation-transamination (Seiler 2004),

The second catabolic pathway involves the direct oxidation of spermine into spermidine by the spermine oxidase enzyme (SMO). This reaction occurs without the need for prior acetylation of the spermine substrate (Vujcic et al., 2002a). The SMO catalyzed reaction produces two by-products, hydrogen peroxide and 3-aminopropanal, which are detoxified by the same combination

of enzymes as the by-products of the acetylation pathway.

2.3.1 Regulation of polyamine catabolism

2.3.1.1 SSAT regulation

The SSAT gene is located in the X chromosome and is encoded by a 2672 bp sequence divided into six exons. The *Sat1* gene transcribes into a 516 bp long mRNA that translates into a 171 amino acid long protein with a mass of 20 kDa. The SSAT enzyme belongs to the general control nonderepressible 5 (GCN5) *N*-acetyltransferase family. SSAT functions as a homodimer providing two active sites. This enzyme catalyzes the acetylation of the polyamines, spermidine and spermine, by adding an acetyl group provided by acetyl-CoA. The acetylation of polyamines is a required step for their further oxidation by APAO. SSAT was originally discovered as a greatly inducible enzyme that accumulated in rodent liver after treatment with carbon tetrachloride (Hölttä et al., 1973). Similarly to ODC, SSAT expression is controlled at the transcriptional, mRNA processing, translational and post-translational levels.

The human *SAT1* promoter lacks the TATAA or CCAAT boxes. However, it has many potential binding sites for Sp-1, AP-1, E2F, AP2, PEA-3 (Fogel-Petrovic et al., 1993), CCAT/enhancer binding protein-β (C/EBPβ), CREB, NF-κβ, and peroxisome proliferator activated receptors (PPARs) (Fogel-Petrovic et al., 1993; Choi et al., 2006; Babbar et al., 2003). The *Sat1* is transcriptionally regulated by a polyamine response element (PRE) located at -1522 bp before the gene transcription start site (5´-TATGACTAA-3´) (Wang et al., 1998). Under conditions of high polyamine loads, the PRE interacts with the transcription factor NF-E2-related factor 2 (Nrf-2) and then recruits the polyamine-modulated factor 1 (PMF 1) that activates SSAT transcription (Wang et al., 2001a).

The *Sat1* mRNA is controlled by alternative splicing. Thus, when high levels of polyamines are present, enhanced catabolism is needed and the original mRNA splices into the form encoding for the catalytically functional SSAT. However, when polyamine levels are low, an extra 110bp exon is transcribed between exons three and four, producing the SSAT-X mRNA variant. This extra exon contains multiple premature termination codons that, if translated, would produce a truncated SSAT version of only 71 amino acids. The SSAT-X mRNA is rapidly targeted to the nonsense mediated mRNA decay pathway (Hyvönen et al., 2006). Moreover, *Sat1* mRNA translation is regulated by an unknown inhibitory protein that associates with the coding part of the mRNA. Under high polyamine levels, this unknown protein dissociates from the SSAT mRNA, meaning that translation can proceed (Fogel-Petrovic et al., 1996; Butcher et al., 2007).

The SSAT activity is usually very low in normal cells, and its half-life is very short, being less than 15 minutes in the rat liver after thioacetamide treatment (Matsui and Pegg, 1981; Persson and Pegg, 1984). SSAT is degraded by the 26S proteasome after polyubiquitination. SSAT turnover requires the presence of a MATEE sequence found in its carboxyl terminal (Coleman and Pegg, 1997). The association of polyamines or their analogues with the SSAT protein can extend its half-life by preventing its ubiquitination to more than 12 hours (Fogel-Petrovic et al., 1996; McCloskey and Pegg, 2000).

2.3.1.2 APAO regulation

The APAO gene spans 8428 bp and contains seven exons that transcribe into a 1515 bp-long mRNA that codes for a 504 amino acid long protein with an estimated mass of 55.4 KDa. APAO was first purified from rat liver in the late 70's by Hölttä (1977). APAO is a flavin adenine dinucleotide-dependent amine oxidase that preferentially oxidizes mono and di-acetylated polyamines and, to a lesser extent, spermine. However, it does not react with spermidine (relative reactivity: N^1-acetylspermine > N^1,N^{12}-diacetylspermine > N^1-acetylspermidine >> spermine) (Seiler, 1995; Bolkenius and Seiler, 1981). The APAO reaction produces two by-products, hydrogen peroxide and 3-acetaminopropanal. APAO is widely distributed in all vertebrate tissues and localizes in the cytoplasm and the peroxisomes (Hölttä, 1977). Acetylated polyamines are either catabolized by APAO or efficiently excreted out of the cells. This is the reason why acetylated polyamines are rarely detected in biological samples, unless there is acute SSAT activation (Casero and Pegg, 1993).

2.3.1.3 SMO regulation

The murine SMO coding region spans 32230 bp, the murine gene contains six exons (eight in the human gene) that create an mRNA of 1668 bp. The final protein has a length of 555 amino acids and a mass of 61.8 kDa. SMO was recently discovered by Wang et al (2001b), making it the latest enzyme to be incorporated into the known scheme of the polyamine metabolism. SMO was originally named PAOh1, since it was the first human PAO to be cloned. Subsequently, the name was changed to SMO after confirmation that this particular enzyme is able to oxidize non-acetylated spermine (Vujcic et al., 2002b). The human SMO is a flavin-containing oxidase that acts on spermine (Km = 1.6 µM) and N^1-acetylspermine (Km = 51 µM). However, it does not oxidize spermidine (Wang et al., 2003). The products of the SMO reaction are spermidine, hydrogen peroxide and 3-aminopropanal. The human *Smox* gene has been demonstrated to undergo alternative splicing creating up to four different isoforms, with each isoform having different substrate preferences and expression patterns (Murray-Stewart et al., 2002). In contrast to APAO, SMO

expression is highly inducible at the mRNA level by various polyamine analogues, such as N^1, N^{11}-bis(ethyl)norspermine (Wang et al., 2001b). While the APAO inhibitor MDL 72,527 is also able to suppress SMO activity, the oligoamines SL-11144 and SL-11150 have been demonstrated to be more potent inhibitors with IC_{50} values lower than 0.1 µM (Wang et al., 2003). One hypothesis claims that the reason for the existence of SMO could be the production of β-alanine from 3-aminopropanal via aldehyde dehydrogenase (Vujcic et al., 2002b), as happens in the yeast SMO catalyzed reaction (White et al., 2001).

2.4 POLYAMINE TRANSPORT

The vast majority of polyamines found in the cell originate from the diet and/or the gut flora. Therefore, polyamine uptake is a crucial mechanism in maintaining intracellular polyamine levels. The polyamine transporter needs to have three main properties: a high affinity for spermine and spermidine, while the affinity of putrescine needs to be lower; fast transport speed (2-5 nmol/10^6 cells/h); a feedback control (OAZ) (Mitchell et al., 2007).

Recently, Soulet et al (2004) proposed a comprehensive model for the cellular polyamine transport. According to the proposed mechanism, polyamine transport into the cells consists of two differentiated steps. The first step occurs at the plasma membrane, where polyamines are transported into the cytosol through an as yet to be discovered channel or uniporter that only needs an electronegative membrane potential (Poulin et al., 1998). It has also been suggested that the heparan sulfate side chains of glypican-1 can act as polyamine presenters for the polyamine transporter (Belting et al., 2003). In the second step, the intracellular polyamines are transported and stored into polyamine-sequestering vesicles formed from the transgolgi network. The transport into the vesicles is an energy consuming process controlled by a proton gradient maintained by vesicular ATPase, and thus the number of protons to be excreted is equivalent to the total number of charges of the polyamine to be imported. Therefore, the vesicle transporter would be a proton:polyamine antiporter. It is rather likely that the release of polyamines from the polyamine-sequestering vesicles is performed by the same proton:polyamine antiporter.

The polyamine transport mechanism takes up spermine and spermidine with a high affinity (K_m < 1 µM), and putrescine with a lower affinity (K_m = 5-10 µM) (the first and second characteristics of the polyamine transporter), but it appears that these molecules are not the only ones transported into the cell by

this transporter. Many of the known polyamine analogues seem to be transported by the same mechanism as the natural polyamines and with similar K_m values. Moreover, other molecules, such as methylglyoxal bis(guanylhydrazone) or paraquat, with little structural resemblance to polyamines, are transported by the same mechanism (Seiler et al., 1996).

Polyamine uptake is controlled by OAZ through an unknown mechanism (Coffino 2001) (the third characteristic of the polyamine transporter). OAZ is activated when intracellular levels of free polyamines become elevated and functions by inhibiting polyamine biosynthesis and polyamine uptake.

The excretion of polyamines seems to use a different mechanism than their import. The polyamine exporter, named the diamine exporter, is regulated by the transmembrane pH gradient (Xie et al., 1997). Polyamines are excreted either as putrescine or as N^1-acetylated polyamines formed by the SSAT catalyzed reaction. Recently, a putative polyamine exporter has been characterized as the solute carrier 3 A2 transporter (SLC3A2). SLC3A2 couples putrescine excretion with arginine uptake. Moreover, the SLC3A2 colocalizes in the plasma membrane together with SSAT, evidence of a direct link between polyamine catabolism and excretion (Uemura et al., 2008).

2.5 FUNCTIONS OF POLYAMINES

The characteristic chemical structure of the polyamines, namely the backbone with differentially spaced amino groups (*Figure 1*), means that under physiological conditions, most of the intracellular polyamines are positively charged. The positive charges from the polyamines allow them to interact with many different cellular components, such as DNA to RNA and proteins. In fact, it is believed that most of the polyamines in the cell are not free but bound to cellular components (Jänne et al., 2004). The general distribution of polyamines combined with their chemical characteristics means that they are critically involved in many basic cellular functions.

2.5.1 DNA interaction

The positive charges of the polyamines are differentially interspaced along their backbone (*Figure 1*). This particular characteristic is a central feature allowing their interaction with polynucleotides. Polyamines are able to condense DNA, either naked or in the form of chromatin. The formation of chromatin requires the compaction of DNA which folds itself with the aid of histones. However, histones by themselves can only neutralize about 55% of the negative charges on DNA (Smirnov et al., 1988). When histones are combined with polyamines, they

can neutralize an additional 15-30 % of the negative charges, thus, neutralizing most of the negative charges. This means that the DNA folding into chromatin can be accomplished more easily in the presence of polyamines (Balasundaram and Tyagi, 1991). This is achieved in a stochiometric manner, i.e. two polyamine molecules are needed per DNA turn (Smirnov et al., 1988).

More related to the polyamine metabolizing enzymes than to the polyamines themselves, histones can be modified by methylation, acetylation or phosphorylation. Already in 1980, there were reports of histone acetyltransferase enzymes that also possessed the capability to acetylate polyamines (Libby 1980). Accordingly, the SSAT enzyme is a member of the GCN5 acetyltransferases family. GCN5 acetyltransferases are able to acetylate various residues in the tails of yeast histones H3 and H2B (Baker and Grant, 2007), decompressing chromatin and making it transcriptionally active. Therefore, there is a tight relationship between chromatin structure and polyamine metabolism. This was also postulated by Morgan et al. (1987) who proposed that the existence of acetyltransferases capable of acting upon both histones and polyamines serves as evidence for the relationship of these two processes.

Polyamines have also been implicated in the “transformation” of B-DNA (the right-handed helix), the most common form of DNA, into Z-DNA (the left-handed zig-zag). The Z-DNA conformation appears in regions where alternative pyrimidine and purine bases occur. These regions are present regularly at every 3000 base pairs in the human genome, and have been found to be preferentially located near to the transcription initiation sites (Schroth et al., 1992). By themselves, micromolar levels of spermidine or spermine are able to transform DNA from the B to the Z conformation (Thomas et al., 1985). Ha et al. (2005) demonstrated that a single DNA chain could present both B and Z structures at the same time. This is possible when the base at the junction of the two conformations is broken and flips out. DNA in the Z conformation is released from the nucleosome and it cannot reform it. The Z conformation is transcriptionally active as it attracts transcription factors and RNA polymerases that initiate transcription (Liu et al., 1987). Thus, it is possible that the intracellular levels of polyamines, in particular the intranuclear pool, helps to regulate the transcription of genes, by means of controlling the B to Z DNA conformational change.

2.5.2 Eukaryotic cell cycle

The progression of the cell cycle depends on the activation of the different constitutively expressed cyclin dependent kinases (CDKs) by the appropriate regulatory cyclins (reviewed by Malumbres and Barbacid, 2009). Together with

the cyclic behavior of the pairs of CDKs and cyclin, polyamine concentration and their metabolic enzymes follow distinct cyclic patterns during the eukaryotic cell cycle.

The polyamine biosynthetic enzymes ODC and AdoMetDC are first activated during the late G1 phase, and reach their first activity peak at the beginning of the S phase. A second activation occurs during the late S phase, reaching a peak at the G2 phase, not declining until after the G2/M transition (Oredsson, 2003). SSAT is also expressed following the cell cycle progression. SSAT is activated only after the G2/M transition, peaking at the M phase and decreasing rapidly afterwards. In accordance with ODC and AdoMetDC activation, the polyamine content also varies, with peaks at the time when ODC appears activated (Wallace et al., 2003). Whether the alterations in the polyamine metabolism are a cause or an effect of the cell cycle progression is not completely clear. What it is clear is that specific amounts of each individual polyamine are needed to allow cells to progress through the cell cycle, but their particular functions remain undetermined (Oredsson, 2003) (*Figure 3*).

One possible function of polyamines in the cell cycle was proposed by Thomas and Thomas (1994) who showed that polyamines could regulate the degradation of cyclin B1. Therefore, the alterations in the polyamine pools during the cell cycle may be due to their role in cyclin regulation. Irrespective of what the exact functions of polyamines are within the cell cycle, alterations on the polyamine metabolism have profound effects on hyperproliferative diseases.

2.5.3 Hypusine formation

Spermidine has one particular function since it is the precursor for the amino acid hypusine (Shiba et al., 1971). Hypusine is a rather unique amino acid that is needed for the formation of the functional form of the eukaryotic initiation factor 5a (eIF-5a, previously known as eIF-4D) (Park 1989). eIF-5a is produced in a two-step reaction (Park et al., 1982). In the first step, spermidine is hydrolysed by deoxyhypusine synthase (DHS) and the released 4-aminobutyl group is transferred to the ε-amino group of the ^{50}Lys residue from the eIF-5a precursor protein. In the second step, the deoxyhypusinated precursor is hydroxylated by deoxyhypusine hydroxylase (DOHH) producing the mature eIF-5a. The exact function of eIF-5a has remained as elusive as the functions of polyamines themselves. Among other, eIF-5a has been linked to the formation of the first peptide bond during mRNA translation (Benne and Hershey, 1978), it serves as a cofactor for HIV-1 REV (Ruhl et al., 1993), and is involved in the nuclear export (Lipowsky et al., 2000) and mRNA turnover (Zuk and Jacobson, 1998). Due to the lack of a specific function for spermine in the higher eukaryotes, it has been proposed that spermine could serve as a reserve pool for spermidine

used in the formation of hypusine (Hyvönen et al., 2007b).

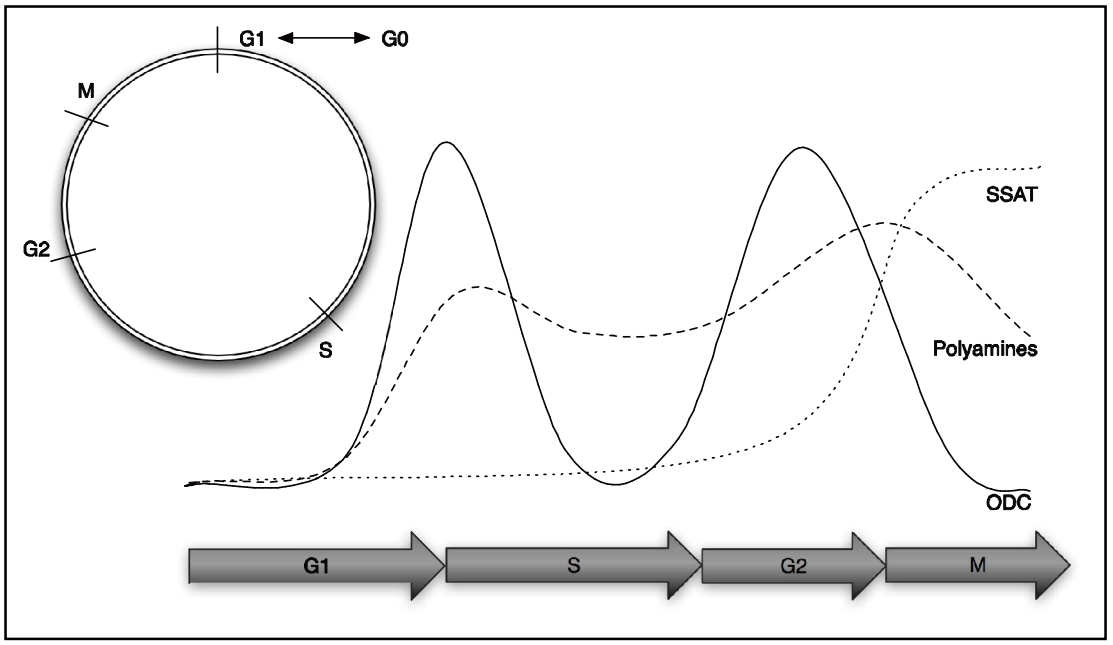

Figure 3. Polyamine levels and main polyamine metabolizing enzymes are altered during the eukaryotic cell cycle (modified from Wallace et al., 2003; Oredsson, 2003).

2.5.4 Ion channels

Polyamines are also capable of interacting with various ion channels, such as potassium inward rectifying (Kir) channels, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, and *N*-methyl-D-aspartate (NMDA) receptors (Johnson, 1996; Williams, 1997). The total charge of the polyamine cation directly correlates to its affinity on the specific ion channel (spermine >> spermidine >> putrescine). In the Kir, AMPA and kainite receptors, polyamines interact with the actual gate, blocking it and making it impossible for other molecules to pass through. On the other hand, in the NMDA receptors, spermine has a triple effect: it potentiates currents in the presence of glycine (a co-activator); increases the affinity of the receptors for glycine; and decreases the affinity for glutamate (Williams, 1997). As a curiosity, some spider and wasp venoms contain toxins that are formed by an aromatic group linked to a polyamine-like tail. These polyamine-derived toxins are able to affect the same group of ion channels as normal polyamines. This property is currently being used in the design of new synthetic selective inhibitors for the

different ion channels (Strømgaard et al., 2005).

2.5.5 Free radical scavenging

Polyamines, in particular spermidine, have been linked with the ability to scavenge free radicals and to protect DNA against reactive oxygen species (ROS) (Ha et al., 1998). Moreover, lipid peroxidation, the process by which free radicals remove negative charges from lipids in the cell membrane producing cellular damage, is inhibited by polyamines (spermine >> spermidine >> putrescine) in protozoa (*Trypanosoma cruzi*) (Hernández et al., 2006), in rat liver homogenates (Pavlovic et al., 1992), and human red blood cells (Farriol et al., 2003).

2.6 POLYAMINES AND DISEASE

The polyamine functions identified so far are many and diverse. Therefore, it could be expected that alterations in polyamine metabolism and their levels would affect, or be affected, in a wide variety of disease states.

2.6.1 Inflammation

Inflammation is the organism's response to cellular damage. Inflammation *per se* is not a disease but a consequence of one. Acute inflammation is the response to cellular damage or injury. During the first steps, resident macrophages and mast cells release inflammatory mediators, such as cytokines, chemokines and eicosanoids. These mediators are used to recruit circulating leukocytes and plasma proteins into the injured area. Neutrophils become activated, either by direct contact with the pathogens or can be activated by the cytokines (chemotaxis), and then these cells move into the injured area and try to remove the pathogens by phagocytosis and/or releasing the contents of their granules (degranulation). The neutrophil granules contain, many potent effectors, including ROS and reactive nitrogen species. These effectors cannot differentiate between the endogenous and exogenous components. Thus, the organism also suffers some damage during the inflammatory process. Once the pathogen responsible for the original damage has been removed, the repair phase can begin. Resident macrophages release lipoxins that enhance the recruitment of monocytes that will remove dead cells and initiate the tissue repair. However, if the inflammation signaling continues to be active, the acute inflammation will be converted into a chronic condition. During chronic inflammation, the constant activity of macrophages produces elevated amounts

of cytokines and ROS evoking DNA and cellular damage (reviewed by Medzhitov 2008). Chronic inflammation has been linked to many different diseases, from type 2 diabetes (Ray et al., 2009), to cardiovascular diseases (Duan et al., 2009), cancer (Rakoff-Nahoum, 2006), pancreatitis (Granger and Remick, 2005) and stroke (Kleinig and Vink, 2009), all of them being also associated with alterations in the polyamine metabolism.

The pro-inflammatory cytokine, tumor necrosis factor α (TNFα), is released upon activation of the inflammation pathway. TNFα activates SSAT in a non-small cell lung carcinoma cell line via the action of nuclear factor $\kappa\beta$ (NF$\kappa\beta$) (Babbar et al., 2006). NF$\kappa\beta$ binds to the NF$\kappa\beta$ response element found in the SSAT promoter region (Choi et al., 2006). The increased SSAT activity after the inflammatory process decreases the polyamine pools and slows down the cell growth rate. As a side effect of the increased SSAT activity, elevated amounts of hydrogen peroxide will be produced this could cause DNA damage and cellular apoptosis leading to persistence of the inflammation (Babbar et al., 2007). On the other hand, spermidine and spermine, which would be depleted after SSAT activation, have been found to inhibit the secretion of TNFα in cultured macrophages (Pérez-Cano et al., 2003) and human peripheral blood mononuclear cells (Zhang et al., 1997). Therefore, they can be considered as having anti-inflammatory effects.

2.6.2 Cancer

Most of the attention that polyamines have attracted comes from their involvement in hyperproliferative disorders. As discussed above, polyamine levels and the activities of the related enzymes are suggested to control the movement of cells through the cell cycle (*Figure 3*). Therefore, it is to be expected that alterations in polyamine metabolism and pools are intimately involved in the development of hyperproliferative diseases.

During the mid 1960s appeared the first reports indicating that polyamines accumulate in rapidly growing tissues, such as chick embryo (Caldarera et al., 1965) or regenerating rat liver (Raina and Jänne, 1968; Russell and Snyder, 1968). The interest in polyamines and cancer originated during the late 1960s and in the early 1970s, when various reports linking alterations in the polyamine levels or polyamine-related enzymes with cancer appeared (Bachrach et al., 1967; Williams-Ashman et al., 1972; O'Brien et al., 1975). Moreover, at the same time it was reported that 70 to 90% of human patients suffering from any form of cancer excreted high levels of polyamines in their urine (Tsuji et al., 1975; Russell 1971), making urinary polyamines levels a good prognostic marker for cancer. This correlation between polyamines and cancer led to the hypothesis that controlling the polyamine cycle could alter the

cancer progression, and this spurred the development of specific polyamine metabolism inhibitors, such as α-difluoromethylornithine (DFMO) (Metcalf et al., 1978). The treatment of cancer by using single enzyme inhibitors has proven unsuccessful, mainly because either these inhibitors were not able to deplete the cells from all three polyamines or they had toxic side effects (Wallace and Fraser, 2003). These initial failures with the single enzyme inhibitors inspired the search for polyamine analogues. Polyamine analogues are designed either to substitute completely the natural polyamines in their cellular functions or to be cytotoxic (Casero and Marton, 2007).

The most detailed account of the relation between polyamine metabolism and cancer is depicted in the mechanism of action of the genetic variant of colon cancer called familial adenomatous polyposis (FAP). Individuals suffering from FAP possess a germline mutation in the tumor suppressor adenomatous polyposis coli (APC) gene. The mutation has a double effect, first it produces the loss of APC function, and second, it induces expression of the c-Myc oncogene (He et al., 1998). The first gene found to be transcriptionally regulated by c-Myc was ODC (Bello-Fernandez et al., 1993). ODC is transcriptionally regulated in FAP cancer through two connected pathways. In the first pathway, functional APC suppresses the activation of c-Myc and activates OAZ, and this combined effect represses ODC activity and increases its degradation by the 26S proteasome (Erdman et al., 1999). This is demonstrated in a FAP mouse model ($APC^{Min/+}$ mouse) that displays increased ODC expression in the intestinal tissue (Erdman et al., 1999). The second pathway involves the activation of V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS). KRAS is usually inactive, but in many types of cancer it becomes mutated and activated, e.g. in colon cancer. Activated KRAS blocks SSAT transcription by means of repressing the PPARγ protein (Ignatenko et al., 2004), a known SSAT activator (Babbar et al., 2003). Moreover, activated KRAS has been demonstrated to block the diamine exporter (SLC3A2) in the HCT116 colon cancer cell line (Uemura et al., 2008). The combination of these processes (ODC activation, SSAT repression and polyamine export blockage) wakes the accumulation of polyamines in the intestinal cells, leading to increased proliferation and finalize with neoplasia (Gerner and Meyskens, 2004) (*Figure 4*).

The study of the FAP colon cancer mechanism allowed the clarification of a possible link between polyamine metabolism and cancer. Similar alterations in the polyamine metabolism appear to occur in most cancer types, as c-Myc is estimated to be upregulated in 70 % of human tumors (Casero and Marton, 2007).

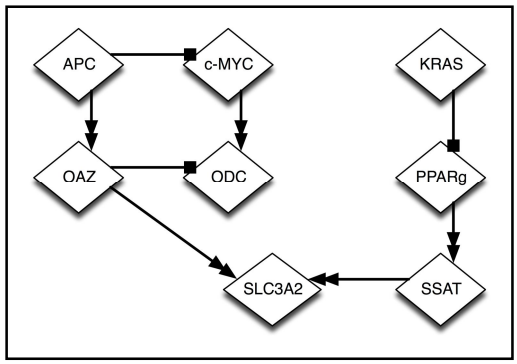

Figure 4. Alterations of polyamine metabolism in FAP colon cancer. Wild-type APC inactivates c-Myc, a known ODC activator, and activates OAZ, the ODC regulator protein. While the mutated APC is not able to block ODC activation by c-Myc. Additionally, mutated and activated KRAS inhibits PPARγ, blocking and decreasing SSAT activity. At the same time, the repressed SSAT negatively regulates the expression of SLC3A2. The combination of all these processes leads to the intracellular accumulation of polyamines, by activating polyamine biosynthesis and repressing polyamine catabolism and excretion, and malignant cellular growth. Lines with a square indicate repression (—■), and lines with a double arrowhead indicate activation (—►►) (modified from Gerner and Meyskens, 2004).

2.6.3 Type 2 diabetes

Type 2 diabetes (T2D) is an endocrinological disorder that arises when pancreatic beta-cells are not capable to secrete enough insulin in response to the serum glucose levels. This imbalance occurs due to a decreased beta-cell mass and/or secretory dysfunction (Rhodes, 2005), combined with the appearance of insulin resistance in peripheral organs, such as adipose tissue, muscle and liver (Laakso, 2001). A combination of both genetic and environmental factors is responsible for the development of T2D. The results from candidate gene studies combined with recent genome-wide association (GWA) studies have identified so far a total of 19 candidate genes related to the development of T2D (McCarthy and Zeggini, 2009). Among the several environmental factors, the activity regime and the diet are the two main causes affecting the development of T2D. In fact, obesity, specifically the amount of abdominal fat, has been positively correlated to insulin resistance in man (Abate et al., 1995). The number of people suffering from T2D was estimated to be around 170 million worldwide in the year 2000, and if the trend continues, it is expected that by the year 2030, it will more than double to about 366 million people (Wild et al., 2004).

Polyamines are found in all living cells, and pancreas and the insulin-producing beta-cells are no exception. In fact, the pancreas is the organ containing the highest concentration of intracellular spermidine and spermine (Rosenthal and Tabor, 1956). It is clear that polyamines have a role in insulin secretion. The two polyamines, spermidine and spermine, are localized in the secretory granules found in the beta-cell cytoplasm (Hougaard et al., 1986). These polyamines can be used as a substrate by the enzyme transglutaminase (Folk et al., 1980), which modulates membrane fusion processes in the beta-cells (Bungay et al., 1984). Moreover, spermidine and spermine seem to be directly involved in the transcriptional control of the insulin gene (Welsh, 1990).

Research performed in our group has recently demonstrated that a transgenic mouse line overexpressing the SSAT gene under its own promoter has 66% reduced perigonadal white adipose tissue (WAT), improved insulin sensitivity and glucose tolerance, and increased expression of PPAR γ co-activator 1α (PGC 1α), which controls many oxidative phosphorylation (Pirinen et al., 2007). Moreover, studies using a knockout mouse line with a disrupted SSAT gene have revealed that these animals become insulin resistant upon ageing (Niiranen et al., 2006) and that they accumulate more adipose tissue than wild-type mice (Jell et al., 2007). A detailed characterization of these two mouse lines showed that the transgenic mice have decreased acetyl and malonyl-CoA levels in the white adipose tissue, and elevated glucose and palmitate oxidation, while the knockout animals have an opposite phenotype, with increased levels of acetyl and malonyl-CoA and decreased glucose and palmitate oxidation (Jell et al., 2007). The decreased level of acetyl-CoA is a direct result of the polyamine catabolism activation, which uses two acetyl-CoA molecules per turn. Moreover, the activation of polyamine metabolism increases consumption of ATP equivalents, in the form of dcAdoMet and acetyl-CoA. This increased energy demand in the SSAT overexpressing animals is thought to be the basis for the increased insulin sensitivity (Pirinen et al., 2007). This association is currently under intensive study by our group and others as it could represent novel approach to the treatment of T2D and obesity.

2.6.4 Pancreatitis

The function of the large pancreatic spermidine pool is not clear but it may be involved either in the active protein synthesis or the secretory mechanism (Jänne et al., 2005), as hypothesized earlier for the insulin secretion.

Transgenic rat and mouse lines harboring the SSAT gene under the control of a mouse heavy metal-inducible metallothionein I promoter have been extensively used in the study of pancreatitis (Alhonen et al., 2000; Herzig et al.,

2005). Upon activation of the SSAT transgene by a non-toxic dose of Zn, the spermidine and spermine pools are depleted while putrescine accumulates, and this is accompanied by acute necrotizing pancreatitis (Alhonen et al., 2000). The activations of cathepsin B and trypsinogen, both early signs of pancreatitis injury, correlate temporally with the spermidine pool depletion. Structurally the pancreas is also affected by the spermidine and spermine depletion, showing dilation of the rough endoplasmic reticulum, appearance of autophagosomes and, later, the disruption of intracellular membranes and organelles (Hyvönen et al., 2007a). Pancreatitis development is linked also with reactive oxygen damage leading to inflammation. Merentie et al. (2007) showed that the MT-SSAT transgenic rats display increased lipid peroxidation, a clear sign of reactive oxygen damage, decreased superoxide dismutase and catalase activities, and activation of pro-inflammatory cytokines, such as IL-1β and TNF-α. More evidence of the direct involvement of polyamines in the development of pancreatitis came from an experiment where SSAT transgenic rats were treated with a polyamine analogue, α-methylspermidine (Räsänen et al., 2002) or bis-α-methylspermine (Merentie et al., 2007), before the induction of pancreatitis. These treatments prevented the development of the pancreatitic damage and the induction of inflammatory factors. Moreover, bis-α-methyl spermine was shown to abolish completely trypsinogen activation in isolated MT-SSAT acini (Hyvönen et al., 2006). Other chemically induced pancreatitis models, such as the taurodeoxycholate (Jin et al., 2008) or gossypol (Räsänen et al., 2003), show similar changes in the polyamine pools.

2.6.5 Protozoan parasites

Polyamines, with the exception of spermine, are also found in protozoan parasites. Spermidine is used by the parasites to produce trypanothione, a spermidine-bis(glutathionyl) conjugate used for protection against oxidative and chemically induced stress. Therefore, drugs aimed at interfering with the polyamine metabolism are promising targets for the fight against protozoan parasites diseases (Heby et al., 2007). During the 1980's, it was demonstrated that treatment with DFMO was able to cure mice infected with *Trypanosoma brucei brucei* (Bacchi et al., 1980), and also humans infected with *Trypanosoma brucei gambiense*, one of the parasites responsible for African trypanosomiasis (African sleeping sickness) (van Nieuwenhove et al., 1985). However, DFMO has not been effective against the trypanosoma strain responsible for the sleeping sickness, *Trypanosoma brucei rhodesiense*. Other parasitic diseases that can be treated with inhibitors of polyamine biosynthesis (DFMO or AbeAdo) include Chagas disease (American trypanosomiasis) caused by the *T. cruzi*, and Leishmaniasis (Heby et al., 2003).

2.6.6 Other diseases

Sequeira et al (2006) identified a correlation between low SSAT expression and suicidal tendencies. A microarray analysis further demonstrated that individuals attempting or committing suicide display lower SSAT levels in the brain. The same group later demonstrated the existence of three different polymorphisms in the promoter region, an insertion/deletion (rs6151267) and two SNPs (rs6526342 and rs928931). These polymorphisms may affect SSAT expression levels even without involving the polyamine response element (PRE) (Fiori et al., 2009). A similar study contrary reported that although successful suicides have lower SSAT expression levels in the brain, there was no correlation between the SSAT expression levels and the SNPs (Guipponi et al., 2008). The differences between the two studies could be due to different areas of the brain that were sampled. Since SSAT can be considered as a stress response protein, Fiori et al. (2009) hypothesized that the lower levels of SSAT found in the SNP carriers could affect their capacity to deal with the consequences of stress, promoting suicidal tendencies on the carriers.

The Snyder-Robinson syndrome (OMIM 309583) is a very rare mental retardation syndrome linked with mutations in the SMS gene. This syndrome is characterized by mildtomoderate mental retardation, asthenic body build, skeletal defects, osteoporosis, and unsteady gait. Since the SMS gene is located in the X chromosome, the syndrome only affects males. There are three reports on three different families affected by the Snyder-Robinson syndrome, and each of these families carries a different mutation in the SMS gene (Cason et al., 2003; de Alencastro et al., 2008; Becerra-Solano et al., 2009).

The rare X-linked disorder keratosis follicularis spinulosa decalvans (OMIM 308800) has been linked with the duplication of a chromosomal region, giving rise to an additional SSAT gene in one single patient. Keratosis follicularis spinulosa decalvans is characterized by follicular hyperkeratosis, progressive cicatricial alopecia, and photophobia. Studies on fibroblasts isolated from the original patient identified an increased putrescine concentration and decreased spermidine and spermine levels (Gimelli et al., 2002). This phenotype bears many resemblances with the symptoms seen in rodent models where there is increased polyamine catabolism, namely hairlessness, wrinkled skin, depletion of spermidine and spermine and accumulation of putrescine (Pietilä et al., 1997; Suppola et al., 1999).

3 Aims of the study

The main aim of this study was to extend the current knowledge on polyamine functions and metabolism. In order to achieve this goal, advantage was taken of a variety of transgenic mouse models. The majority of the present work is based on studies performed on a transgenic mouse model overexpressing the SSAT gene under the control of the metallothionein promoter (MT-SSAT), but also a mouse model overexpressing the SSAT gene under its own promoter and a mouse model with a disrupted SSAT gene (SSATKO) were used.

The specific aims of the study were:

- To test the stability of α-methylated polyamine analogues and whether these analogues are able to substitute natural polyamines *in vivo* in the presence of activated polyamine catabolism (MT-SSAT) (Original Publication I)
- To analyze the hepatic protein expression pattern in a mouse line with activated polyamine catabolism (MT-SSAT) (Original Publication II)
- To characterize the accelerated aging phenotype linked with activated polyamine catabolism seen in the MT-SSAT mouse line (Original Publication III)
- To improve the current methodology used for the polyamine flux studies (Original Publication IV)
- To characterize the link between activated polyamine catabolism and altered insulin secretion (MT-SSAT)

4 Materials and methods

4.1 ANIMAL MODELS (I, II AND III)

The generation of MT-SSAT transgenic mice (UKU181) (*Figure 5*) has been previously described. Briefly, both the metallothionein I promoter and the SSAT coding sequence used for the transgene construct were cloned from a mouse genomic DNA library. The transgenic mice were generated by a standard pronuclear microinjection to zygotes from BALBc x DBA/2 females mated with CD2F1 males (Suppola et al., 1999). The animals were housed in the National Laboratory Animal Center of Kuopio University under controlled temperature (20 ± 2°C) and relative humidity (50 %), a 12-hours light cycle, and were fed a standard chow diet *ad libitum*, unless otherwise stated. All the animal experiments were approved by the Institutional Animal Care and Use Committee at the Provincial Government of Eastern Finland.

4.2 ANALYTICAL METHODS (I, II, III AND IV)

4.2.1 SSAT activity (I, II, III and IV)

SSAT activities were assayed following the method of Libby (1978). In short, tissue and cell homogenates were centrifuged for 30 min at 13,000 rpm at + 4°C (Heraeus Biofuge Fresco, Thermo Scientific, Walthman, MA, USA). A volume of 10 µl of the supernatant was transferred into 0.5 ml tubes. Samples were incubated in duplicates for 10 min at 37°C with 100 mM Tris-HCl pH 7.8, 10 mM spermidine, 1 mM DTT, and 0.5 nCi [^{14}C]-acetyl-CoA (0.5 µCi/ml, GE Healtcare, Uppsala, Sweden) in a final volume of 100 µl. After the incubation, 20 µl of hydroxylamine hydrochloride were added and the mixture was further incubated at 99°C for 3 min in order to inactivate the enzyme. The tubes were centrifuged for 3 min at 13,000 rpm to remove any protein, and 40 µl of the supernatant were spotted on a phosphocellulose P81 paper (1 cm^2) piece. The papers were dried for 10 min and then extensively washed with MilliQ water and ethanol. Finally, the papers were dried and placed in scintillator tubes with 3 ml of Optiphase Hisafe 2 (Perkin Elmer, Shelton, CT, USA) and measured in a

liquid scintillator counter (1450 Microbeta Plus, Perkin Elmer).

4.2.2 ODC activity (III)

ODC activities were assayed with the method of Jänne and Williams-Ashman (1971). Cellular extracts were centrifuged for 30 min at 13,000 rpm at + 4°C. A total of 50 µl of the supernatant was transferred into glass tubes, preparing duplicates per sample. 200 µl of a solution containing 125 mM Tris-HCl pH 7.4, 5 mM EDTA, 5 mM DTT, 250 nM L-ornithine, 0.5 mM pyridoxal phosphate, and 0.2 µCi [^{14}C]-L-ornithine (0.02 µCi/µl, GE Healtcare) was added to each of the samples, and a piece of Whatman paper soaked in Soluene (Sigma-Aldrich, Stenheim, Germany) was attached to the top of the tube. The reaction tubes were incubated for 30 min at 37°C with constant shaking. After the incubation, 1 ml of 2 M citric acid was added and the mixture was further incubated at 37°C for 5 min in order to terminate the reaction and liberate the remaining CO_2 from the solution. The papers were placed into scintillator tubes with 3 ml of Optiphase Hisafe 2 and measured in liquid scintillator counter.

Figure 5. Two MT-SSAT transgenic mice (hairless) together with a non-transgenic littermate of the same age.

4.2.3 Polyamine levels (I, II, III, IV and V)

Polyamine levels in the experiments presented in manuscripts I, II, III and V

were measured using the HPLC-based method of Hyvönen et al. (1992). The polyamine levels in study IV were measured using the LC/MS-MS-based method of Häkkinen et al. (2008). The protein contents of the tissue and cell samples were precipitated in a 5 % sulfosalicylic acid solution containing 10 µM diaminoheptane (DAH), used as internal standard. The samples were vortexed and then centrifuged for 30 min at 13,000 rpm at + 4°C. The supernatants were filtered through a 0.22 µm filter and transferred into HPLC vials.

4.2.4 Nucleoside levels (II and III)

AdoMet and AdoHcy were measured using the HPLC-based method of She et al. (1994). The samples were prepared in the same way as the polyamine samples.

4.2.5 ATP levels (II)

ATP levels were measured using the ATP Lite 1 step kit (Perkin Elmer). Tissues were homogenized in 10 volumes of 5 % perchloric acid. The homogenates were centrifuged for 20 min at 13.000 rpm at + 4°C, and the obtained supernatant was neutralized to pH 7 using 3.75 M K_2CO_3. The neutralized supernatant was centrifuged for 15 min at 13,000 rpm, and their supernatants were diluted to 1/100 prior to the ATP measurement. Each sample was run in triplicate.

ATP levels from pancreatic islets were measured in triplicate from 10 islets placed in 100 µL of Hank's Buffered Salt Solution (HBSS, Gibco, Invitrogen, United Kingdom) containing 0.5 % BSA (Sigma-Aldrich) and 20 mM HEPES.

4.2.6 Protein carbonyl content (III)

The protein carbonyl content was measured as the absorbance at 370 nm produced by the carbonyl groups after they have reacted with 2,4-dinitrophenylhydrazaine (DNPH, Sigma-Aldrich) (Levine et al., 1990).

4.2.7 Catalase activity (II and III)

Catalase activity was measured from tissue homogenates as the decrease of absorbance at 240 nm (Aebi 1984).

4.3 TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (2D-PAGE) (II)

The 2D-PAGE is based on the separation of proteins using two of their physicochemical properties. The first property is the protein isolectric point (pI),

the pH at which the protein net charge becomes 0 and the second property is the protein mass. The two separations are performed in perpendicular directions, therefore, creating a 2D map of the proteome. The detailed protocol used for the 2D-PAGE analysis is detailed in original publication II.

4.4 HISTOLOGICAL ANALYSES (III)

Histology samples were placed on a tissue cassette and submerged in formalin for at least 24h. After the formalin fixation, the samples were embedded in paraffin and cut into sections of different thicknesses. The histological sections were stained with hematoxylin-eosin.

4.5 WESTERN BLOT (II AND III)

Tissues were homogenized in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % SDS, 0.5 % deoxycholic acid, 10 % glycerol, 1 mM sodium vanadate, and complete EDTA free cocktail tablet (Roche, Penzberg, Germany)). In order to remove the cellular debris, the homogenates were centrifuged for 15 min at 13,000 rpm at + 4°C. The protein content was measured using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). The Western blot samples contained 10 or 20 µg of protein in sample loading buffer. The samples were boiled for three to five minutes before loading them into a 1.5 mm thick 8, 10 or 12 % polyacrylamide gel with a 4 % stacking gel. The gels were run at 80 V for 20 min and then at 160 V. The gels and PVFD membranes (Immobilon-FL, Millipore, Billerica, MA, USA) were equilibrated in semi-dry transfer buffer (25 mM Tris, 192 mM glycine, 10 % methanol, and 0.01 % SDS) for at least 15 min. The transfer of the proteins from the polyacrylamide gel to the membrane was performed on a Trans-Blot SD semi-dry transfer cell (Bio-Rad) at 15 V. The membrane was blocked with either 5 % non-fat milk or 2 % ECL Advance blocking agent (GE Healthcare) in TBS containing 0.1 % Tween-20 for 1 h at room temperature with constant shaking. The primary antibody was diluted in TBS-Tween, and the incubation was performed overnight at +4°C on constant shaking. The membranes were washed in TBS-Tween using the SNAP i.d. system (Millipore), and further incubated in secondary antibody diluted in TBS-Tween for 1 h at room temperature on constant shaking, and finally the membranes were washed again in TBS-Tween. Detection was achieved using the ECL plus (GE Healthcare) and the membranes were scanned in the Typhoon 9410 imager (GE Healthcare). The images were

analyzed and the bands quantified with the ImageQuant TL (GE Healthcare).

4.6 QUANTITATIVE RT-PCR (QRT-PCR) (II)

Two-step qRT-PCR (Saiki et al., 1985) was used for the quantification of messenger RNA levels for genes of interest from liver, muscle and WAT. Total mRNA was extracted by the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) using the commercial reagent TRIzol (Invitrogen, United Kingdom). An adequate amount of each tissue was homogenized in 1 ml of TRIzol. Total RNA was further purified by means of a chloroform extraction, and finally precipitated with the aid of isopropyl alcohol. In order to remove any trace of genomic DNA, the extracted total RNA was treated with DNAse I (Ambion Inc., Austin, TX, USA). The RNA quality was assessed spectrophotometrically and by running the samples in an 1% agarose gel. The good quality samples were further processed with a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). Twelve ng of the produced cDNA (RNA equivalents) were used as templates for the qRT-PCR. qRT-PCR reactions were run in triplicate together with a known standard curve in 96-well plates in an ABI Prism 7700 instrument (Applied Biosystems). The running conditions for the qRT-PCR were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15s at 95°C and 1min at 60°C. Gene-specific primers and Taqman probes were designed using Applied Biosystems assay-by-design tool (Applied Biosystems, https://www5.appliedbiosystems.com/tools/cadt).

4.7 INTRAPERITONEAL GLUCOSE, INSULIN AND ARGININE TOLERANCE TESTS AND INSULIN RELEASE (II)

The intraperitoneal glucose tolerance tests were performed on four and eight month old mice. The mice were fasted for 12 hours prior to the test. A single dose of 2 mg/g body weight of D-glucose was injected intraperitoneally and blood from the tail vein was taken at 0, 15, 30, 60, and 120 min for glucose and insulin analyses. In the insulin release assay, a dose of 3 mg/g body weight D-glucose was injected intraperitoneally and blood from the tail vein was taken at 0, 2, 5, 15, and 30 min for glucose and insulin analyses.

The insulin tolerance tests were performed on fed animals. A single dose of 0.15 mU insulin/g body weight (Actrapid, Novo Nordisk, Denmark) was

injected intraperitoneally and blood from the tail vein was sampled at 0, 20, 40 and 80 min for glucose analysis.

The arginine tolerance test was performed on four hours fasted animals. A dose of 2 mg/g body weight of arginine (Sigma-Aldrich) was injected intraperitoneally and blood was collected from the tail vein at 0, 2, 5, 15, 30 and 60 min for glucose and insulin analysis.

Plasma glucose levels were analyzed with the fluorometric method (Passonneau and Lowry, 1993), and the insulin levels were measured using a rat insulin ELISA with mouse insulin standards (Christal Chem Inc., Chicago, IL, USA).

4.8 ISOLATION OF PANCREATIC ISLETS

Fed mice were killed by CO_2 and the pancreata were intraductally perfused with a solution of 0.75 mg/ml of collagenase XI (Sigma-Aldrich). The perfused pancreata were transferred into 15 ml tubes containing 2 ml of HBSS (Gibco) containing 0.5 % BSA (Sigma-Aldrich) and 20 mM HEPES, and incubated on constant shaking at 37 °C for 10 min. After the incubation, the tubes were transferred on ice and 10 ml of HBSS solution were added, the islets were allowed to pellet by gravity and washed again with another 10 ml of HBSS solution. After the last wash, the digestions were left in 10 ml of HBSS solution and the islets were hand-picked (Gotoh et al., 1985). The islets were incubated overnight in RPMI 1640 (Sigma-Aldrich) containing 1 % BSA (Sigma-Aldrich), 2 mM glutamine (Sigma-Aldrich) and 10000 U/ml penicillin and 10 mg/ml streptomycin (Euroclone. Italy). The intact islets were hand-picked on the next morning and used for the experiments.

4.9 CELL CULTURE (IV)

E14.5 MT-SSAT embryos from C57Bl/6J females were used to isolate embryonic fibroblasts. The cells were grown in DMEM (Sigma-Aldrich) containing 10 % fetal bovine serum, 2mM glutamine, and 50 µg/ml gentamycin (Sigma-Aldrich). The incubator was set to 37°C with a 5 % CO_2 flux.

The cells were seeded in 100mm plates and let to grow for 24 h prior treatment. The labeled precursors, [$^{13}C_6$,$^{15}N_4$]arginine (Isotec. Sigma-Aldrich), [$^{13}C_5$,^{15}N]methionine (Isotec. Sigma-Aldrich) or [$^{13}C_5$]ornithine (Cambridge Isotope Laboratories Inc., Andover, MA, USA), were diluted to a concentration of 0.03 g/l in DMEM without methionine (Gibco). The cells were collected at

different time points by trypsinization and lyzed in 5 % sulfosalicylic acid. The cells were sonicated and then centrifuged for 25 min at 13,000 rpm in a temperature-controlled centrifuge. The supernatants were filtered using 0.22 µm filters (Millipore) and used to measure polyamines using the MS-based method. The pellets were used for measurement of DNA.

4.10 STATISTICAL ANALYSIS (I, II, AND III)

The data from the different experiments were analyzed by Student's *t*-test when two groups were compared. When more than two groups were analyzed, the one-way analysis of variance (ANOVA) with Dunnet's post hoc test for multiple groups comparisons was used. Data are expressed as the mean ± standard error of the mean (S.E.M.), unless otherwise stated. Statistical analyses were performed with the aid of the SPSS v. 16.0 (SPSS Inc, Chicago, IL, USA).

5 Results

5.1 IN VIVO TESTING OF **A**-METHYLATED POLYAMINE ANALOGUES (I)

An improved methodology for the synthesis of α-methylspermidine, bis-α-methylspermine and α-methylspermine in the gram scale was devised and presented. In order to perform *in vivo* studies with such analogues large amounts of these are needed. The different analogues were tested *in vivo* using the MT-SSAT transgenic mice and transgenic rats, also *in vitro* tests using fibroblasts derived from the transgenic rats were performed.

The in vitro study with immortalized fibroblasts derived from the MT-SSAT rats showed that α-methylspermidine and bis-α-methylspermine were well tolerated up to concentrations of one millimolar. However, α-methylspermine was shown to be quite toxic at concentrations higher than 10 µM. All three analogues dramatically reduced the natural polyamine pools in the treated fibroblasts. Bis-α-methylspermine was the most potent of the analogues in depleting the natural polyamines (*I Figure 3*). The depletion of the natural polyamines was coupled with a slight SSAT activation in the transgenic fibroblasts, while no signs of SSAT activation were visible in the non-transgenic cells (*I Table S18A-B*).

During the *in vivo* tests, the different analogues were tested at several doses well below the LD_{50} limit of their natural counterparts. The *in vivo* test using the MT-SSAT transgenic rats indicated that all of the analogues were able to decrease the natural hepatic and kidney spermidine and spermine pools in a concentration-dependent manner. The pancreas did not seem to be strongly affected by the analogue treatment since only a minor decrease in the spermine pool was observed after treatment (*I Table S17*). Moreover, only bis-α-methylspermine treatment induced SSAT (*I Table S16*).

The *in vivo* test using the MT-SSAT transgenic mice was more exhaustive than the rat experiment. In the mice experiment the plasma α-amylase and ALAT activities were not affected by the different analogues (*I Tables S1, S6 and S11*). Only the treatment with α-mehtylspermine slightly decreased the plasma α-amylase activity in the non-transgenic group (*I Table S11*). SSAT activity was greatly induced in the liver, pancreas and kidneys from both genotypes after

α-methylspermidine treatment. Bis-α-methylspermine treatment induced SSAT in the pancreas of the non-transgenic line, but had no effect in the MT-SSAT mice. α-Methylspermine treatment affected the non-transgenic renal SSAT activity and the transgenic hepatic and pancreatic SSAT activities. Although all three analogues decreased the natural hepatic spermidine and spermine pools, the total polyamine pool remained stable, indicating that the different analogues were quite able to substitute for the natural polyamines (*I Tables S3, 8 and S13*). The spermidine analogue was not metabolized during the *in vivo* experiment. However, both spermine analogues, α and bis-α-spermine, appeared to be catabolized into α-spermidine. The spermine analogues were metabolized only in the livers of transgenic animals (*I Tables S13 and S17*).

5.2 PROTEIN EXPRESSION ANALYSIS FROM THE LIVERS OF MT-SSAT TRANSGENIC MICE (II)

The inducible MT promoter used for the generation of the MT-SSAT transgenic mouse line targets the expression of the transgenic construct mainly, but not limited, to the pancreas and liver. All of the studied organs from the MT-SSAT mice displayed increased SSAT expression (*Figure 6*). As expected, higher SSAT expression was detected in the pancreas and liver, followed by the small intestine (represented by the jejunum and ileum). When comparing the changes at the expression level, the organs with the greatest differences were pancreas (35-fold) and liver (34-fold), followed by heart (10-fold) and BAT (7-fold), in other organs, the increase of SSAT expression ranged from 1.5 to 5.5-fold (*Figure 6 Inset*).

In order to maintain the cellular polyamine levels, the transgenic cells co-activated the polyamine biosynthesis. The simultaneous activation of the biosynthetic and catabolic creates a futile cycle. This futile cycle can influence the associated metabolism, such as the metabolism of methionine, urea cycle and acetyl-CoA. The generation of both dcAdoMet and acetyl-CoA are energy driven processes, and thus the increased utilization of both metabolites in the MT-SSAT transgenic mice correlated with the 54% reduced hepatic AdoMet levels and with the 59% reduced hepatic ATP pools (*II Figures 3b and 3c*).

The detailed protein expression analysis from the livers of the MT-SSAT transgenic mice detected a total of 23 proteins differentially expressed. Due to the inherent technical limitations of the 2D-PAGE and MS techniques, only 21 spots could be positively identified. The expression profiles of the identified proteins showed that 13 were repressed, seven were overexpressed, and one protein was expressed below the detection limit in the transgenic line. The

complete list of the identified proteins was divided into protein families with similar function (*II Table 1*). As would be expected when analyzing a transgenic mouse line with activated polyamine catabolism, most of the identified proteins (14) had been previously described to function in polyamine and/or the xenobiotic metabolism. The rest of the proteins (7) did not appear to be directly related with the polyamine and/or xenobiotic metabolisms.

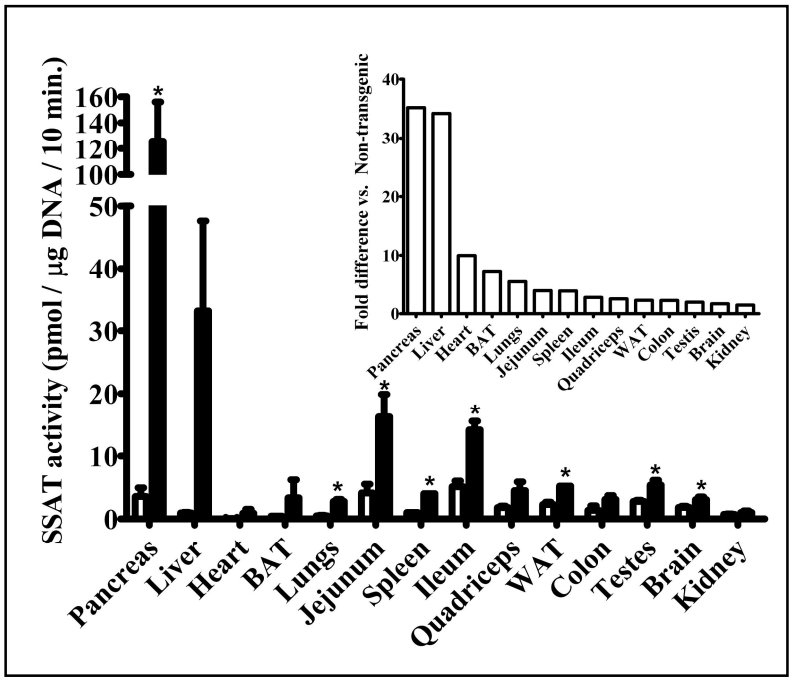

*Figure 6. SSAT expression pattern in different organs from the MT-SSAT transgenic mice. The graph shows the actual expression levels. Values are expressed as means ± SEM from three mice per line. White bars represent the non-transgenic mice, and black bars represent the MT-SSAT transgenic mice. Values were analyzed with Student's mice. t-test. * $p < 0.05$. The inset represents the fold differences in the activities between the non-transgenic and the MT-SSAT transgenic mice.*

The detailed protein expression analysis from the livers of the MT-SSAT transgenic mice detected a total of 23 proteins differentially expressed. Due to the inherent technical limitations of the 2D-PAGE and MS techniques, only 21 spots could be positively identified. The expression profiles of the identified proteins showed that 13 were repressed, seven were overexpressed, and one protein was expressed below the detection limit in the transgenic line. The complete list of the identified proteins was divided into protein families with similar function (*II Table 1*). As would be expected when analyzing a transgenic

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Among the proteins related to the polyamine metabolism, it was noted that MAT1 was 1.6-fold overexpressed. AdoMet functions as a methyl donor in most of the cell methylation reactions, a small amount is decarboxylated and can thereafter only be used as an aminopropyl donor in the polyamine biosynthesis. The greatly reduced AdoMet pools seen in the MT-SSAT transgenic mice could be part of a positive feedback and induce the MAT1 expression in order to replenish its pools. Other enzymes involved in the polyamine metabolism and xenobiotic metabolism were GSTpi (identified in two separated spots and 68% and 66% reduced), aldehyde dehydrogenase (2.5-fold overexpressed), and catalase (67% reduced). The acetylation polyamine catabolic pathway produces hydrogen peroxide and 3-acetaminopropanal as toxic by-products. The fact that aldehyde dehydrogenase was elevated in the transgenic liver was expected on the grounds that elevated intracellular levels of aldehydes were released by the hugely activated polyamine catabolism. However, it was completely unexpected that catalase and GSTpi, which detoxify hydrogen peroxide and protect the cell against ROS, were repressed in the transgenic mice. It was hypothesized that as a result of the elevated oxidative stress, induced due to the activation of the polyamine catabolism, these two enzymes should become activated in order to protect the transgenic cells. Nonetheless, the analysis of their enzymatic activities matched nicely with the 2D-PAGE results, showing that catalase activity was reduced to 65%, while the activity of the glutathione S-transferases was reduced to 80% in the transgenic mice (*II Figure 4a and 4b*).

Previous reports have shown a correlation between decreased GST pi and selenium binding protein, and activation of PPARα, which is a recognized target for PGC 1α (Chu et al., 2004; Giometti et al., 2000). Further analysis of the PGC 1α expression demonstrated that it was clearly induced in both mRNA (4.3-fold) and protein (3.5-fold) levels in the livers from the MT-SSAT transgenic mice (*II Figure 5a*). Moreover, PGC 1α is known to control the hepatic gluconeogenesis, and accordingly, two of the genes regulating this factor, namely phosphoenolpyruvate carbokinase (Pepck) and glucose-6-phosphatase (G6PC-1), were found to have increased mRNA expression in the transgenic livers (*II Figure 5b*). Although activation of the gluconeogenic program is one of the hallmarks of insulin resistance (Koo et al., 2004), the transgenic mice appeared to be hypoglycemic and hypoinsulinemic (*II Figure 5c*). Moreover, intraperitoneal insulin and glucose tolerance tests revealed that although the MT-SSAT displayed elevated hepatic PGC 1α expression, the transgenic mice were clearly more insulin-sensitive and

glucose-tolerant than their non-transgenic littermates (*Figure 7*).

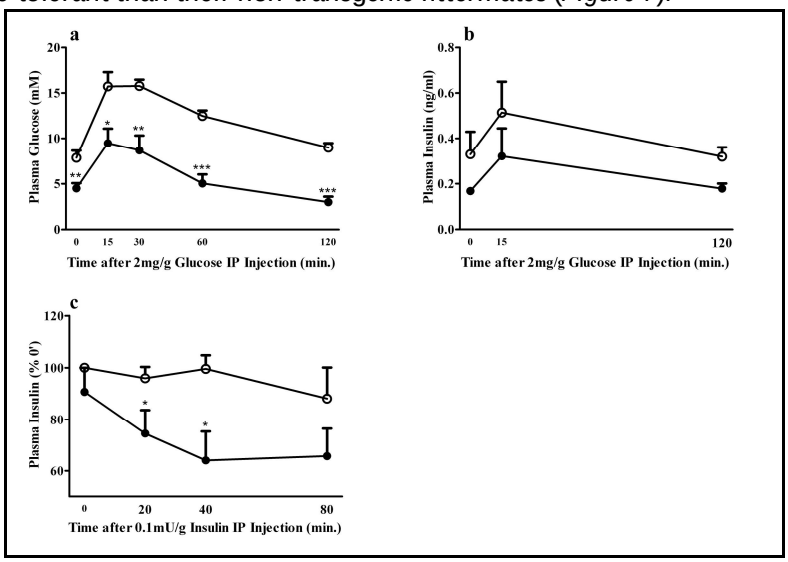

*Figure 7. Intraperitoneal glucose and insulin tolerance tests in four months old MT-SSAT transgenic mice. a Plasma glucose (mM) and b insulin (ng/ml) levels after the glucose tolerance test using 2mg of D-glucose/g weight. c Plasma insulin values (% time point 0 min) after the insulin tolerance test using 0.1mU of insulin/g weight. Data is expressed as mean ± SEM of ten mice per genotype. Non-transgenic values are represented by the open circles (○) and MT-SSAT values are represented by the filled circles (●).Values were statistically analyzed using Student's t-test. * p < 0.05, ** p <0.01 and *** p < 0.001.*

5.3 PROTECTION AGAINST HEPATOTOXICITY AT THE COST OF ACCELERATED AGING (III)

Polyamine catabolism has been proposed as a source of oxidative stress. The acetylation-oxidation polyamine catabolic pathway produces hydrogen peroxide and 3-acetaminopropanal (Alhonen et al., 2000; Pegg 2008). The protein carbonyl content, used as a measure of the oxidative state of an organism, was found to be 13-fold increased in the MT-SSAT transgenic mice (non-transgenic: 0.20 ± 0.07 nmol/mg protein; and MT-SSAT: 2.72 ± 0.47 nmol/mg protein; $p = 0.0015$). Surprisingly, the analysis of antioxidant enzymes during the hepatotoxicity studies showed that catalase and Cu, Zn-superoxide dismutase were reduced in the transgenic line by 42% and 23%, respectively (*III Table 1*). These results supported the previous observations

made during the 2D-PAGE analysis of the livers from the MT-SSAT mice line (Cerrada-Gimenez et al., 2009). Both of these enzymes are used in the degradation of hydrogen peroxide, one of the by-products of the polyamine catabolism. Moreover, reduced glutathione (GSH), which is used to provide cellular protection against toxins, was greatly increased in the transgenic line, 1.5-fold in males and almost 2-fold in females. At the same time, the oxidized glutathione (GSSG) pools were slightly increased in both genders. These changes elicited the elevation in the GSH/GSSG ratio in the transgenic mice, 1.5-fold in females and 1.3-fold in males (*III Table 1*). The analysis of the liver by DNA microarray confirmed the presence of oxidative stress and the data revealed increased transcription of many genes involved in the cellular response to oxidative stress, such as A170 stress-induced protein and cytoskeletal keratin 18.

Treatment with thioacetamide or carbon tetrachloride indicated that the MT-SSAT transgenic mice were protected against the hepatic toxicity produced by these two chemicals. Thioacetamide treatment clearly activated the polyamine metabolism in both genotypes. The effects of carbon tetrachloride on polyamine metabolism were less evident, with only a minor ODC activation. The polyamine pools were modified, reducing the levels of spermine and spermine and causing an accumulation of putrescine (*III Table 2 and 3*). The intraperitoneal injection of 100 g/kg of thioacetamide evoked 33% mortality in the non-transgenic mice, while none of the transgenic animals died during the experiment. Moreover, treatment with thioacetamide increased the number of binuclear cells 2-fold in the transgenic mice compared to the wild-type mice. Carbon tetrachloride treatment produced a similar outcome, with histological preparations showing only mild signs of edema at 24 hours post treatment in the transgenic mice, while the non-transgenic group had clear signs of periportal necrosis (*III Figure 3*).

The MT-SSAT transgenic mice also showed clear signs of accelerated aging. In a long-term survival study, it appeared that the MT-SSAT transgenic mice median life was 57 weeks, and the maximum life span was 72 weeks, about 50 % lower that the non-transgenic mice (*III Figure 2*). The accelerated aging phenotype in the MT-SSAT transgenic mice was associated with increased expression of transcription factor p53 (*III Figure 1*). One of the functions of p53 is to help in the cellular protection against oxidative stress. In order to combat the deleterious effects of oxidative stress, transcription factor p53 is known to become activated through the Arf pathway. However, constant activation of p53 would deplete the stem cell pool and promote accelerated aging (Matheu et al., 2008), as seen in the MT-SSAT transgenic mice.

Aging compromises the activation of antioxidant enzymes, thus, the decreased activities from different antioxidant enzymes seen in the MT-SSAT

transgenic line. Moreover, the toxic effects of thioacetamide and carbon tetrachloride are mediated through the accumulation of their metabolic by-products. Therefore, the actual protection of the MT-SSAT mice against the effects of these two hepatotoxic compounds is likely to be linked to the accelerated aging phenotype and the lower activity of the antioxidant enzymes involved in the detoxification of these toxins. A summary from the hypothesized events leading to the phenotype seen in the MT-SSAT transgenic mice is depicted in *Figure 8*.

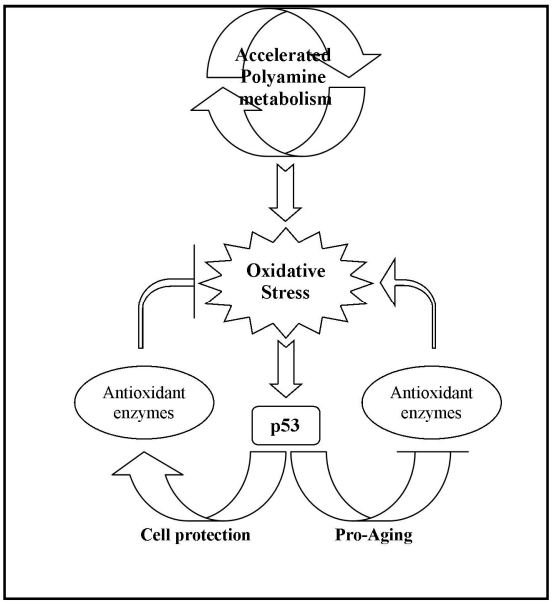

Figure 8. Schematic representation from the course of events leading to accelerated aging and hepatotoxic protection in the MT-SSAT transgenic mice line.

5.4 POLYAMINE FLUX STUDIES (IV)

The involvement of polyamines in many different disease states makes the study of their metabolism essential for the better understanding of each one of the diseases with which they have been linked. In the past, the study of polyamine flux has been based on using HPLC to measure the incorporation of the label from fluoro-ornithine into the polyamine pools (Kramer et al., 2008). This method suffers many drawbacks, such as different biochemical characteristics between the fluoro-labelled and natural compounds, and the

limited ability of the HPLC technique to separate and quantify highly related compounds, such as fluoro-labeled and natural polyamines. In order to overcome these problems, it was decided to use a combination of ^{13}C, ^{15}N labeled precursors with LC-MS/MS as a quantification methodology (Häkkinen et al., 2008).

Three different mouse fetal fibroblasts cell lines were selected to represent different states of polyamine metabolism activation: wild-type cells; MT-SSAT transgenic cells overexpressing the SSAT enzyme; and SSATKO cells that have a disrupted SSAT gene. The cells were cultured in a methionine-free cell media with one of the three labeled precursors used, [$^{13}C_6$,$^{15}N_4$]arginine, [$^{13}C_5$,^{15}N]methionine or [$^{13}C_5$]ornithine, or in the presence of unlabeled precursors.

Due to the different biochemical characteristics of each of the labeled precursors, their incorporation into the polyamine pools was different. [$^{13}C_5$]ornithine was incorporated at higher rates than [$^{13}C_5$,^{15}N]methionine (*IV Table 1 and 2*), while [$^{13}C_6$,$^{15}N_4$]arginine was the label incorporated at the lowest rate in the polyamine pools (*IV Supplementary Table 4*). Nonetheless, each of the labeled precursors reached levels close to its theoretical incorporation rate in the control and MT-SSAT fibroblasts. With all three precursors, the control and the MT-SSAT fibroblasts behaved in a similar fashion, while the SSATKO cells incorporated slightly less label, especially during the [$^{13}C_5$,^{15}N]methionine or [$^{13}C_5$]ornithine treatments.

Theoretically, the analysis of the flux could be adapted to estimate the energy consumption in each cell type. If we consider that one full turn of the polyamine cycle should consume four ATP equivalents (*Figure 2*) (Pirinen et al., 2007; Cerrada-Gimenez et al., 2009) the following approximations could be used for approximating the polyamine metabolism energy consumption: “the synthesis of one pmol/µg DNA of spermidine, acetyl-spermidine, spermine, or acetyl-spermine, consumes one pmol /µg DNA of ATP”.

However, there exists a small caveat in estimating these approximations. During the polyamine flux studies the incorporation of the label provided by each precursor into the polyamine pools followed a three phase process. In the first phase, the label was poorly incorporated while the cell reservoirs of the non-labeled precursor are being depleted. During the second phase, the label was exponentially incorporated until reaching the maximum incorporation rate, the third phase. Therefore, calculations to approximate the energy consumption of each cell line can only be performed during the exponential phase of label incorporation. If not, the calculations would be misleading.

The most crucial point in designing an experiment to follow the polyamine flux was the time point selection. Polyamine flux is highly dependent on the speed of the polyamine metabolism. The transgenic MT-SSAT fibroblasts

with activated polyamine metabolism reached the maximum incorporation at 6 hours post treatment with [$^{13}C_5$,^{15}N]methionine, and it was evident that for this particular cells the time points selected (24, 48, and 72h) for the other two labeled precursors were well after the maximum incorporation time point. The control cells reacted somewhat more slowly than the transgenic cells, while the SSATKO fibroblasts were much slower at incorporating each of the labels.

Interestingly, when the [$^{13}C_6$,$^{15}N_4$]arginine or [$^{13}C_5$]ornithine treatments were combined with the APAO inhibitor MDL 72,527 the levels of excreted acetylated-spermidine were greatly increased in the control and MT-SSAT cells. This together combined with the recent report indicating that the polyamine exporter SLC3A2 colocalizes with SSAT in the plasma membrane of colon cancer cells (Uemura et al., 2008) supports the argument that the SSAT enzyme is needed for an efficient polyamine export from the cell.

5.5 ALTERED INSULIN SECRETION (UNPUBLISHED RESULTS)

The metallothionein promoter used in the MT-SSAT mouse line targeted the transgene expression mainly to the pancreas and liver (*Figure* 6). The pancreas is the organ that contains the largest pools of spermidine and spermine in the mammalian body (Rosenthal and Tabor, 1956). Therefore, it was decided to undertake a detailed study of the pancreas from the MT-SSAT transgenic mice. Studies on how the same transgene construct could affect the development of pancreatitis on transgenic rats have been previously published (Alhonen et al., 2000; Hyvönen et al., 2007a) (*see 2.6.4*). However, due to the recent discovery that activated polyamine catabolism could protect against the development of insulin resistance (Niiranen et al., 2006; Pirinen et al., 2007; Cerrada-Gimenez et al., 2009), it was also important to elucidate at how the increased polyamine catabolism could affect insulin secretion.

In the intact pancreas and in pancreatic islets, the massive pancreatic SSAT activation dramatically altered the polyamine pools. There was extensive accumulation of putrescine, and acetylated spermidine, but the spermidine and spermine pools decreased (Table 2). Morphologically the transgenic pancreas did not differ from its non-transgenic counterpart. However, the detailed analysis of the pancreatic islet composition did reveal a number of differences. The transgenic animals had a 1.6-fold enlarged beta-cell area (non-transgenic: 0.0019 ± 0.0003; MT-SSAT: 0.0031 ± 0.0004; $p = 0.032$). Although the beta-cell area was enlarged, no visible changes were seen in the alpha-cell area. Therefore, the ratio of beta to alpha cells appeared 4-fold increased in the MT-SSAT transgenic

mice. Moreover, the enlarged beta-cell area did not lead to greatly increased pancreatic insulin levels. On the contrary, the MT-SSAT transgenic mice had lower amounts of total pancreatic insulin (non-transgenic: 5.91 ± 0.21 pg insulin/µg protein; MT-SSAT: 5.11 ± 0.37 pg insulin/µg protein; $P = 0.11$). This was further confirmed when the gene expression of the two murine insulin genes was analyzed. Expression of both Ins1 and Ins2 was reduced by 50 % in the transgenic pancreatic islets.

Table 2. Polyamine pools in the pancreas and pancreatic islets from non-transgenic and MT-SSAT transgenic mice.

	Genotype	Putrescine	Spermidine	N^1-Ac spermidine	Spermine
		(pmol/µg DNA)			
Pancreas	Non-transgenic	ND	3932 ± 49	ND	866 ± 36
	MT-SSAT	5200 ± 1169***	1166 ± 104***	127 ± 103***	276 ± 50***
Islets	Non-transgenic	39 ± 20	133.8 ± 44.7	ND	20 ± 4
	MT-SSAT	318 ± 85**	40 ± 8.7*	106 ± 67***	12 ± 1*

*Polyamines are expressed as means ± SD of three animals per genotype. Statistical significance vs. non-transgenic, * p < 0.05, ** p < 0.01 and *** p < 0.001.*

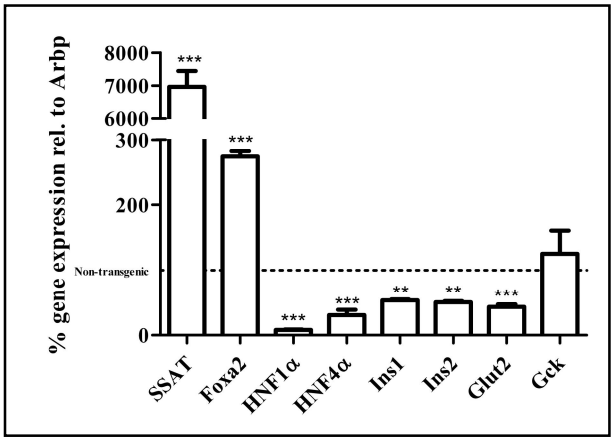

*Figure 9. Gene expression analysis from the pancreatic islets of the MT-SSAT transgenic mice. The acidic ribosomal phosphoprotein P0 was used to normalize the expression data. Data are expressed as mean ± SEM of three different pools of two to three mice per pool. Data are transformed to the percentage of expression relative to the non-transgenic expression (100 %). Values were statistically analyzed using Student's t-test. * p < 0.05, ** p <0.01 and *** p < 0.001.*

The expression level of Foxa2, which is one of the main transcription factors in pancreatic islets, was upregulated in the MT-SSAT transgenic mice (2.7-fold). Foxa2 controls the expression of genes involved in glucose-stimulated insulin secretion. The first step in glucose metabolism in pancreatic islets is the glucose uptake, which is mediated through the Glut2 transporter. The Glut2 gene expression was repressed by 67 % in the MT-SSAT transgenic mice. Inside beta-cells, glucose enters the glycolytic pathway after its phosphorylation into glucose-6-phosphate by Gck. Although Gck is believed to be regulated by Foxa2, no changes were seen in the transcription level between the two genotypes. Foxa2 negatively regulates the transcription factors HNF1α and HNF4α, and in the transgenic line, the expression of both HNF1α and HNF4α was greatly repressed (*Figure* 9). Insulin gene expression is positively regulated by the transcription factor HNF1α, which appeared greatly downregulated in the MT-SSAT mice. Therefore, the cascade initiated by the upregulation of Foxa2 ended up regulating the expression of Ins1 and Ins2 through the HNF1α repression. This cascade is shown in *Figure* 10.

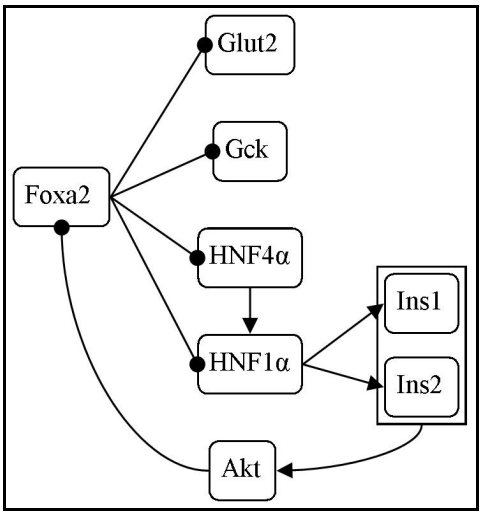

Figure 10. Schematic representation of the regulatory cascade of Foxa2 in the MT-SSAT transgenic mice. Foxa2 negatively regulates (—●) the expression of Glut2 and Gck, involved in the glucose stimulated insulin secretion; HNF1α and HNF4α activate (—▶)the transcription of the two insulin genes (Ins1 and Ins2). Insulin then inhibits Foxa2 through AKT phosphorylation.

The morphological and gene expression changes observed in the transgenic pancreas were the impetus to analyze the pancreatic endocrine function. The insulin secretory patterns were assayed by performing an *in vivo* insulin release test (*Figure 11*). The glucose bolus had similar effects on both

phenotypes, with the plasma glucose increasing steadily until 15 minutes and decreasing afterwards (*Figure 11*). However, the insulin secretory patterns appeared to be totally disrupted in the MT-SSAT transgenic mice. The expected first peak of insulin secretion, which usually appears at two minutes, was clearly seen in the non-transgenic animals but was clearly delayed in the MT-SSAT transgenic mice (*Figure* 11). The insulin secretion peak in the MT-SSAT mice was achieved at five minutes, and decreased thereafter. In contrast, in the non-transgenic mice insulin secretion remained stable, slightly increasing until the end of the experiment. A combination of the results in the insulin and glucose graphs revealed that the MT-SSAT mice were able to reduce the circulating glucose levels similarly as the non-transgenic mice even though they displayed a decreased and very disturbed insulin secretion.

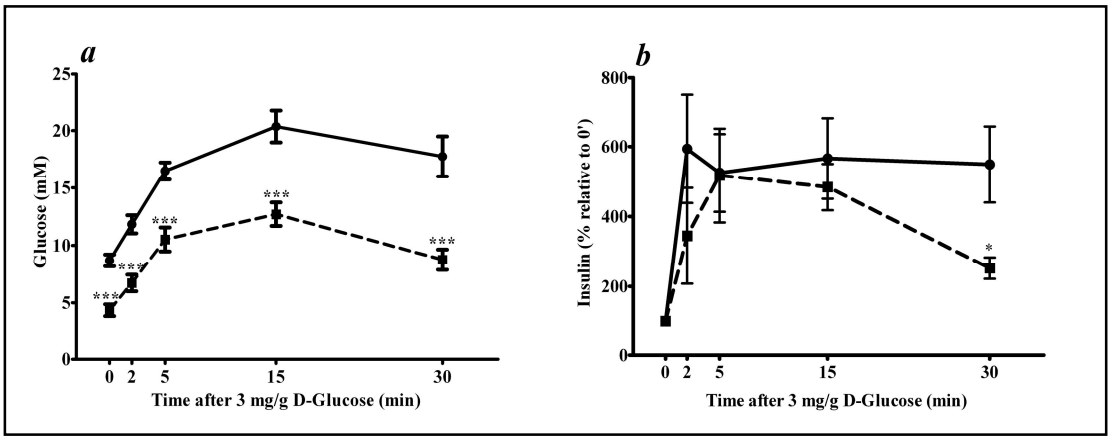

*Figure 11. In vivo insulin release test performed on four months old male non-transgenic and MT-SSAT transgenic mice. Data are expressed as mean ± SEM of ten mice per genotype. The continuous line represents the non-transgenic mice while the broken line represents the MT-SSAT mice. Values were statistically analyzed using Student's t-test. *P < 0.05 and ***P < 0.001.*

6 Discussion

Polyamines and their metabolic pathways have been known for a long time. However, the complex relationship between polyamines and many basic cellular functions has hampered the complete clarification of both the functions of polyamines and how their metabolism is regulated. Polyamines have a wide variety of actions, e.g. interactions with DNA, RNA and proteins (Jänne et al., 2004). Polyamine homeostasis is tightly controlled to maintain precise intracellular levels. Alterations in polyamine metabolism and in their intracellular levels have been linked with many different disorders, such as cancer (Gerner and Meyskens, 2004), pancreatitis (Alhonen et al., 2000) and diabetes (Pirinen et al., 2007). The design, production and phenotyping of transgenic mouse lines are a valuable asset for the polyamine researcher since it helps to overcome these natural limitations. However, gaining new insights was limited by the state of the art techniques. Therefore, novel techniques for the synthesis of polyamine analogues and for studying the polyamine flux needed to be developed.

I

Polyamine analogues have changed from being a group of therapeutic agents that structurally resemble the basic polyamines into a group of compounds that at the same time can be exploited for basic polyamine research (Casero and Woster 2009). The α-methylated compounds represent a subclass of polyamine analogues that contain a methyl group at the α-position of spermidine or spermine chains. This subtype of polyamine analogues were first synthesized during the mid 1980's (Nagarajan and Ganem, 1986). The original synthetic methods gave poor yields, making it difficult to obtain enough material to perform *in vivo* studies (Lakanen et al., 1992). Therefore, improved synthetic methods were needed. The new methods presented here allowed production of α-methylspermidine, α-methylspermine and bis-α-methylspermidine on the gram scale. Additionally, the purity of the compounds obtained was good (99%). Therefore, taking advantage of these new synthetic methods, it was possible to design and test the different α-methylated polyamines *in vivo* and *in vitro*. The *in vivo* assays were performed on MT-SSAT transgenic mice and transgenic rats, while the *in vitro* assays were performed on fibroblasts isolated from MT-SSAT rats.

The three different analogues accumulated in the animal tissues in a dose dependant manner, with α-methylspermine having the highest toxicity both *in vivo* and *in vitro*. Two known characteristics of α-methylated polyamine analogues are that they induce a depletion of the natural polyamine pools (Lakanen et al., 1992) and that they are metabolically more stable than their natural counterparts (Järvinen et al., 2005). The current results confirmed both of these previous observations. α-Methylspermidine was quite stable, while the two spermine analogues appeared to be slowly catabolized into α-methylspermidine. SMO was expected to be the responsible for the catabolism of bis-α-methylspermine into α-methylspermidine (Hyvönen et al., 2007b). *In vivo*, all three analogues produced a decrease in hepatic spermidine and spermine pools and a slight increase in the putrescine pool. However, SSAT was not always induced by the analogue treatment. Treatment with α-methylspermidine greatly induced the SSAT enzyme in both mouse lines and in the three studied organs, liver, pancreas and kidney. α-Methylspermine affected the SSAT activity in the MT-SSAT transgenic mice. Bis-α-methylspermine had barely any effect on SSAT. Nonetheless, the alterations seen in the natural polyamine pools argue in favor of SSAT being responsible for these changes. Moreover, the decreased levels of natural spermidine and spermine were replaced by the accumulation of the analogues, resulting in the fact that the total polyamine pools remained unaffected.

This study also served to show for the first time the specificity of the APAO enzyme for the R-isomer of acetylated-α-methylspermidine. Recently, Hyvönen et al (2009) noted that the stereospecificity could play an important role in the regulation of ODC, OAZ, AdoMetDC and SSAT expression by the analogues. Moreover, the different isomers of the α-methylspermidine analogues were also proven to differently replace the natural polyamines in their common functions, such as the formation of hypusine (Hyvönen et al., 2007b) and DNA condensation (Nayvelt et al., 2010).

II

The liver and the pancreas were the organs most affected by the MT-SSAT transgene. The acute activation of polyamine catabolism had profound effects on cellular metabolism. In order for the cells to survive and grow normally, they need adequate polyamine levels, thus the MT-SSAT transgenic mice show a compensatory activation of the polyamine biosynthesis (Suppola et al., 1999). The activation of polyamine biosynthesis was reflected in the decreased hepatic levels of AdoMet and ATP. The activated polyamine flux in the MT-SSAT

transgenic mice would consume AdoMet in the form of dcAdoMet for the spermidine and spermine synthesis, while acetyl-CoA would be used for their catabolism. ATP is used as an energy source, both in the production of AdoMet from methionine and the synthesis of cytosolic acetyl-CoA.

To perform an analysis of the hepatic protein expression we used a 2D-PAGE approach. However, the 2D-PAGE technique is not devoid of limitations. Analyzed proteins will be between 15kDa to 150-200KDa. Moreover, the analyzed proteins will be limited to those present on a detectable amount, and with an isoelectric point fitting the selected IPGs (Görg et al., 2009). Despite this limitation, the 2D-PAGE coupled with MS protein detection is still a valid proteomics technique. The 2D-PAGE analysis displayed relatively few differences between the non-transgenic and the MT-SSAT mice. Only 21 proteins were found to be differentially expressed between the two genotypes. Majority of the differentially expressed proteins were directly involved in polyamine and/or xenobiotic metabolism. However, it was quite unexpected that most of the identified xenobiotic enzymes, such as catalase, GSTpi and carbonic anhydrase III, were downregulated in the transgenic mice. It has previously been hypothesized that increased polyamine catabolism elevates oxidative stress (Ha et al., 1997). The elevated oxidative stress would be caused by the accumulation of hydrogen peroxide and 3-acetamidopropanal, the by-products from the SSAT and APAO-catalyzed reactions. The fact that most of the xenobiotic metabolizing enzymes were indeed repressed in the transgenic mice was further confirmed by assaying their activities (II and III) and by the results of a DNA microarray study (III). The DNA microarray results showed activation of A170 stress-induced protein and cytoskeletal keratin 18, both of them involved in the cellular response to oxidative stress (Ishii et al., 1996; Ku et al., 1996). Therefore, although it was expected that there would be upregulation of xenobiotic metabolizing enzymes in the transgenic mice, these enzymes were actually found downregulated (discussed in greater detail in III).

The activated polyamine flux seen in the MT-SSAT transgenic mice produced an increased energy demand. In accordance, the hepatic expression of the 60-kDa heat-shock protein was increased in the transgenic mice. This protein has been previously linked with an increased ATP recovery rate in rat cardiac myocytes after ischemic injury (Lin et al., 2001). Similarly, the 60-kDa heat-shock could be used in the transgenic animals to confer protection against lowered ATP levels due to activated polyamine flux.

The results of the proteomics study served to confirm that activation of polyamine flux is linked with elevated PGC 1α expression as previously detected by Pirinen et al. (2007; 2009). PGC 1α is a ubiquitous transcription factor that links the transcription machinery with its energy state (Puigserver et al., 2003). The expression of various identified proteins matched with the

patterns found upon activation of PGC 1α or its targets. Moreover, the activation of PPARα, which is a known PGC 1α target (Vega et al., 2000), could be the responsible for the lower GSTpi and selenium binding protein 2 seen in the transgenic mouse line as shown in two different model of PPARα activation (Chu et al., 2004; Giometti et al., 2000).

In conditions of low energy in the cells, PGC 1α activates the three key enzymes of gluconeogenesis, PEPCK, fructose 1,6-bisphosphatase and glucose-6-phosphatase (Yoon et al., 2001). In the transgenic mice, hepatic expression of PEPCK and glucose-6-phosphatase were clearly induced already in the fed state. The combination of all these results favorably argues for the activation of PGC 1α in response to the activated polyamine flux in the MT-SSAT transgenic mice. Activation of hepatic gluconeogenesis by PGC 1α has been linked with the development of T2D (Koo et al., 2004). However, it was recently demonstrated that a transgenic mouse line harboring the SSAT gene under its own promoter presented elevated PGC 1α expression in the white adipose tissue (Pirinen et al., 2007) and liver (Pirinen et al., 2009). This specific mouse line represents an opposite phenotype to T2D, with enhanced insulin sensitivity and glucose tolerance, similarly to the MT-SSAT transgenic mice. Moreover, SSAT knockout mice show a completely opposite phenotype, becoming insulin resistant upon ageing (Niiranen et al., 2006). Therefore, overexpression of the SSAT gene produces phenotypic changes that protect the organism against the development of T2D. Since the SSAT transgene was overexpressed ubiquitously, it would be expected that ATP depletion would occur similarly in the whole organism. Therefore, the general lower energy levels would activate PGC 1α in all organs producing the particular phenotype seen in the SSAT overexpressing mice.

III

The lifelong activation of polyamine catabolism, that the MT-SSAT transgenic animals endure, produced a high level of oxidative stress, as reflected in the 13-fold elevated protein carbonyl content. In order to counteract the oxidative stress, the transgenic mice would be anticipated to activate anti-oxidant mechanisms. p53 is a transcription factor involved in the protection of the organism against cellular damage. If there is oxidative stress then p53 becomes active, and triggers a cascade aimed at the repair or elimination of damaged cells (Matheu et al., 2008). The controlled activation of p53 helps the organism repair/remove damaged cells and may lead to prolong the lifespan. However, long-term activation of p53 causes to depletion of the stem cell pool and leads to

accelerated aging (Matheu et al., 2008). Interestingly, p53 was found to be greatly activated in the MT-SSAT line. Moreover, a long-term survival study performed on the MT-SSAT mouse line revealed that the transgenic mice had a 50% reduced lifespan. In fact, the MT-SSAT mouse line shared several similarities with another mouse model with activated p53 expression ($p53^{+/m}$) (Tyner et al., 2002). Both lines present reduced lifespan, reduced hair growth and decreased subcutaneous fat deposits. Moreover, both lines are also resistant to tumor development (Pietilä et al., 2001). Therefore, the chronic SSAT activation produced enhanced oxidative stress that lead to activation of the p53 transcription factor as a measure to minimize the stress in the transgenic mice concluding in an accelerated aging phenotype.

Not all effects of chronically activated p53 in the transgenic mice are beneficial, with the exception of protection from tumor development. The expression/activities of most of the antioxidant enzymes analyzed from the MT-SSAT transgenic mice were decreased, such as GST pi, catalase, aldehyde dehydrogenase, and CYP450 2E1. The decreased antioxidant capacity of the MT-SSAT transgenic mice was associated to their accelerated aging (Semsei et al., 1989; Brown-Borg and Rakoczy, 2000). Although decreased activity of the various antioxidant enzymes was likely to have harmful effects, it had an unexpected positive effect. The toxicity exhorted by many different hepatotoxins, such as thioacetamide and carbon tetrachloride, appears after their metabolization (Manibusan et al., 2007). Therefore, the low drug metabolizing capability of the MT-SSAT transgenic mice helped to protect the animals against these hepatotoxins.

IV

Previous methods to analyze polyamine flux were based on the use of unnatural labeled polyamine precursors (fluoro-ornithine) and the analysis of the incorporation of the fluoro label into the polyamines by HPLC (Kramer et al., 2008). This combination does not invariably produce optimal results. Firstly, due to the different biochemical properties of the natural and fluoro-labeled polyamine it is not known how fluoro-ornithine could affect polyamine metabolism. Secondly, the HPLC-based methodology is not able to separate properly the natural and fluoro-labeled polyamines. The combination of labeled natural precursors (^{13}C and ^{13}C, ^{15}N) with a newly developed LC/MS-MS method (Häkkinen et al., 2008) allowed overcoming these problems. On one hand, the labeled natural precursors ensured that polyamine metabolism was not affected by their addition. On the other hand, the LC/MS-MS methodology

allows for an efficient quantification of the labeled and natural polyamine pools. Moreover, this method is highly versatile, having the appropriate standards, the number of compounds to be analyzed can be easily increased.

The methodology was tested using three labeled polyamine precursors in three different cell lines, non-transgenic, MT-SSAT and SSATKO cells. Of the tested precursors, i.e. arginine, methionine and ornithine, arginine was incorporated at the lowest rates. The lower arginine incorporation was probably due to the dilution of the label with the unlabeled arginine present in the growth media. The use of a methionine-free cell culture medium provided the greater incorporation of the label derived from methionine.

Although the present methodology is an improvement over previous HPLC-based methods (Kramer et al., 2008), the study of polyamine flux still encounters some difficulties. The label incorporation starts with a phase of poor incorporation, followed by an exponential phase until the incorporation reaches a maximum or plateau phase. The analysis of the polyamine flux is only viable during the second phase, the exponential phase. Once the label accumulation reaches the maximum level (plateau phase), it is not possible to differentiate whether the label is being transferred from outside the cells or is being recirculated inside the polyamine metabolism. The time that each cell line needs to reach the plateau phase of label incorporation depends on the particular cell line and the precursors used. Therefore, extra care needs to be taken when selecting the different time points for obtaining data. This point was particularly obvious during the $^{13}C_5$, $^{15}N_1$-methionine treatment. At the 24 hours time point, the non-transgenic and SSATKO cells were accumulating label in an exponential manner, although the SSATKO cells were somewhat slower. However, the MT-SSAT cells had already reached the plateau phase at the 6 hours time point. The rate of label incorporation into the polyamine pools clearly showed that polyamine flux speed was altered according to the level of SSAT expression (MT-SSAT >> non-transgenic >> SSATKO). Moreover, the treatment of the different cell lines with the APAO inhibitor MDL 72,527 clearly induced export of acetylated polyamines in the non-transgenic and MT-SSAT cells. Therefore, similarly as with the polyamine flux speed, the export rate clearly correlated with the level of SSAT activation.

Unpublished results

All previously published research related with studies on the MT-SSAT transgenic mice and rats pancreas has focused on the exocrine aspect of it. When the same transgene construct was activated using a non-toxic dose of a heavy

metal in a MT-SSAT transgenic rat line, it greatly depleted the spermidine and spermine pools, and the appearance of acute necrotizing pancreatitis followed (Alhonen et al., 2000). The pancreatic structure suffered from polyamine depletion, with the presence of autophagosomes and disruption of intracellular membranes and organelles (Hyvönen et al., 2007a). Moreover, pre-treatment with polyamine analogues, such as α-methylspermidine or bis-α-methylspermine, was able to prevent the onset of the symptoms (Räsänen et al., 2002; Merentie et al., 2007). Therefore, considering that the pancreas is the organ containing the largest spermidine pools (Rosenthal and Tabor, 1956) and that the basal pancreatic SSAT activity was greatly activated in the MT-SSAT line it was not surprising that the endocrine pancreas was also affected by the transgene.

The MT-SSAT transgenic mice exhibited no signs of pancreatic dysfunction other than the enlarged beta-cell area found. The alpha-cell area, as well as the number of beta and alpha cells remained unchanged. Thus, the enlargement of the beta-cell area was due to hypertrophy. Shantz et al (2001) showed that the cardiac hypertrophy was caused by activating ODC. Moreover, there are other reports about the effects of polyamines on hypertrophy in different organs, such as kidneys and heart (reviewed in Manteuffel-Cymborowska 1993). Although the beta-cell area was enlarged in the MT-SSAT transgenic mice, the total insulin level in the pancreas was decreased in the transgenic mice. Thus, each transgenic beta-cell produced less insulin than their non-transgenic counterparts did. It is not clear whether this is due to a reduced insulin requirement in the insulin-sensitive transgenic mice or is attributable to direct regulation of insulin production by polyamines themselves. In fact, polyamines tend to accumulate upon glucose stimuli. Hougaard et al (1986) and Welsh (1990) observed a correlation between increased spermine levels and elevated insulin transcription. In the MT-SSAT transgenic mice, the 70% reduced islet spermine pool was accompanied by a slight reduction of insulin mRNA and protein levels.

Polyamines have been localized in the cytoplasmic secretory granules within the beta-cells (Hougaard et al., 1986). This suggests a function for polyamines in the insulin secretory mechanism. Polyamines may be substrates for the Ca^{2+} responsive transglutaminase. Transglutaminases have been implicated in the receptor-mediated endocytosis (Davies et al., 1980; Levitzki et al., 1980) and directly linked with glucose-mediated insulin secretion (Gomis et al., 1983; Lindsay et al., 1990). Therefore, the co-localization of polyamines, insulin and transglutaminase in beta-cells suggest a common function for all of them (Bungay et al., 1986).

Insulin secretion is biphasic, with the first peak of secretion taking place in less than 5 minutes post-glucose load, followed by the second secretory phase

that lasts for 20-30 minutes. There is a small group of granules that are part of the immediately releasable pool that is responsible for the fist secretory phase. The first phase peak is a K-ATP channel-dependent process, while the second phase is regarded as being K-ATP channel-independent (Straub and Sharp, 2002). The insulin release test showed that while the non-transgenic mice followed the expected biphasic pattern, the MT-SSAT transgenic mice showed a single insulin secretion peak that reached its maximum at 5 minutes post-glucose load and decreased steadily thereafter. However, although the MT-SSAT transgenic mice presented a disturbed insulin secretion, their plasma glucose was cleared from circulation much faster than in the non-transgenic mice. Whether the altered insulin secretion observed in the MT-SSAT transgenic mice was due to their lower insulin levels/production, or the direct effect of polyamines on the glucose-stimulated insulin secretion, or the combination of the two is not known. Nonetheless, despite the badly disturbed insulin secretion, the transgenic mice remained insulin-sensitive, clearing the glucose bolus as fast as the non-transgenic mice.

As in the other of tissues where polyamine flux is activated, the pancreatic islets from the MT-SSAT transgenic mice showed decreased ATP levels. During low energy states, or fasting, the transcription factor Foxa2 is activated and this controls hepatic gluconeogenesis (Zhang et al., 2005), fatty acid oxidation and ketone body production (Wolfrum et al., 2004). In the pancreas, Foxa2 controls the differentiation of primordial stem cells into alpha and beta-cells (Lee et al., 2005). When phosphorilation of Foxa2 by the insulin-activated Akt occurs, Foxa2 is excluded from the nucleus and cannot regulate its targets (Wolfrum et al., 2003). Moreover, Pirinen et al (2009) showed that hepatic activation of polyamine flux repressed Akt and activated bile acid synthesis, which has been shown to be regulated by Foxa2 (Bochkis et al., 2008). Thus, a similar mechanism of Foxa2 activation in the MT-SSAT transgenic islets could occur, where Foxa2 appeared to be upregulated. In confirmation, Foxa2 targets, such as Glut2 (Lee et al., 2002), HNF1α, HNF4α, and Ins1 and 2 (Wang et al., 2002), appeared to be repressed at the mRNA level in the MT-SSAT transgenic islets. Moreover, Foxa2 is also involved in the glucose-dependent insulin secretion (Friedman and Kaestner, 2006; Kitamura and Kitamura, 2007). Therefore, the activated polyamine flux would induce the insulin-sensitivity seen in the MT-SSAT transgenic mice through the constantly decreased energy levels. The MT-SSAT insulin-sensitive mice had lower circulating insulin levels, and thus, were not able to repress Foza2 via Akt-dependent phosphorylation. Thus, the activated Foxa2 would repress insulin production and altering its glucose-dependent secretion.

7 Summary and conclusions

The partial understanding of the polyamine metabolism and polyamine functions drove the production of different transgenic rodent lines in order to complete our understanding. The characterization of the MT-SSAT transgenic mouse line, which represents activated polyamine catabolism, produced novel information about the regulation of polyamine metabolism. This set of studies on the polyamine biology in transgenic mouse models were performed using novel techniques, such as improved polyamine analogue synthesis protocols, proteomics and LC/MS-MS. This allowed expanding our initial knowledge in different aspects of polyamine metabolism:

I.1 A novel methodology for producing large (gram) quantities of the α-methylated polyamine analogues was developed. The synthesis method allowed for the first time the *in vivo* testing of the α-methylated polyamine analogues.

I.2 The α-methylated polyamine analogues were able at the same time to activate the polyamine flux and substitute for the natural polyamine pools *in vivo*. The analogues were non-toxic and metabolically more stable than natural polyamines. Even thought, the two spermine analogues, α-methylspermine and bis-α-methylspermine, were catabolized into α-methylspermidine.

II The hepatic protein pattern in the MT-SSAT transgenic mice displayed the signs of activated polyamine catabolism. Moreover, the changes in expression of some proteins indicated hepatic activation of the transcription factor PGC 1α. However, although PGC 1α was activated in the liver, the MT-SSAT transgenic mice showed improved insulin sensitivity and glucose tolerance.

III.1 Acute activation of the polyamine flux increased the oxidative stress. Accordingly, the transcription factor p53 became activated, resulting in accelerated aging in the transgenic mice.

III.2 Aging was accompanied with a reduction in xenobiotic metabolizing enzymes activities, including catalase, aldehyde dehydrogenase, and CYP450 2E1. The lower activities of these xenobiotic metabolizing enzymes were associated with protecting the transgenic mice from hepatotoxic damage such as treatment with thioacetamide or carbon tetrachloride.

IV.1 The use of labeled natural polyamine precursors combined with the LC/MS-MS method represented a major improvement over previous methods for studying polyamine flux.

IV.2 Polyamine flux was directly correlated with the levels of SSAT activation. Moreover, efficient polyamine excretion needed the presence of the SSAT enzyme.

V The MT-SSAT transgenic pancreas presented an enlarged beta-cell area, and the transgenic animals showed an altered insulin secretion pattern. Activation of the polyamine flux decreased the available ATP, the lower energy content activated the transcription factor Foxa2. Activated Foxa2 in turn controlled many different genes involved in the insulin production and the glucose-stimulated insulin secretion pathways.

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Development and Use of Tools to Study Metabolic Consequences of Altered Polyamine Catabolism

Although polyamines were originally discovered during the XVIIth century, their importance for the organism is still not completely understood. The use of molecules that resemble polyamines in their structure and function, i.e. polyamine analogues, and transgenic mice are the most used tools for the study of polyamines and their metabolism. The original techniques used for the analysis of polyamine functions and metabolism present limitations. Therefore, the development of new techniques to be used in the polyamine metabolism research could provide the necessary tools to advance our knowledge in this field.

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