POLYAMINE HOMEOSTASIS

Cellular responses to perturbation of
polyamine biosynthetic enzymes

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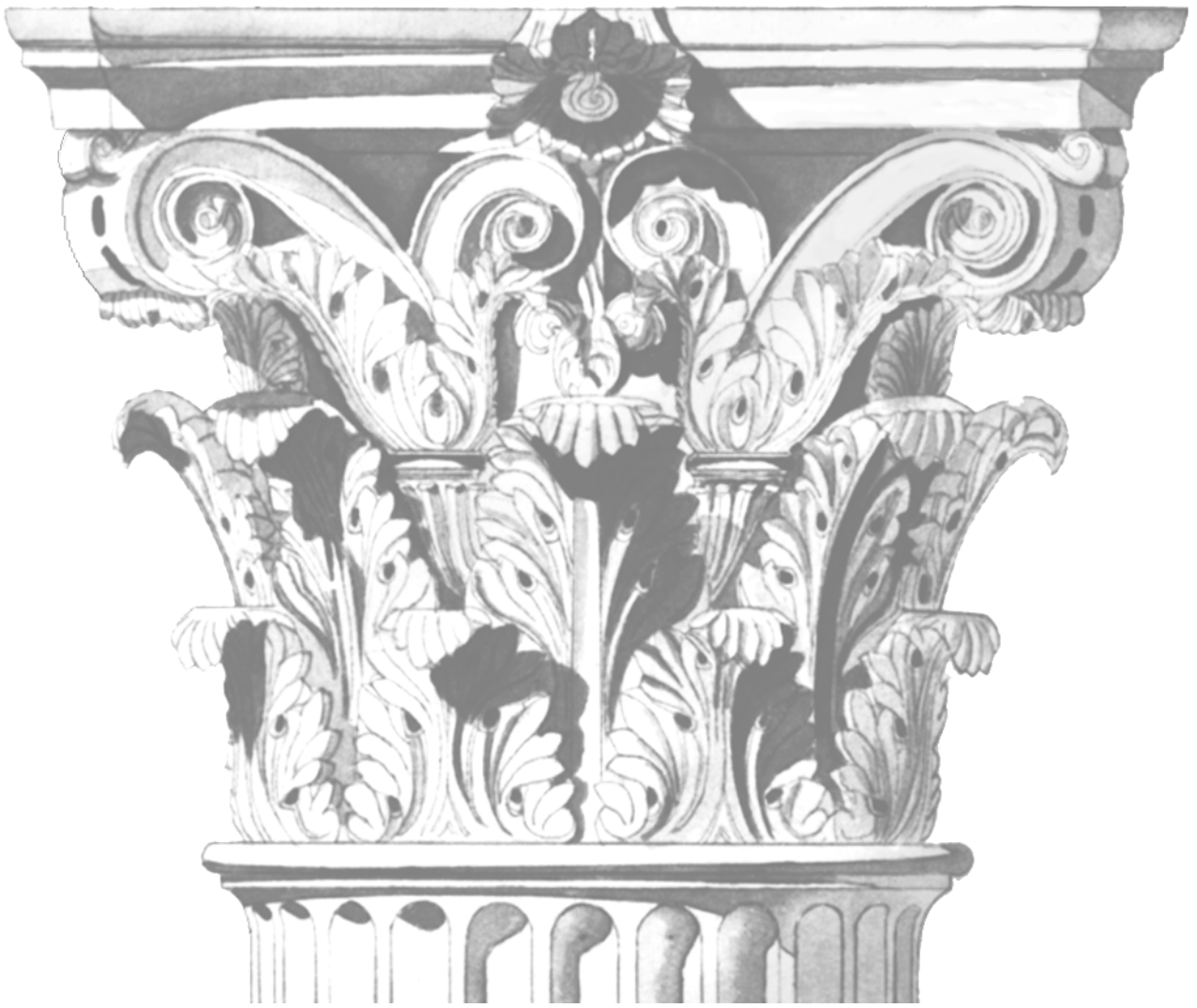

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Abstract

The polyamines putrescine, spermidine and spermine are highly regulated polycations present in virtually all cells of higher eukaryotes. They are essential for proper cell growth and differentiation by participating in various physiological processes including DNA, RNA and protein synthesis, apoptosis and interactions with ion-channels. The complexity of polyamine metabolism and the multitude of compensatory mechanisms that are invoked to maintain polyamine homeostasis argue that these molecules are critical for cell survival.

The primary aim of this study was to gain a better understanding of the mode of action of polyamines and the regulatory mechanisms in which they are involved. Transgenic mice overexpressing the polyamine biosynthetic enzymes S-AdoMetDC and ODC were found to maintain their polyamine pools by acetylation of spermidine and spermine and an increased export of these acetylated compounds. The expression of various genes was studied as a response to polyamine deprivation in cell- and kidney organ culture. Among these genes *acetyl-CoA synthetase* and *ornithine decarboxylase* were demonstrated to be developmentally regulated. Changes in gene expression patterns, with most of the transcripts upregulated in the polyamine-depleted samples, indicated selective stabilization of mRNAs. Polyamines were shown to play an important role in kidney organogenesis as their depletion results in a reduction of ureteric branching and retardation of tubule formation. The selective changes of various genes in the ureteric bud and mesenchyme indicate that polyamines might have a role in the regulation of epithelial-mesenchymal interactions during mouse kidney development.

Keywords: epithelial-mesenchymal interactions, polyamine homeostasis, polyamines

"Ebben a dalban van négy akkord
Ebben a dalban van két gitár
Ebben a dalban van egypár barát
És egy kicsit benne vagyok én"

(Sztevanovity Dusán, Presser Gábor: Egészen egyszerű dal)

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Végül, de nem utolsó sorban szüleimet köszöntöm. Köszönöm nektek, hogy felneveltetek, majd engedtétek, hogy a saját utamat járjam akkor is, ha épp nem arra mentem, amerre ti láttátok jónak. Remélem, hogy ez a kis könyv nektek is örömet szerez.

Oulu, Finland, February 2005

Abbreviations

ABC	ATP binding cassette
AceCS	AMP-forming acetyl-CoA synthetase
ANT	adenine nucleotide translocator
BCR	B cell antigen receptor
BrdU	5-bromo-2'-deoxyuridine
cAMP	cyclic adenosine monophosphate
Cdk	cyclin-dependent kinase
CHENSpm	N^1-cycloheptylmethyl-N^{11}-ethylnorspermine
CHX	cycloheximide
CK2	casein kinase II
CRP	cAMP receptor protein
CSRE	carbon source-responsive element
DAB	3,3'-diaminobenzidine
DENSPM	N^1,N^{11}-diethylnorspermine
DFMO	α-difluoromethylornithine
eIF	eukaryotic initiation factor
ERK	extracellular signal-regulated kinase
EST	expressed sequence tag
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gy	gyro
HSF	heat-shock factor
IAP	inhibitors of apoptosis
IPENSpm	(S)-N^1-(2-methyl-1-butyl)-N^{11}-ethyl-4,8-diazaundecane
IRES	internal ribosome entry site
JNK	c-Jun kinase
Kir channels	inward rectifier K^+ channels
MAT	methionine adenosyltransferase
MGBG	methylglyoxal bis(guanylhydrazone)
NF	nuclear factor
NOHA	N^{ω}-hydroxy-L-arginine
ODC	ornithine decarboxylase
OppA	oligopeptide-binding protein

ORF	open reading frame
Orn	ornithine
PAO	polyamine oxidase
PHEX	phosphate regulating gene
PMF-1	polyamine-modulated factor-1
PMT	plasma membrane transporter
pRb	retinoblastoma protein
PRE	polyamine-responsive element
Put	putrescine
Sir2	silent information regulator protein
SMO	spermine oxidase
Spd	spermidine
ROS	reactive oxygen species
S-AdoMet	S-adenosylmethionine
S-AdoMetDC	S-adenosylmethionine decarboxylase
SREBPs	sterol regulatory element-binding proteins
SSAT	spermidine/spermine N^1-acetyltransferase
TNF-α	tumor necrosis factor α
TRAMP	transgenic adenocarcinoma of mouse prostate
UTR	untranslated region
VA	vesicular polyamine-H^+ antiporter
V-ATPase	vacuolar-ATPase
WT1	Wilms' tumor suppressor
$\Delta\Psi_m$	mitochondrial inner transmembrane potential

List of original articles

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

I Heljasvaara R, Veress I, Halmekytö M, Alhonen L, Jänne J, Laajala P & Pajunen A (1997) Transgenic mice overexpressing ornithine and S-adenosyl-methionine decarboxylases maintain a physiological polyamine homeostasis in their tissues. Biochem J 323 (2), 457-62.*

II Veress I, Haghighi S, Pulkka A & Pajunen A (2000) Changes in gene expression in response to polyamine depletion indicates selective stabilization of mRNAs. Biochem J 346, 185–191.*

III Loikkanen I, Haghighi S, Vainio S & Pajunen A (2002) Expression of cytosolic acetyl-CoA synthetase gene is developmentally regulated. Mech Dev 115:1-2 139-41. **

IV Loikkanen I, Lin Y, Railo A, Pajunen A & Vainio S (2005) Polyamines are involved in murine kidney development controlling expression of *c-ret*, *Pax2/8* and *E-cadherin* genes. *Submitted.*

Contents

1 Introduction

The polyamines putrescine spermidine and spermine are puzzling molecules. They are involved in various physiological processes and are crucial to the growth and proliferation of mammalian cells. Yet, despite extensive research during the last forty years, their exact function in these processes is still elusive. High polyamine concentrations are required for rapid cell growth and low polyamine content is typical for quiescent cells, suggesting that drugs that inhibit the synthesis of polyamines could prevent cancer and may be used for therapeutic purposes. However, the development of polyamine synthesis inhibitors has had disappointing results due to the unexpected compensatory mechanisms such as an increase in polyamine uptake from the circulation. Cells have a complex regulatory system, involving biosynthesis, catabolism and transport, to ensure tight control of intracellular polyamine pools to avoid cytotoxicity caused by high spermidine and spermine concentrations. A general approach to the study of the mechanisms in which polyamines are involved is to inhibit polyamine biosynthetic enzymes using specific inhibitors or overexpress these enzymes by generating genetically engineered animals.

The present study focused on cellular responses following perturbations in the function of polyamine biosynthetic enzymes using various models. Mechanisms by which transgenic mice overexpressing the polyamine biosynthetic enzymes S-AdoMetDC and ODC maintain their polyamine pools were clarified. Depletion of polyamines was shown to alter the expression of a panel of genes in cell- and also kidney organ culture, of which *acetyl-CoA synthetase* and *ornithine decarboxylase* were demonstrated to be developmentally regulated. Changes in the gene expression patterns indicated selective stabilization of mRNAs. Polyamines were shown to have an important role in kidney organogenesis being involved in inductive epithelial-mesenchymal tissue interactions.

2 Review of the literature

2.1 Polyamines

The history of polyamines is long; the first observation dating back to 1678, when Leeuwenhoek observed the crystallization of spermine phosphate in human semen (Cohen 1998). More than 300 years have passed, but still no one can definitively describe the function of spermine or the other natural polyamines. The natural polyamines (shown in figure 1) include, in addition to the tetraamine spermine, the triamine spermidine and its precursor, the diamine putrescine.

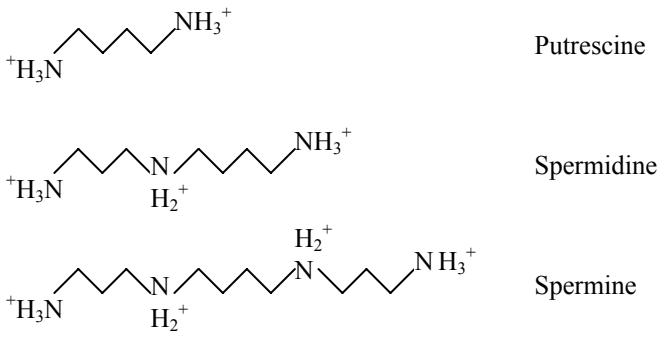


Fig. 1. Natural polyamines

Spermidine and spermine are simple aliphatic amines containing two and three flexible carbon chains, respectively, separated by secondary amines with primary amines at either end (Usherwood 2000). Although putrescine is a primary diamine it is customary to treat it as a polyamine (Raina & Jänne 1975).

The amino groups are fully protonated at physiological pH, which allows polyamines to participate in many cellular processes through binding to RNA, DNA, nucleotide triphosphates and other acidic substances (Igarashi & Kashiwagi 2000).

2.1.1 Polyamine metabolism in mammals

Polyamines are involved in the regulation of cell growth. Their concentrations are high in actively proliferating cells, during periods of tissue growth and in mature tissues with highly active protein synthesis. Intracellular polyamine levels are strictly regulated by multiple metabolic processes including biosynthesis, catabolism and transport (reviewed in Pegg & McCann 1982, Wallace *et al.* 2003, Jänne *et al.* 2004, Seiler 2004) (Figure 2).

2.1.1.1 Biosynthesis

In eukaryotic cells polyamines are synthesized from two amino acids, L-arginine and L-methionine in a series of enzymatic reactions. L-ornithine is cleaved from L-arginine by arginase II (EC 3.5.3.1) or taken up in the diet. L-ornithine is decarboxylated by ornithine decarboxylase (ODC; EC 4.1.1.17) to form the diamine putrescine. Methionine adenosyltransferase (MAT; EC 2.5.1.6) converts L-methionine to S-adenosylmethionine (S-AdoMet), which is further decarboxylated by S-adenosylmethionine decarboxylase (S-AdoMetDC; EC 4.1.1.50). The resulting decarboxylated S-AdoMet serves as the aminopropyl donor for spermidine and spermine synthesis. The aminopropyl moiety of S-AdoMet is transferred to putrescine by spermidine synthase (EC 2.5.1.16) and to spermidine by spermine synthase (EC 2.5.1.22) to produce spermidine and spermine respectively. ODC and S-AdoMetDC are considered the rate-limiting enzymes in polyamine biosynthesis. Both have a fast turnover rate with a half-life less than 1 hour (Berntsson *et al.* 1999) and their translation is negatively regulated by polyamines (Kahana & Nathans 1985, Shantz *et al.* 1992, Shantz & Pegg 1999).

ODC is a homodimer with two active sites located at the interface between the subunits (Tobias & Kahana 1993, Coleman *et al.* 1994). Its regulation occurs at the levels of transcription, translation and degradation. *ODC* is a delayed early gene whose transcriptional activation requires ongoing protein synthesis in the stimulated cells (Tobias *et al.* 1995). Its promoter contains response elements for several *trans*-acting factors, including the Wilms' tumor suppressor WT1 (Moshier *et al.* 1996), several Sp1 sites (Kumar *et al.* 1995) and a cAMP response element (Palvimo *et al.* 1991, Abrahamsen *et al.* 1992). ODC is a transcriptional target of the proto-oncogene c-Myc through c-Myc/Max heterodimers, in association with induction of cell cycle progression, cell proliferation and transformation (Bello-Fernandez *et al.* 1993, Pena *et al.* 1993, Auvinen *et al.* 2003).

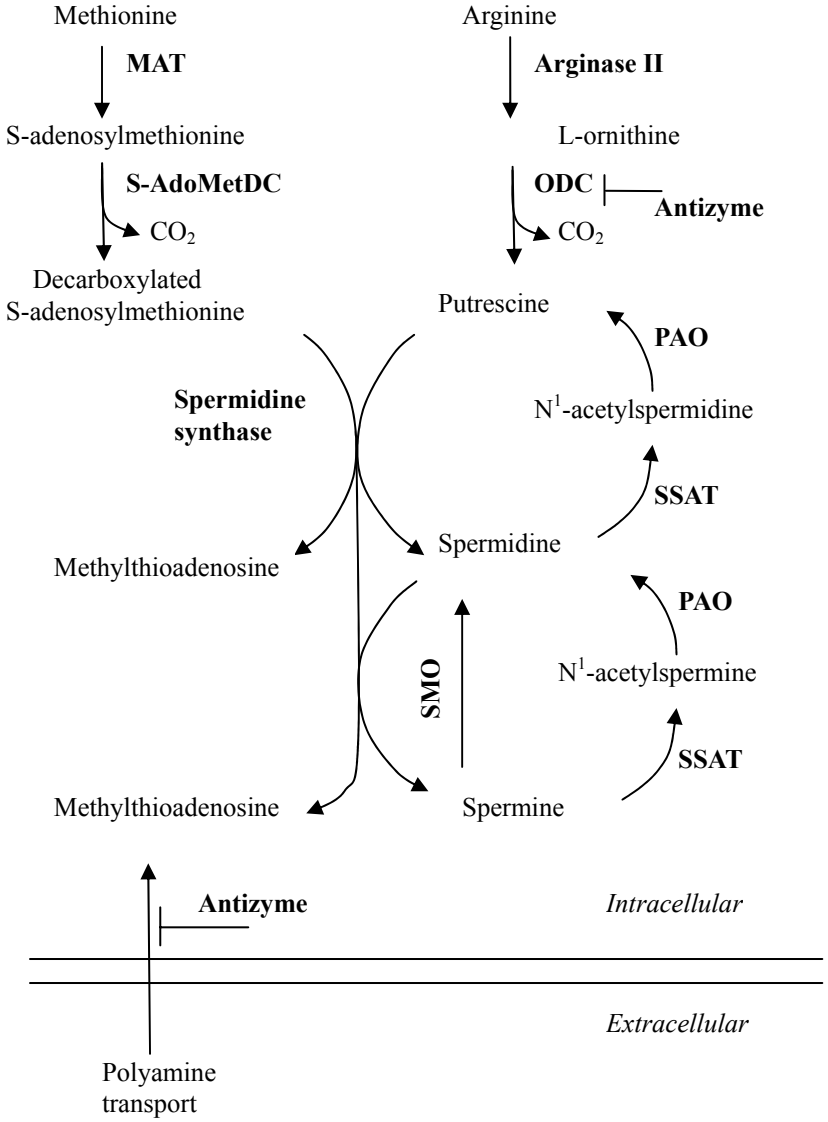


Fig. 2. Pathways of polyamine metabolism. Enzymes are marked with bold font type. Abbreviations: MAT, methionine adenosyltransferase; S-AdoMetDC, S-adenosylmethionine decarboxylase; ODC, ornithine decarboxylase; PAO, polyamine oxidase; SSAT, spermidine/spermine N^1-acetyltransferase; SMO, spermine oxidase

The translation of ODC mRNA is regulated by its 5'-untranslated region (UTR). This GC-rich, long 5'UTR has the potential for stable hairpin formation that has inhibitory effect on ODC mRNA translation (Manzella & Blackshear 1990). Interaction of the

5'UTR with the 3'UTR reverses this inhibition and enhances translation (Grens & Scheffler 1990, Lorenzini & Scheffler 1997). Within the 5'UTR of the ODC mRNA there is a small internal open reading frame (ORF). The sequence and length of the predicted protein differs between species and its role in translational regulation has not yet been established (Shantz & Pegg 1999). ODC mRNA can be translated by both cap-dependent and independent mechanisms (Hayashi *et al.* 2000, Pyronnet *et al.* 2000). The eukaryotic initiation factor (eIF) 4E has been reported to enhance the cap-dependent translation at the level of translation initiation (Rousseau *et al.* 1996, Shantz *et al.* 1996), whereas eIF-4G stimulates both cap-dependent and independent protein synthesis (Hayashi *et al.* 2000). Low levels of spermidine (0.2 mM) stimulate, whereas high levels (0.6-1mM) inhibit ODC translation via the 5'UTR at the level of initiation complex formation (Ito *et al.* 1990, Shimogori *et al.* 1996).

ODC is degraded by the 26S proteasome in an unusual ATP-dependent, but ubiquitin-independent, manner (Bercovich *et al.* 1989, Murakami *et al.* 1992). The degradation is accelerated by antizyme, the enzyme inhibitor of ODC. Antizyme binds to the ODC monomer (Mitchell & Chen 1990) and directs the proteasome to degrade the enzyme. Antizyme expression is regulated by a special +1 translational frameshift that is induced by polyamines in a concentration-dependent manner (Rom & Kahana 1994, Matsufuji *et al.* 1995). (For details see 2.1.3.4.) In addition to its effects on ODC, antizyme also negatively regulates polyamine transport into cells (Mitchell *et al.* 1994, Suzuki *et al.* 1994, Zhu *et al.* 1999). These processes together form a complete feedback loop: when polyamine levels rise, they induce antizyme production. Antizyme inhibits and degrades ODC which causes a decline in polyamine biosynthesis.

S-AdoMetDC is a pyruvoyl-dependent enzyme, synthesized as an inactive proenzyme that undergoes autocatalytic cleavage into two subunits. The mature enzyme consists of a dimer of these subunits (Pajunen *et al.* 1988). The autocatalytic cleavage reaction also forms the pyruvoyl cofactor from an internal serine residue at the amino terminus of the α subunit (Stanley *et al.* 1989, Tolbert *et al.* 2003). Regulation of the mammalian enzyme occurs at multiple levels; the cleavage reaction and the catalytic activity of the mature enzyme are both stimulated by putrescine (Kameji & Pegg 1987a, Stanley & Pegg 1991, Stanley *et al.* 1994), whereas the synthesis of the proenzyme is repressed by spermidine and spermine (Kameji & Pegg 1987b). The promoter region of the mammalian *S-AdoMetDC* gene contains DNA elements for the binding of transcription factors Sp1, AP-1, AP-2, CREB and multiple steroid receptors (Maric *et al.* 1992, Pulkka *et al.* 1993, Nishimura *et al.* 1998).

Like ODC, S-AdoMetDC mRNA also has a long 5'UTR with a short internal ORF (Shantz & Pegg 1999). The ORF in the S-AdoMetDC 5'UTR is a mediator of the polyamine-dependent regulation of the enzyme synthesis (Nishimura *et al.* 1999, Raney *et al.* 2000). This well conserved ORF, which encodes the peptide sequence MAGDIS (Shantz & Pegg 1999), suppresses the translation of the downstream cistron by stalling ribosomes close to the terminal codon of the ORF and thus limiting the number of scanning ribosomes that can reach the downstream start site. The ribosome stalling is affected by polyamine levels: high levels causing more effective stalling, and by the peptide sequence encoded by the upstream ORF (Hill & Morris 1993, Law *et al.* 2001, Raney *et al.* 2002).

The degradation of S-AdoMetDC is an important control mechanism maintaining polyamine levels. S-AdoMetDC activity can be lost by substrate-mediated transamination when an amine group from the substrate S-AdoMet is transferred to the pyruvoyl cofactor of the enzyme, converting it to alanine (Anton & Kutny 1987, Xiong *et al.* 1999). It was recently shown that S-AdoMetDC is degraded by the 26S proteasome in an ubiquitin-dependent manner and the degradation is accelerated by substrate-mediated transamination (Yerlikaya & Stanley 2004).

Spermidine and spermine synthases, stable enzymes and present in excess have not received much attention in polyamine research. Their activities are regulated mainly by the amount of enzyme protein and the availability of their substrate, decarboxylated S-AdoMet (Pegg 1986). Nevertheless the first human polyamine deficiency syndrome was recently characterized as a defect in the X-linked spermine synthase gene. The defect results from a splice mutation, and is associated with the Synder-Robinson syndrome, an X-linked mental retardation disorder (Cason *et al.* 2003).

2.1.1.2 Catabolism

Although the reactions catalyzed by spermidine and spermine synthase are practically irreversible, spermidine and spermine can be reconverted to putrescine *in vivo*. Cytosolic spermidine/spermine N^1-acetyltransferase (SSAT; EC 2.3.1.57) is the first enzyme in the interconversion process, using acetyl-CoA to produce N^1-acetylspermidine and N^1-acetylspermine. These intermediates are either exported from the cells or they become substrates of the FAD-dependent peroxisomal polyamine oxidase (PAO; EC 1.5.3.11). As PAO prefers acetylated polyamines as substrates, acetylation is the rate-limiting step in this interconversion. Spermine can be directly converted to spermidine in the absence of SSAT by a recently discovered spermine oxidase (SMO) (Wang *et al.* 2001b).

SSAT is a highly regulated inducible enzyme. Its short biological half-life (less than one hour) is comparable to that of ODC and S-AdoMetDC (Seiler 2004). The active enzyme is either a homodimer (Ragione & Pegg 1982, Shinki & Suda 1989) or a tetramer (Casero & Pegg 1993, Fukuchi *et al.* 1994) with binding sites for acetyl-CoA and polyamines (Coleman *et al.* 1995, 1996, Lu *et al.* 1996).

The TATA-less promoter of the human SSAT gene contains binding sites for several transcription factors, including Sp1, GAGA factor, heat-shock factor (HSF), AP1, NF-κB and the polyamine-responsive element (PRE)-binding Nrf-2 (Tomitori *et al.* 2002). Nrf-2 has been shown to regulate polyamine-dependent SSAT expression by acting in cooperation with the polyamine-modulated factor-1 (PMF-1) (Wang *et al.* 1999, Wang *et al.* 2001a). In addition to transcriptional control, SSAT regulation includes stabilization of the mRNA (Fogel-Petrovic *et al.* 1993) and the protein (Fogel-Petrovic *et al.* 1997, Marverti *et al.* 2004), regulation of the mRNA translation (Parry *et al.* 1995) as well as protein degradation (Coleman & Pegg 1997).

SSAT is normally present in very small amounts in the cell and induced by a wide variety of factors, such as the polyamines themselves (Shappell *et al.* 1993), polyamine analogs (Casero *et al.* 1990, Fogel-Petrovic *et al.* 1996), hormones (Seidel & Snyder

1989), growth factors (Desiderio *et al.* 1998) and different toxic agents (Matsui & Pegg 1982). SSAT belongs to the heat-shock protein family because the enzyme is rapidly induced by heat-shock, an induction negatively regulated by polyamines (Fuller *et al.* 1990).

SSAT is degraded via the proteasomal/ubiquitin pathway. Proteasomes interact with the terminal MATEE motif at the carboxyl end of the ubiquitinated SSAT protein. Degradation is prevented in the presence of polyamines or polyamine analogs which cause conformational changes in the SSAT protein and decrease its ability to serve as a substrate for efficient ubiquitination (Coleman & Pegg 1997, 2001).

2.1.1.3 Transport of polyamines

The uptake and release of polyamines are integral parts of the regulatory machinery that adjusts polyamine levels according to the needs of cells. The uptake of a particular polyamine is often stimulated when its synthesis is inhibited. To date polyamine carriers on the plasma membrane have been molecularly identified only in prokaryotes, whereas in eukaryotic cells the genes encoding polyamine transporters have not yet been isolated (Igarashi & Kashiwagi 1999, Gugliucci 2004). Polyamine uptake is a saturable, carrier-mediated and energy-dependent process. In *Escherichia coli* three different polyamine transport systems have been characterized (Figure 3). Uptake is mainly catalyzed by two ABC (ATP binding cassette) transporters, one is spermidine-specific (Figure 3a) and the other prefers putrescine (Figure 3b). These two polyamine transporters consist of four different proteins, a substrate-binding protein (PotD or PotF), an ATPase (PotA or PotG) and two channel-forming proteins (PotB, PotC or PotH, PotI) (Furuchi *et al.* 1991, Pistocchi *et al.* 1993). The third uptake system (Figure 3c,d) involves the PotE protein, which can catalyze both the uptake and excretion of putrescine. Putrescine uptake (Figure 3d) is dependent on membrane potential whilst excretion (Figure 3c) is catalyzed by a putrescine/ornithine antiporter activity of PotE (Kashiwagi *et al.* 1992, Igarashi & Kashiwagi 1999).

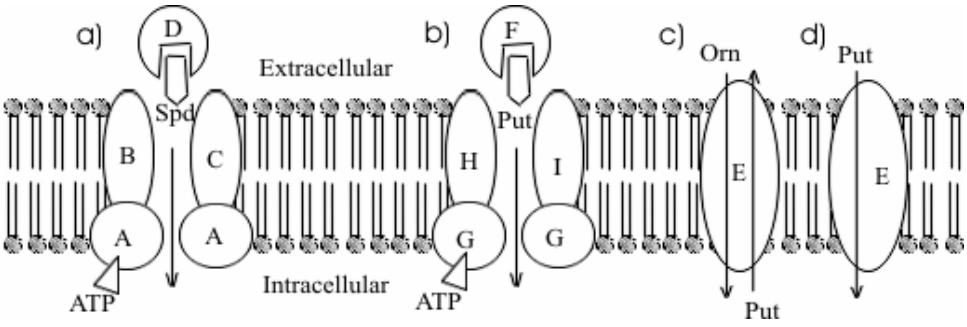


Fig. 3. Polyamine transport systems in *E. coli*. Spd, spermidine; Put, putrescine; Orn, ornithine

Although the genes responsible for eukaryotic polyamine carriers are still unknown, the properties and physiological function of the polyamine transport system has been studied in different cell types. Polyamine uptake is subject to hormonal regulation (Lessard *et al.* 1995), pH- and membrane-potential-dependent, Na^+-independent and requires extracellular divalent cations such as Ca^{2+} or Mg^{2+} (Poulin *et al.* 1995a). Polyamine transport is negatively regulated by antizyme, the enzyme inhibitor of ornithine-decarboxylase (Suzuki *et al.* 1994, Zhu *et al.* 1999). Antizyme not only inhibits polyamine uptake but also stimulates polyamine and acetylpolyamine excretion (Sakata *et al.* 2000). In addition to the natural polyamines polyamine transport systems are capable of transporting a significant range of polyamine analogs. Based on experiments with fluorescent polyamine conjugates (Cullis *et al.* 1999, Soulet *et al.* 2002) two models have been proposed concerning polyamine uptake mechanisms in mammalian cells: polyamines are internalized by a plasma membrane carrier and then sequestered into pre-existing vesicles (model A) or polyamines are directly captured by polyamine receptors and undergo endocytosis (model B). The results of recent studies support model A, indicating that polyamine transport occurs in two steps via a vesicular H^+/polyamine carrier (Soulet *et al.* 2004).

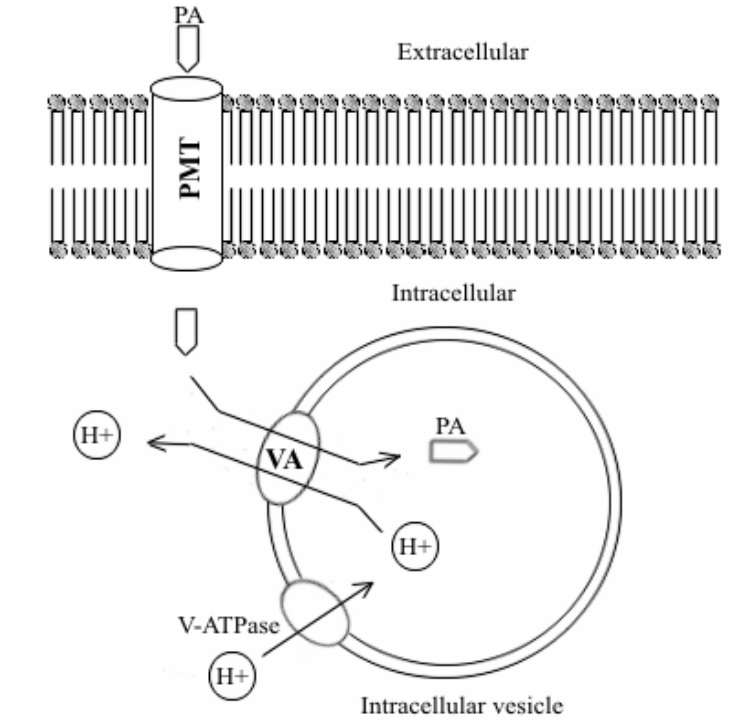


Fig. 4. Model A for the intravesicular polyamine accumulation in eukaryotic cells. PA, polyamines; PMT, plasma membrane transporter; VA, vesicular polyamine-H^+ antiporter; V-ATPase, vacuolar ATPase

2.1.2 Manipulation of the polyamine homeostasis

Proper polyamine homeostasis is essential for cell growth and replication. Failure to maintain individual polyamine concentrations leads to cell-cycle-arrest, cell death or transformation. Cancer cells show elevated ODC and S-AdoMetDC activities as well as increased polyamine levels. Agents inhibiting polyamine biosynthesis prevent, or at least limit cell growth. These facts made the polyamine pathway a promising target for the development of antiproliferative drugs. (Wallace *et al.* 2003.)

2.1.2.1 Polyamine inhibitors and analogs

A number of single enzyme inhibitors have been developed to block the polyamine pathway. The first effective, rationally designed drug that is still in use for polyamine depletion was α-difluoromethylornithine (DFMO) (Metcalf *et al.* 1978). DFMO is cleaved by ODC but the product is not released. It remains in the active site and irreversibly inactivates the enzyme (Wallace & Fraser 2004). *In vitro* DFMO prevents cell growth through depletion of putrescine and spermidine without affecting spermine levels (Meyskens & Gerner 1999). In experimental animal models it was especially effective in the treatment of carcinogen-induced epithelial cancers of many organs (Weeks *et al.* 1982, Nigro *et al.* 1987). Despite the promising *in vitro* and animal studies, DFMO as a single drug proved to be disappointing in human cancer therapy resulting in cytostatic rather than cytotoxic effects *in vivo*. This failure is mainly due to the compensatory increases in the uptake of polyamines from the circulation. However, due to its low toxicity, DFMO recently became a potential drug for cancer prevention (Meyskens *et al.* 1998, Wallace *et al.* 2003).

DFMO has been more successful in the treatment of African sleeping sickness caused by *Trypanosoma brucei gambiense*. Trypanosomes are more sensitive to the drug than human cells, possibly due to the slower turnover of ornithine decarboxylase in these organisms. DFMO treatment leads to the complete inhibition of ODC activity and depletion of polyamines making these parasites more vulnerable to the host immune system (Bacchi *et al.* 1980, Burri & Brun 2003, Docampo & Moreno 2003).

Another important polyamine biosynthetic enzyme inhibitor is methylglyoxal bis(guanylhydrazone) (MGBG), a potent competitive inhibitor of S-adenosylmethionine decarboxylase. It inhibits cell growth, lowers spermidine and spermine concentrations but causes a significant accumulation of putrescine. In addition to inhibition of S-AdoMetDC, MGBG also inhibits diamine oxidase, making it impossible to deplete polyamines in tissues with high diamine oxidase activity. Although it has been efficient in the treatment of several leukemias and lymphomas, the use of MGBG as an anticancer drug is limited by its extreme toxicity and profound antimitochondrial action.

Studies with single enzyme inhibitors indicate that the successful inhibition of tumor growth by the manipulation of the polyamine pathway requires the impairment of several reactions at the same time. (Jänne *et al.* 1991, Wallace & Fraser 2004.)

Polyamine analogs target more than one reaction in the polyamine pathway. They are designed to be sufficiently similar in structure to the parent compound to allow their recognition and uptake by the polyamine transporter and downregulate ODC and S-AdoMetDC, but dissimilar enough not to induce compensatory changes in metabolism (Wallace *et al.* 2003). Polyamine analogs can be divided into two main categories based on their ability to change the polyamine pool size. Antimetabolites activate catabolism and export of natural polyamines leading to cell growth arrest and cell death. The first generation of polyamine analogues, the symmetrical, terminally alkylated bis(ethyl) analogues of spermidine or spermine (Fukuchi *et al.* 1992) or the second generation, asymmetrically alkylated, spermine analog (S)-N^1-(2-methyl-1-butyl)-N^{11}-ethyl-4,8-diazaundecane (IPENSpm) (Fraser *et al.* 2002), belong to this group.

The polyamine mimetics, like the N^1-cycloheptylmethyl-N^{11}-ethylnorspermine (CHENSpm) (McCloskey *et al.* 2000), decrease cell growth but do not necessarily deplete polyamine pools.

Recently a third generation of polyamine analogs has been developed, including oligoamines, amines with inserted double bonds and amines with internal cyclic structures (Valasinas *et al.* 2001). These compounds have also shown some anti-proliferative effects in human tumor cells. Regarding the future it seems that the use of polyamine analogs as multisite inhibitors in cancer therapy is still a promising approach as it exploits the cell's own regulatory mechanisms to deplete polyamines and induce growth arrest and apoptosis (Wallace *et al.* 2003).

2.1.2.2 Genetic engineering of polyamine metabolism

The genes for almost every reaction of polyamine metabolism have been genetically engineered in transgenic animals and embryonic stem cells. To better understand their physiological function *in vivo*, *ODC*, *antizyme*, *S-AdoMetDC*, *SSAT*, *spermidine* and *spermine synthase* have been generally or tissue-specifically overexpressed or disrupted (Jänne *et al.* 2004).

Polyamine biosynthesis has been activated in transgenic mice overexpressing the human *ornithine decarboxylase* gene under its own promoter (Halmekytö *et al.* 1991c). ODC activity and polyamine content showed great deviations between different tissues with the highest ODC activity and subsequent dramatically increased putrescine levels in the testis and brain associated with male infertility. However, with the exception of testis, spermidine and spermine levels were not markedly affected in any of the studied tissues of these animals, suggesting there is an effective compensatory mechanism to prevent the toxic accumulation of spermidine and spermine (Halmekytö *et al.* 1991a, Halmekytö *et al.* 1993).

ODC has been tissue-specifically overexpressed in the skin of transgenic mice driven by the bovine keratin promoter (Megosh *et al.* 1995, O'Brien *et al.* 1997) and in the heart using the α-myosin-heavy-chain promoter (Shantz *et al.* 2001). These animals showed elevated putrescine and spermidine levels in the target tissues. Elevated ODC activity caused clear phenotypic abnormalities such as the development of dermal follicular cysts,

skin wrinkling, hair loss, spontaneous tumor development (Megosh *et al.* 1995) and severe cardiac hypertrophy following β-adrenergic stimulation (Shantz *et al.* 2001), respectively.

Transgenic mice generally overexpressing *spermidine* (Kauppinen *et al.* 1993) and *spermine synthase* (Ikeguchi *et al.* 2004) have been generated as well. In the *spermidine synthase* overexpressing mice, the elevated enzyme activity did not affect the tissue polyamine pool sizes (Kauppinen *et al.* 1993), whereas in the mice overexpressing *spermine synthase* there was a significant decrease in spermidine and increase in spermine content. However, the latter changes in the tissue polyamine levels were smaller than expected on the basis of the elevated spermine synthase activity. Crossing of transgenic mice generally overexpressing *spermine synthase* with a mouse line with targeted *S-AdoMetDC* expression in the heart resulted in a lethal phenotype. This indicates that decarboxylated S-AdoMet is the limiting factor in spermine synthesis. (Ikeguchi *et al.* 2004.)

Polyamine catabolism has been activated by generally overexpressing its rate-limiting enzyme, SSAT. These mice accumulated N^1-acetylspermidine, normally not present in mouse tissues, and putrescine, whereas spermidine and/or spermine pools decreased. The animals lost hair at the age of three to four weeks, their skin was heavily wrinkled and females were infertile (Pietilä *et al.* 1997). When *SSAT* and *ODC* overexpressing mouse lines were crossbred, the putrescine levels in the offspring increased further but the diminished spermidine and spermine pools were not replenished, suggesting that catabolism is the overriding control mechanism in polyamine metabolism (Suppola *et al.* 2001).

To further study the possible physiological roles of the polyamine biosynthetic enzymes, recently two knockout mouse lines have been generated by the disruption of the *ODC* (Pendeville *et al.* 2001) and the S-adenosylmethionine decarboxylase encoding *Amd1* (Nishimura *et al.* 2002) genes respectively. *ODC* heterozygous mice did not exhibit visible pathology, at least by 1 year of age. Embryos that lacked *ODC* developed normally to the blastocyst stage and implanted at embryonic day E3.5 but died shortly thereafter, before the onset of gastrulation (Pendeville *et al.* 2001). Similarly, *Amd1* heterozygous mice were normal and fertile, but homozygous embryos died early in embryonic development, between E3.5 and E6.5 (Nishimura *et al.* 2002).

The gyro (Gy) mouse line represents a model for X-linked dominant hypophosphatemic rickets. Gy mice had a contiguous gene deletion, containing part of the *PHEX* (phosphate regulating gene) and *spermidine synthase* genes. Gy male mice were infertile and showed a marked decrease in their hepatic and pancreatic spermine pools (Lorenz *et al.* 1998). Studies with cardiac myocytes derived from Gy mice indicate that spermidine and spermine are major controllers of inward rectification at potassium channels (Lopatin *et al.* 2000).

2.1.3 Physiological roles of polyamines

Polyamines have been implicated in a large number of cellular processes, including cellular growth, apoptosis, functioning of ion-channels, DNA replication and packaging, transcription and translation. Despite the intensive research, the exact molecular mechanisms describing their physiological functions in cells still remain to be clarified.

2.1.3.1 DNA-polyamine interactions

The positively charged polyamines interact readily with the anionic compounds of the cell such as DNA and RNA. Polyamines are associated with highly condensed mitotic chromosomes (Hougaard *et al.* 1987), inducing more stabilizing than regulating effects on the chromatin structure during the cell cycle (Morgan *et al.* 1987, Snyder 1989, Laitinen *et al.* 1998). The polyamine-mediated stabilization of highly condensed chromosomal fibers is inhibited by histone hyperacetylation, which confirms the hypothesis that polyamines act as transcriptional repressors via condensed chromatin stabilization (Pollard *et al.* 1999). Spermidine and spermine protect DNA from oxidative stress- (Muscari *et al.* 1995, Ha *et al.* 1998a, Ha *et al.* 1998b) or radiation-induced (Douki *et al.* 2000) strand breakage and subsequent mutations. Polyamines can promote DNA conformational changes such as conversion of the right-handed B-DNA form to left-handed Z-DNA (Thomas & Messner 1986). The positively charged polyamines readily generate ionic bonds with cellular phosphate anions and form aggregates. These polycationic aggregates are able to interact with genomic DNA and protect its integrity with much higher efficiency than single polyamines (D'Agostino & Di Luccia 2002). Several models for the interaction between polyamines and DNA have been proposed based on different biochemical, physical and chemical techniques. Recent studies with Fourier transform spectroscopy suggest that putrescine forms intrastrand complexes with DNA as well as interacts electrostatically with the exo-groove phosphate moiety. Spermidine binds inter- and intrastrand whereas spermine favors interstrand attachment to the DNA duplex. The ability of higher polyamines to form interstrand interactions with DNA could explain their higher protection against strand breaks. (Ruiz-Chica *et al.* 2001, Ouameur & Tajmir-Riahi 2004.)

2.1.3.2 Cell growth

Polyamines are necessary for normal cell growth. Investigations using polyamine biosynthetic inhibitors indicate that depletion of cellular polyamines blocks cell growth, and polyamine-depleted cells are stimulated to grow in the presence of exogenous polyamines (Luk *et al.* 1981, Herr *et al.* 1984, Pegg 1984, Kapyaho *et al.* 1985, Regenass *et al.* 1992, He *et al.* 1995).

All the regulatory proteins of polyamine metabolism undergo coordinate changes in their activities during cell-cycle progression (Bettuzzi *et al.* 1999), but alterations in the polyamine levels do not always correlate with fluctuation in the levels of their biosynthetic enzymes (Fredlund *et al.* 1995). The activity of the rate-limiting polyamine biosynthetic enzyme, ornithine decarboxylase (ODC) is increased at the G_1/S transition (Fuller *et al.* 1977, Kaczmarek *et al.* 1987, Bettuzzi *et al.* 1999) and at G_2/M (Fredlund *et al.* 1995, Pyronnet *et al.* 2000) (Figure 5). Earlier studies (Fredlund *et al.* 1995) reported a possible second peak of ODC activation at the S/G_2 transition.

Polyamine depletion with α-difluoromethylornithine (DFMO), a specific inhibitor of ODC, prolongs the S phase within one cell cycle in CHO cells (Fredlund & Oredsson 1996) due to a decreased rate of DNA elongation (Alm & Oredsson 2000). DFMO arrests untransformed IEC-6 cells in the G_1 phase inhibiting the activity of Cdk2 and activating p53, $p27^{Kip1}$ and $p21^{Waf1/Cip1}$ (Ray *et al.* 1999, Ray *et al.* 2001). On the other hand, fibroblasts transformed by ODC overexpression show increased Cdk2 activity and a profound decrease in $p27^{Kip1}$ activity as well as the loss of $p27^{Kip1}$ from the cyclin E/Cdk2 complex (Ravanko *et al.* 2000). The increase in the $p21^{Waf1/Cip1}$ expression following polyamine depletion is at least partly due to the increased JunD level. JunD transactivates $p21^{Waf1/Cip1}$ -promoter activity and enhances expression of the *p21*$^{Waf1/Cip1}$ gene (Li *et al.* 2002). Polyamine depletion can also induce G_1 arrest in human melanoma cells by activating the p53-$p21^{Waf1/Cip1}$ -pRb pathway (Kramer *et al.* 1999, Kramer *et al.* 2001).

S-AdoMetDC-deficient *S.pombe* cells do not grow in the absence of added spermidine. Spermidine deprivation from the culture medium causes an overall delay in the cell cycle progression with an accumulation of cells in the G_1 phase (Chattopadhyay *et al.* 2002).

Cyclin B1 mRNA, which is normally degraded in early G_1, is stabilized by DFMO (Thomas and Thomas 1994). These studies suggest that polyamines are required to traverse the restriction point in G_1 at which cells become committed to enter the S-phase and complete the cell cycle.

At the G_2/M transition the protein synthesis is strongly inhibited by the repression of cap-dependent translation initiation. The short-lived protein products required in mitosis (ODC, c-myc, etc.) are produced by a cap-independent translation mechanism through internal ribosome entry sites (IRES). The second increase in ODC activity at the G_2/M transition most likely provides polyamines needed for mitotic chromosome alterations (Pyronnet *et al.* 2000).

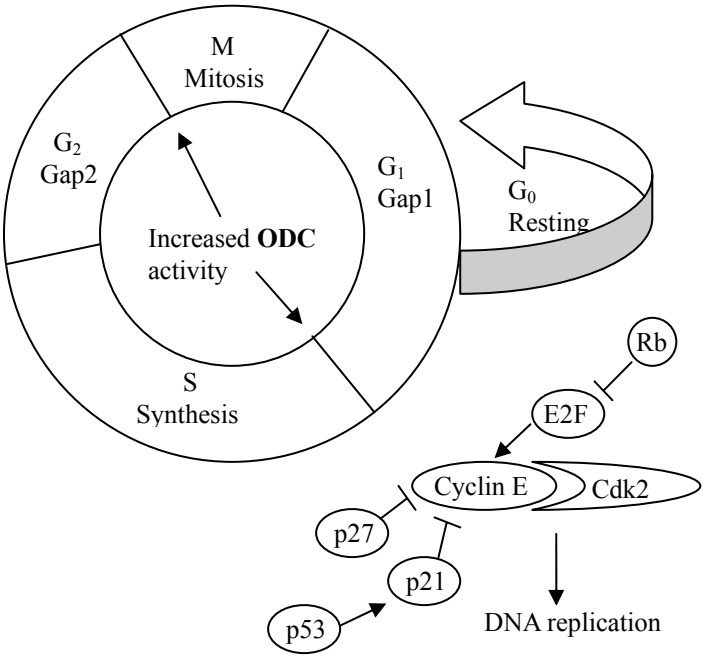


Fig. 5. The cell cycle. DFMO induces G_1 arrest inhibiting the activity of Cdk2 and activating p53, p27 and p21.

2.1.3.3 Apoptosis

Eukaryotic cells possess a genetic program to commit cellular suicide, a phenomenon also known as apoptosis. The characteristics of apoptosis include mitochondrial structural changes, activation of caspase proteases, chromatin condensation and DNA fragmentation. Failure of apoptosis can lead to auto-immune diseases or cancer. There is an emerging body of literature indicating the involvement of polyamines in this process by several mechanisms (Susin *et al.* 1998, Schipper *et al.* 2000) (Figure 6). Both a decrease (Desiderio *et al.* 1995, Grassilli *et al.* 1995, Penning *et al.* 1998) and an increase (Poulin *et al.* 1995c, Tobias & Kahana 1995, Tome *et al.* 1997, Xie *et al.* 1997) in polyamine levels have been reported to trigger apoptosis, and in several cases polyamines, particularly spermine, appear to protect cells against apoptotic cell death (Harada & Sugimoto 1997).

Ornithine decarboxylase is an effector of c-Myc-induced apoptosis. Under conditions in which ODC expression is normally down-regulated, elevated ODC enzyme activity is sufficient to induce apoptosis (Packham & Cleveland 1994). On the other hand, c-Myc, through ODC, has a role in protecting cells from death during periods of cellular stress.

Many cellular insults that induce c-Myc expression also enhance ODC expression. The inductions of ODC and c-Myc seem to be mediated by reactive oxygen species (ROS) (Park *et al.* 2002). The protective role of ODC is most likely due to the production of spermidine and spermine which prevents the DNA strand breakage induced by ROS (Brune *et al.* 1991, Muscari *et al.* 1995, Ha *et al.* 1998b).

Spermine stabilizes DNA and chromatin structure (Penning *et al.* 1998) and interferes with different ion channels such as K^+ channels or NMDA receptors (Harada & Sugimoto 1997), that can explain its protective action on programmed cell death.

Putrescine overaccumulation induces apoptosis (Tobias & Kahana 1995) by a mechanism involving suppression of hypusine formation in the eukaryotic initiation factor (eIF) 5A. The formation of hypusine, in which the butylamine moiety of spermidine is transferred to the lysine residue in eIF-5A, is an important part of the normal function of spermidine (For details see 2.1.3.5) Excess putrescine seems to act post-transcriptionally to reduce eIF-5A levels (Tome *et al.* 1997).

During B cell receptor (BCR)-mediated apoptosis intracellular polyamine levels decrease. The addition of exogenous spermine prevents mitochondrial inner transmembrane potential ($\Delta\Psi_m$) collapse but does not completely prevent apoptotic changes (Nitta *et al.* 2001).

Spermine and spermidine have been shown to trigger caspase activation in cell-free models of apoptosis (Stefanelli *et al.* 1999) and to cause a selective, rapid and saturable leakage of cytochrome c from isolated mitochondria (Stefanelli *et al.* 2000).

During apoptosis the Bax protein undergoes a transformational change at its NH_2 terminus, translocates from the cytosol to the mitochondria, inserts itself into the outer mitochondrial membrane and induces the release of cytochrome c (Goping *et al.* 1998, Murphy *et al.* 2000). Polyamine depletion inhibits the translocation of Bax to the mitochondria and consequently prevents cytochrome c release (Yuan *et al.* 2002). Bid, a proapoptotic Bcl-2 family protein, is cleaved by caspase 8. The truncated Bid also translocates to the mitochondria and induces cytochrome c release (Li *et al.* 1998, Luo *et al.* 1998).

Exogenous L-ornithine antagonizes the apoptotic effect of N^{ω}-hydroxy-L-arginine (NOHA) in releasing cytochrome c from the mitochondria and all subsequent steps downstream leading to cell death, but does not inhibit the activation of caspase-8 and cleavage of Bid (Singh *et al.* 2002). On the other hand polyamine depletion decreases the cleavage of Bid, that contributes, at least partially, to the decrease in release of cytochrome c from the mitochondria (Yuan *et al.* 2002).

Extracellular signal-regulated kinases (ERKs) 1 and 2 can act in signal transduction pathways leading to apoptosis. In etoposide-treated fibroblasts, polyamine depletion inhibits ERK 1/2 phosphorylation and the subsequent caspase activation (Stefanelli *et al.* 2002).

Polyamines are negative regulators of nuclear factor (NF)-κB activation. Depletion of cellular polyamines increases the basal level of NF-κB proteins, induces NF-κB nuclear translocation, and activates the sequence-specific DNA-binding activity (Li *et al.* 2001). NF-κB is an inducible transcription factor and is thought to be the central regulator of transcription of genes involved in apoptosis. It can play a proapoptotic or antiapoptotic role depending on the death stimulus and the cell type. In intestinal epithelial cells, decreased polyamines activate NF-κB and stimulate expression of the inhibitors of

apoptosis (IAP) genes, leading to the accumulation of IAP proteins. The increased IAPs inhibit the activation of caspases and protect cells from TNF-α/CHX induced apoptosis (Zou *et al.* 2004).

The serine-threonine kinase Akt is a multifunctional signaling intermediate in the regulation of cell cycle progression, apoptosis, and energy metabolism. Akt activation suppresses apoptosis induced by different death-stimuli in a variety of cell types. In normal intestinal cells polyamine depletion induces Akt activation mediating suppression of apoptosis at least partly through inhibition of caspase-3 (Zhang *et al.* 2004).

In intestinal epithelial cells, polyamine depletion inhibits the activation of NH_2-terminal c-Jun kinase (JNK), and subsequently prevents the activation of caspases-6, -8, -9 and -3 (Bhattacharya *et al.* 2003).

These results indicate that polyamines are involved in the transduction of the cell-death message.

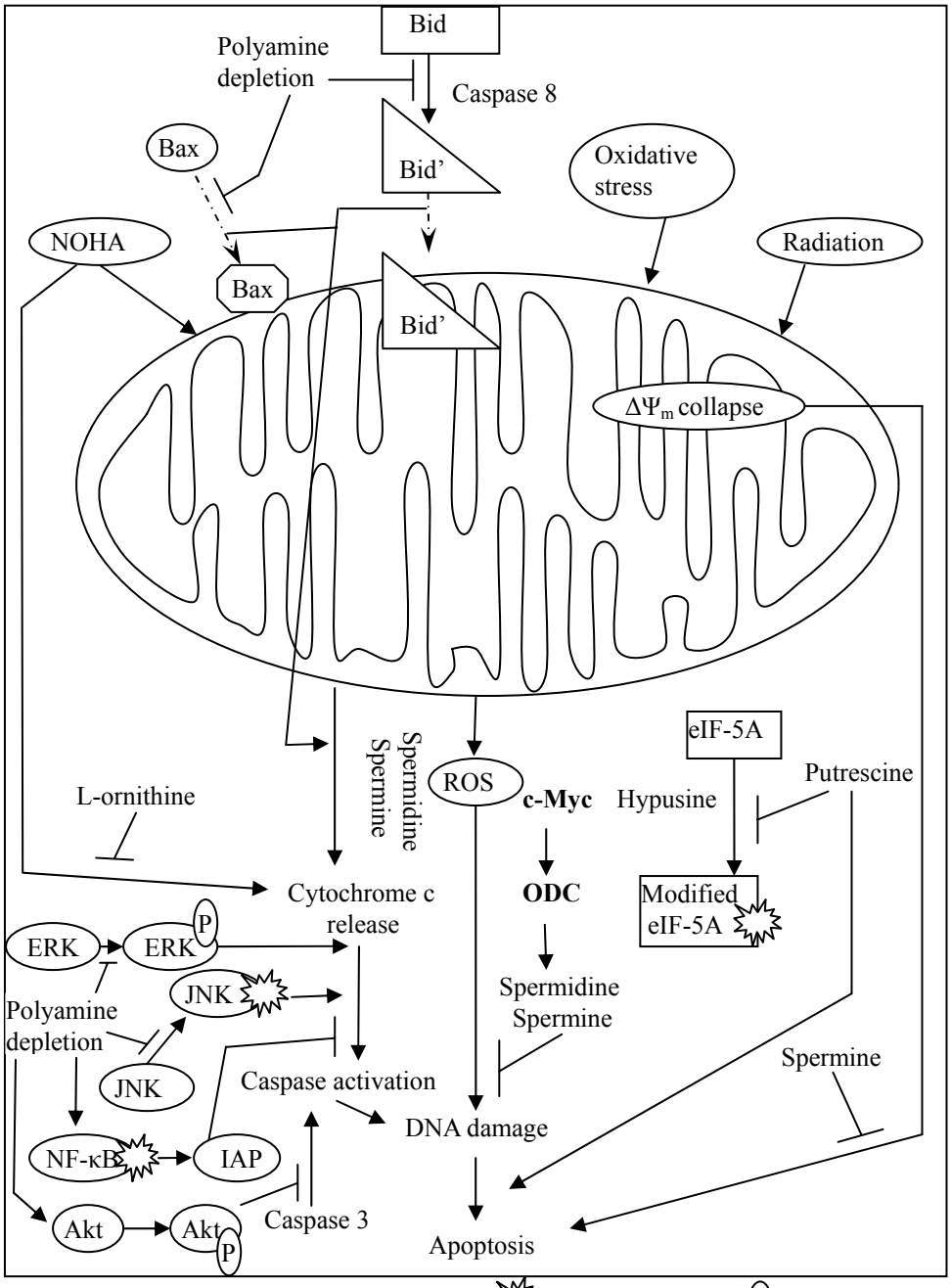


Fig. 6. Involvement of polyamines in apoptosis. ✸, activated protein; (P), phosphorylated protein, ROS, reactive oxygen species; eIF, eukaryotic initiation factor; $\Delta\Psi_m$, mitochondrial inner transmembrane potential; Bid', truncated Bid; NOHA, N^{ω}-hydroxy-L-arginine; ERK, extracellular signal-regulated kinase; NF, nuclear factor; IAP, inhibitors of apoptosis; JNK, c-Jun kinase

2.1.3.4 DNA and protein synthesis

Increased polyamine levels stimulate (Ginty *et al.* 1989, Tantini *et al.* 2001) and conversely the deprivation of polyamines inhibits DNA, RNA and protein synthesis (Oredsson *et al.* 1990). Polyamine deficient cells are not able to ligate Okazaki-fragments which causes cessation of cellular DNA synthesis (Pohjanpelto & Hölttä 1996). Polyamines might regulate the expression of many genes via DNA bending required for transcription initiation (Childs *et al.* 2003). Polyamines were shown to participate in protein synthesis in various ways, such as enhancing the synthesis and the activity of RNA polymerases (Jänne *et al.* 1975, Moruzzi *et al.* 1975, Jacob & Rose 1976, Yoshida *et al.* 2002), stimulating the synthesis of specific proteins (Atkins *et al.* 1975, Watanabe *et al.* 1981, Kashiwagi *et al.* 1990), decreasing misincorporation of amino acids during polypeptide synthesis (Jelenc & Kurland 1979, Ito & Igarashi 1986), stimulating the assembly of ribosomal subunits (Kakegawa *et al.* 1986), influencing tRNA methylation (Leboy 1971, Mach *et al.* 1982) and stimulating the formation of Ile-tRNA (Igarashi *et al.* 1978, Kusama-Eguchi *et al.* 1991).

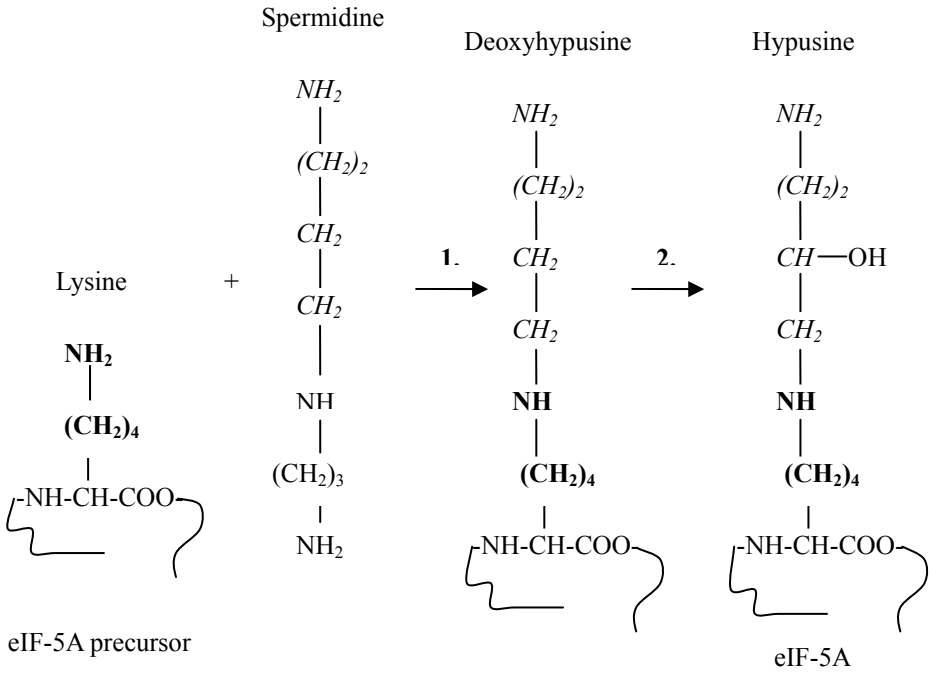
The addition of polyamines to a cell-free protein-synthesis system results in a 3-to 5-fold stimulation of amino acid incorporation and increases the synthesis of high molecular weight proteins (Atkins *et al.* 1975). Polyamine regulation of protein synthesis depends on the size and base composition of the 5'-UTR of the mRNA. There is a tendency for polyamines to regulate the translation of mRNAs having long 5'-UTR in both prokaryotes and eukaryotes (Igarashi *et al.* 1997). Spermidine regulation (stimulation at low concentrations and inhibition at high concentrations) of protein synthesis directed by mRNA having a GC-rich 5'-UTR occurs at the level of initiation complex formation of Met-$tRNA_i$, mRNA and ribosomes (Shimogori *et al.* 1996).

Polyamines also participate in a rare autoregulatory mechanism of protein synthesis, called translational frameshifting. Antizyme mRNA contains two overlapping open reading frames, ORF1 and ORF2. ORF1 contains sequences required for the initiation of translation but ORF2 encodes most of the protein. The functional protein can be translated only by starting at ORF1, having a frameshift one codon just before its stop codon, and continuing translation in the +1 frame until reaching the stop codon of ORF2. Polyamines have a stimulatory effect on this frameshifting process producing their negative regulator antizyme. (Matsufuji *et al.* 1996, Coffino 2001.)

2.1.3.5 Hypusine formation

Eukaryotic translation initiation factor 5A (eIF-5A) is a small, acidic protein, highly conserved from yeast to mammalian cells (Childs *et al.* 2003). The inactive eIF-5A precursor is post-translationally processed by the synthesis of an unusual amino acid, hypusine (Park *et al.* 1981). The biosynthesis of hypusine occurs via two consecutive enzymatic steps (Figure 7) (Murphey & Gerner 1987, Park & Wolff 1988). In the first step of this process, deoxyhypusine synthase (EC 2.5.1.46) catalyzes the NAD-dependent transfer of the butylamine moiety of the donor spermidine to the amino group of a

specific lysine residue of the acceptor eIF-5A precursor (Chen & Dou 1988, Wolff *et al.* 1995, Lee *et al.* 2001). This intermediate is then hydroxylated by deoxyhypusine hydroxylase (EC 1.14.99.29) to complete the synthesis of hypusine and the maturation of eIF-5A (Abbruzzese *et al.* 1986). Hypusine synthesis is one of the most specific post-translational modifications and the only biological function for a polyamine described at the molecular level to date. Mature eIF-5A has an essential role in eukaryotic cell proliferation. Gene disruption studies in the yeast, *Saccharomyces cerevisiae*, show that inactivation of the two eIF-5A genes (Schnier *et al.* 1991) or of the single-copy deoxyhypusine synthase gene (Sasaki *et al.* 1996, Park *et al.* 1998) results in a loss of cell viability. In addition, inhibitors of deoxyhypusine synthase (Park *et al.* 1994) or deoxyhypusine hydroxylase (Hanauske-Abel *et al.* 1994) suppress growth in various mammalian cells. However, eIF-5A is not required for general protein synthesis, as eIF-5A-depleted yeast cells are still able to synthesize the major part of their proteins (Kang & Hershey 1994). Although the exact function of eIF-5A is still unclear, its highly conserved structure in eukaryotes and unique post-translational modification machinery suggest a critical biological role.



1. deoxyhypusine synthase
2. deoxyhypusine hydroxylase

Fig. 7. Biosynthesis of eukaryotic initiation factor 5A (eIF-5A)

2.1.3.6 Interactions with ion channels

Spermidine and spermine play a role in blocking and modulating various ion channels, such as the inward rectifier K^+ channels (Kir channels) and different glutamate receptors (Williams 1997a). In the case of K^+ channels, inward rectification means that the inward flow of K^+ ions at negative membrane potentials is always greater than the outward flow for the opposite driving force. Strong inward rectification is essential to maintain the resting potential of cells and permit prolonged depolarization, a feature of the cardiac action potential (Nichols & Lopatin 1997). Inward rectification may result from two independent mechanisms, an instantaneous block by Mg^{2+} and a phenomenon called intrinsic gating caused by intracellular spermidine and spermine (Ficker *et al.* 1994, Lopatin *et al.* 1994). Intrinsic gating involves a slow decrease in the current (50-100 ms) at depolarized membrane potentials. Spermine was shown to enter a Kir channel and bind electrostatically to its negatively charged residues at various depths, thus blocking the channel pore and the outward K^+ flow in a voltage dependent manner (Kubo & Murata 2001, Xie *et al.* 2002).

Glutamate receptors mediate fast synaptic transmission in the central nervous system. They are classified on the basis of their selectively activating agonists into three groups: NMDA-, AMPA- and kainate receptors (Williams 1997a). NMDA- (Traynelis *et al.* 1995, Masuko *et al.* 1999) and kainate receptors (Mott *et al.* 2003) are regulated by protons at ambient pH (pH 7.3). Spermine is able to relieve proton inhibition and stimulate these receptors possibly via binding to their LIVBP-like domain (Mott *et al.* 2003).

Intracellular polyamines control the rectification of the Ca^{2+}-permeable AMPA and kainate receptors as well (Donevan & Rogawski 1995, Williams 1997b). Bowie *et al.* (1998) have suggested that polyamines modulate these glutamate receptors dynamically via both open- and closed-channel blocking mechanisms.

Activation of NMDA receptors requires binding of glutamate and glycine at separate sites on the receptor. Extracellular spermidine and spermine have multiple effects on NMDA receptors. They facilitate the binding of glycine to the receptor complex (glycine-dependent stimulation) (Sacaan & Johnson 1989, McGurk *et al.* 1990, Ransom & Deschenes 1990) as well as potentiate NMDA currents via increase in frequency of channel opening (glycine-independent stimulation) (Rock & MacDonald 1992, Araneda *et al.* 1993).

Polyamines also block responses of the NMDA receptors by reducing conductance in a voltage-dependent manner (Araneda *et al.* 1993). Spermine reduces NMDA single-channel currents, and blocks and permeates NMDA receptor channels from both the extracellular and intracellular sides (Araneda *et al.* 1999). Recently it was shown (Turecek *et al.* 2004) that intracellular spermine modulates the activity of NMDA receptors by a direct mechanism involving a decrease in the probability that receptor channels were open.

2.1.3.7 Embryonic development

The role of polyamines in invertebrate development was studied by using the polychete *Ophryotrocha labronica* (Heby & Emanuelsson 1981) and the nematode (or round worm) *Caenorhabditis elegans* (MacRae *et al.* 1998) as model systems. DFMO treatment of fertilized *Ophryotrocha labronica* eggs prevents putrescine accumulation at the beginning of gastrulation. This polyamine limitation results in a developmental arrest at gastrulation, prevents nucleolar formation and suppresses ribosomal activity (Heby & Emanuelsson 1981). *C.elegans odc-1* null mutant worms with no detectable ODC activity show two different phenotypes in polyamine-free medium, depending on the developmental stage at which it was imposed. Polyamine depletion at the early L1 stage results in animals that are morphologically adult but do not contain or lay eggs. If mutant larvae are transferred to polyamine-deficient medium at the L4 stage, they develop and lay eggs normally, but the embryos fail to hatch and arrest at about the 550-cell stage (MacRae *et al.* 1998).

In polyamine research the most widely used model systems for vertebrate development are *Xenopus* (Shiokawa *et al.* 2000), chick (Goyns 1979, Lowkvist *et al.* 1980, Heby & Emanuelsson 1981), rat (Russel & McVicker 1972) and mouse (Fozard *et al.* 1980a, Jotova *et al.* 1999, Pendeville *et al.* 2001, Nishimura *et al.* 2002). S-AdoMetDC mRNA injection into *Xenopus* fertilized eggs activates a maternally preset apoptotic program at the early gastrula stage. S-AdoMetDC overexpression induces an S-adenosylmethionine deficient state, that in turn induces the inhibition of protein synthesis. The fact, that various other agents which damage DNA, RNA and protein synthesis switch on the apoptotic program at the same developmental step (at the early gastrula stage, shortly after the midblastula transition), indicates the existence of an important developmental check-point at this stage (Shiokawa *et al.* 2000).

Administration of DFMO into fertilized chick eggs also blocks embryonic development at gastrulation (Lowkvist *et al.* 1980, Heby & Emanuelsson 1981). ODC inhibition interferes with nucleolar formation with a reduction of the fibrillar component of the nucleolus and segregation of the nucleolar material (Lowkvist *et al.* 1983).
DFMO treatment in mice at early pregnancy abolishes increases in uterine ODC activity and in putrescine and spermidine concentrations seen during normal gestation, but markedly increases S-AdoMetDC activity. As a consequence, decidualization takes place normally after implantation, but embryonic development is arrested at a stage typical of days 6 to 7 of normal gestation that corresponds to the onset of gastrulation. Rats and rabbits show similar responses to DFMO (Fozard *et al.* 1980b).

The ODC and the S-adenosylmethionine decarboxylase knock-out mouse lines (Pendeville *et al.* 2001, Nishimura *et al.* 2002) were discussed more in detail in 2.1.2.2. Briefly, homozygous embryos were not viable, dying before the onset of gastrulation, between E3.5 and E6.5. These knockout studies confirm the generally accepted view that polyamine homeostasis plays an essential role in development and cell growth. However, the generation of conditional knockout mouse lines is needed to further clarify the role of polyamines and their biosynthetic enzymes during vertebrate embryogenesis.

2.1.3.8 Polyamines in the urogenital organs

Polyamines have a critical role in germ cell development. Putrescine, spermidine and spermine concentrations show continuous and cell type specific changes during testicular maturation that are consistent with fluctuations of their biosynthetic enzymes (Shubhada *et al.* 1989b). In rodent testes, ornithine decarboxylase (ODC) mRNA levels increase during late meiosis and early spermiogenesis and decrease to background levels in later stages of spermatid development and in spermatozoa suggesting that polyamines are needed during haploid gene expression (Alcivar *et al.* 1989, Shubhada *et al.* 1989a, Kaipia *et al.* 1990). Transgenic mice overexpressing *ODC* exhibit male infertility and dramatic morphological changes in testicular tissue with grossly impaired spermatogenesis. These mice have more than a 20 fold higher putrescine concentration in their testes when compaired with their nontransgenic littermates (Halmekytö *et al.* 1991a, Halmekytö *et al.* 1991c). Putrescine has a selective mode of action during mitotic and meiotic cell cycles in spermatogenesis, strongly stimulating the last mitosis before the onset of meiosis in type B spermatogonia and inhibiting meiotic DNA synthesis. Aberrantly high putrescine levels may lead to decreased fertility in the *ODC*-overexpressing transgenic mice (Hakovirta *et al.* 1993). During sperm development, ODC expression is regulated by antizyme 3, an ODC antizyme expressed only in the haploid germ cells in the testis between early spermiogenesis and the late spermatid phase (Ivanov *et al.* 2000, Tosaka *et al.* 2000). The expression pattern of antizyme 3 suggests that it provides spatial and temporal regulation of ODC during spermatogenesis sharply limiting polyamine accumulation in cells that have completed meiotic reduction and are about to be remodeled into mature spermatozoa (Coffino 2000).

In the developing chick ovary ODC has two activity peaks: one in the early developmental stages correlated with early morphological development and germ cell proliferation and another related to the maturation of a large population of follicular cells. Polyamine levels follow the changes in ODC activity (Teng & Teng 1980). Interestingly, transgenic mice overexpressing spermidine/spermine N^1-acetyltransferase (SSAT) are characterized by permanent hair loss and female infertility. In transgenic animals, primary and small secondary follicles are present but larger developing follicles and corpus luteum formation are absent suggesting a role for polyamines in follicular development (Pietilä *et al.* 1997).

In the adult murine kidney, ODC is expressed mainly in the proximal tubules (Levillain & Hus-Citharel 1998) and its expression is regulated by androgens at multiple levels, including transcription, posttranscription and translation (Seely *et al.* 1982, Isomaa *et al.* 1983, Berger *et al.* 1986, Watson & Paigen 1988). Testosterone enhances ODC expression causing a significant difference in renal ODC activity and consequently in putrescine levels between sexes, being high in males and low in females (Goldstone *et al.* 1982). This renal sexual dimorphism is manifested after weaning, simultaneously with an increase in plasma testosterone concentration (Sanchez-Capelo *et al.* 1994, Sanchez-Capelo *et al.* 1999). Despite the low ODC level in female kidneys, antizyme 1 expression levels are similar to those of males suggesting a continuous degradation process of the ODC protein in females (Murakami *et al.* 1988, Levillain *et al.* 2003).

2.2 Acetyl-CoA synthetase

Acetyl-CoA synthetase activates acetate to acetyl-CoA, an essential molecule utilized in various metabolic pathways including the fatty acid and cholesterol synthesis as well as the tricarboxylic acid cycle. There are two different forms of acetyl-CoA synthetase, the ADP-forming and the AMP-forming enzymes, which are encoded by two independent, nonhomologous genes (Starai & Escalante-Semerena 2004). The ADP-forming acetyl-CoA synthetase (EC 6.2.1.13) catalyzes the reversible reaction of acetate+ ATP→ ADP + P_i + acetyl-CoA. This type of enzyme is restricted to lower organisms, such as anaerobic protists and some archeal halophytes and thermophiles. AMP-forming acetyl-CoA synthetase (AceCS, EC 6.2.1.1) synthesizes acetyl-CoA in two steps via an acyl-AMP intermediate. The simplified reaction is acetate+ ATP→ (acetyl-AMP)→ acetyl-CoA + PP_i + AMP. AceCS has a broad distribution from prokaryotes to human (Karan *et al.* 2001, Starai & Escalante-Semerena 2004). The characteristics of this latter enzyme will be discussed more in detail below.

2.2.1 Regulation of AMP-forming acetyl-CoA synthetase

The coding regions of the AMP-forming acetyl-CoA synthetase (AceCS) are well conserved among different species (Karan *et al.* 2001). *AceCS* gene expression is controlled by complex regulatory mechanisms as a function of carbon flux including a posttranslational NAD^+/sirtuin-dependent protein acetylation/deacetylation system.

In *E.coli AceCS* is the promoter-proximal gene of an operon involved in acetate metabolism. The *AceCS* gene is cotranscribed with the acetate transporter coding *actP* gene, and with the *yjcH* gene whose function is unknown to date (Gimenez *et al.* 2003). *AceCS* is induced through the actions of different transcription factors such as the carbon regulator cAMP receptor protein (CRP), the oxygen regulator FNR, the glyoxylate shunt repressor IclR and its activator FadR, (Kumari *et al.* 2000a). *AceCS* transcription occurs as a function of sigma factor σ^{70} activity (Kumari *et al.* 2000b).

Eukaryotes possess two isoforms of AceCS, one in the mitochondria, participating in the energy generation processes and a second one in the cytosol supplying acetyl-CoA for lipid biosynthesis. In *S. cerevisiae* ACS1 is the mitochondrial enzyme, only expressed during respiratory and respirofermentative growth, whereas *ACS2* is expressed in the cytosol during anaerobic growth on glucose (Starai & Escalante-Semerena 2004). Transcriptional regulation of the two genes differs strongly.

ACS1 is repressed by high concentrations of glucose and other fermentable carbon sources (de Jong-Gubbels *et al.* 1997). Induction of *ACS1* on non-fermentable ethanol or acetate is positively controlled by a carbon source-responsive element (CSRE) and an alcohol dehydrogenase regulator, Adr1p in the promoter region whereas binding of the Ume6p protein to the URS1 motifs exerts negative control on the *ACS1* promoter (Kratzer & Schuller 1995, 1997).

ACS2 is coregulated with structural genes of lipid biosynthesis via binding of the heterodimeric activator Ino2p/Ino4p protein to the ICRE regulatory motif as well as

binding of the pleiotropic transcription factor Abf1p to the *ACS2* promoter region (Hiesinger *et al.* 1997).

Mammalian acetyl-CoA synthetase also has two isoforms with an unfortunate nomenclature. Mammalian cytoplasmic *AceCS* is called *AceCS1* and the mitochondrial isoform is *AceCS2*, which is the opposite and confusing numbering compared to yeast.

Cytosolic acetyl-CoA synthetase is involved in generating acetyl-CoA for lipid biosynthesis. *AceCS1* transcription is negatively regulated by sterols (Luong *et al.* 2000) and induced via binding of the sterol regulatory element-binding proteins (SREBPs) and Sp1 or Sp3 to the promoter region (Ikeda *et al.* 2001). *AceCS1* is also regulated by insulin and the diabetic status of the individual. It also responds to dietary changes possibly under the control of SREBP-1 (Sone *et al.* 2002).

AceCS2 is a mitochondrial enzyme and its acetyl-CoA product is mainly used for oxidation by the citric acid cycle. *AceCS2* transcripts are abundant in the heart and skeletal muscle of mouse. Under ketogenic conditions such as starvation and diabetes *AceCS2* is induced via unknown mechanisms (Fujino *et al.* 2001).

2.2.2 Posttranslational regulation of AceCS

AceCS expression is further regulated by a sirtuin-dependent posttranslational control mechanism. Sirtuin is the collective name of the silent information regulator protein (Sir2) and its homologs involved in gene silencing and chromosome stability. Sir2 protein has a genetically conserved NAD^+-dependent protein deacetylase activity (Smith *et al.* 2000) and one of its substrates is AceCS. Deacetylation by Sir2 activates AceCS whereas a single acetylation is enough to block enzyme activity. The site of acetylation is a lysine residue in a well conserved motif of the AMP-forming enzymes suggesting a common regulatory mechanism in pro- and eukaryotes (Starai *et al.* 2002, Starai *et al.* 2004, Takasaki *et al.* 2004).

3 Aims of the research

To date, over 60.000 articles have been published involving polyamines, but it is still not possible to define exactly how polyamines function in cells. While it is difficult to find a single cellular function in which they would not be involved, polyamines were studied mostly because of their role in malignant cell growth. Polyamines accumulate in cancerous tissues and their concentration is elevated in the body fluids of cancer patients. However, cells have a sophisticated regulatory machinery to maintain their polyamine pools that makes polyamine research very complicated. To date, inhibitors of virtually all of their biosynthetic enzymes are available and transgenic and knock-out animals have been generated for both their biosynthetic and catabolic enzymes to study their cellular functions and interactions with cellular components. In this work we have used a variety of approaches from cell culture to transgenic animals with tools of molecular and developmental biology to elucidate the function of polyamines and specify mechanisms in which they are involved.

The specific aims of the present work were:

1. to clarify mechanisms by which transgenic mice overexpressing polyamine biosynthetic enzymes maintain their polyamine pools;
2. to identify genes responsive to changes in polyamine synthesis;
3. to characterize mammalian cytosolic *acetyl-CoA synthetase* (*AceCS1)*
4. to gain a better understanding of the regulatory functions of polyamines using the embryonic kidney as a model organ

4 Materials and methods

Detailed description of the materials and methods are presented in the original publications I-IV.

4.1 Mouse lines (I, II, III, IV)

A transgenic mouse line overexpressing S-AdoMetDC (UKU99) was obtained by the standard pronuclear microinjection technique (Hogan *et al.* 1986). A 19.5 kb fragment consisting of the entire rat S-AdoMetDC gene with 3kb of 5'- and 0.9 kb of 3'- flanking sequences was injected into fertilized mouse oocytes. A hybrid transgenic mouse line was generated by mating the UKU99 mice with UKU2, a transgenic mouse line which overexpresses the human ODC gene (Halmekytö *et al.* 1991a) (I, II).

For whole-mount (III, IV) and organ culture (IV) experiments whole embryos or embryonic tissues were isolated from the CD-1mouse line, with day 0.5 being the day of detection of the vaginal plug.

The effect of DFMO on *Pax-2* epithelial expression was studied by using a *Pax-2/lacZ* mouse line, which expresses a β-galactosidase gene under the control of a 8.5 kb-long *Pax-2* promoter (Kuschert *et al.* 2001) (IV).

4.2 Cell culture (I, II, IV)

To study mechanisms by which hybrid mice overexpressing *ODC* and *S-AdoMetDC* maintain their polyamine pools (I), and for the differential display analysis (II) early-passage mouse embryonic transgenic and non-transgenic fibroblasts from UKU2/UKU99 cross mated mice hybrids were grown in DMEM with 20% (v/v) fetal bovine serum in 5% CO_2/95% air at 37°C. In the pulse labeling experiments (I) subconfluent cells were incubated for 2 hours with 0.4 μCi/ml of L-[U-^{14}C] ornithine. After labeling, the cells

were grown for 4-24 hours, and the concentrations and radioactivity of the polyamines were determined.

For differential display analysis (II), Rat-2 cells (ATCC, Rockville, MD, USA) were grown in DMEM supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 5% (v/v) fetal bovine serum in 5% CO_2/95% air at 37°C. Cells were treated with 5 mM DFMO for 90 min or 48 h prior to differential display and for 48 hours before exposure to 4 µM actinomycin D for the mRNA half-life assays. To study the effect of DFMO on the turnover of Tex261 mRNA (II), subconfluent cells were transfected with a pBK-CMV-Tex261 construct using a standard calcium-phosphate transfection protocol (Sambrook *et al.* 1989) and grown for 48 hours in a medium containing 5 mM DFMO and/or 5µM MGBG.

For the analysis of the minimal Pax-2 promoter (IV), HEK 293 cells (ATCC) were grown in Eagle minimal essential medium supplemented with 2mM glutamine, 1mM sodium-pyruvate and 10% (v/v) heat-inactivated horse serum in 5% CO_2/95% air at 37°C. Subconfluent cells were transfected with luciferase reporter plasmids using Fugene 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions and grown for 48 hours in the presence or absence of 10 mM DFMO before harvesting for the luciferase assay.

4.3 Polyamine determination (I, II)

Polyamine concentrations were determined essentially as described earlier (Gilbert *et al.* 1991). Briefly, samples were passed over using a weakly acidic cation exchange resin, Biorex-70. After concentration (freeze-drying), samples were derivatized with dansyl-chloride and analyzed by high performance liquid chromatography (HPLC) on a reversed phase Ultrasphere ODS column using a methanol-acetonitrile-water gradient. The detector was a Waters 470 fluorescent scanning detector at an excitation wavelength of 330 nm and an emission wavelength of 510 nm. To standardize the different cell samples, DNA was quantified by the method described by Giles & Myers (1965).

4.4 Northern blot analysis (I, II, III)

Total RNA (Chomczynski & Sacchi 1987) was fractionated (15-20 µg/lane) on a 1% agarose gel containing formaldehyde (Meinkoth & Wahl 1984), blotted onto nitrocellulose and hybridized with cDNA fragments labeled with [α-^{32}P]dCTP using the random-priming method. The amount and the quality of total RNA loaded in the gels were checked by staining the transferred membrane with methylene blue (Herrin & Schmidt 1988).

4.5 Differential display (II)

Differential display was performed essentially as described previously (Liang & Pardee 1992, Liang *et al.* 1993). Briefly, total RNA was used in a reverse-transcription reaction using each of the four 1-bp-anchored 3´ oligo(dT) primers (Liang *et al.* 1994). The reverse-transcribed cDNA was used for each PCR reaction, performed using the same 1-bp-anchored 3´ oligo(dT) primer and four 5´ arbitrary oligonucleotides of 10 nucleotides in length. The parameters for PCR were 40 cycles of denaturation at 94 °C for 30 s, annealing at 40 °C for 2 min, and extension at 72 °C for 30 s. The PCR reaction mixture was loaded onto an 8% sequencing gel, and the differentially amplified PCR fragments were visualized by exposing the dried sequencing gel to X-ray film. Candidate PCR products were excised from the gel and DNA eluted from the gel slices by boiling in TE buffer for 10 min. The eluted DNA fragments were re-amplified by using the same primer pair and subsequently cloned into a TA cloning vector. Inserts were sequenced by cycle sequencing on an ABI sequencer and DNA sequences analyzed using the FASTA and BLAST computer programs. The isolated cDNA fragments were used as probes to confirm differential expression by northern-blot analyses.

4.6 Whole mount and section in situ hybridization (III, IV)

Whole-mount and section *in situ* hybridization was performed based on standard procedures (Wilkinson 1992). Digoxigenin labeled *AceCS1* antisense and sense probes were prepared from the mouse clone AF216873 in the pBluescript SK- vector (III). Full-length cDNAs from *Pax-2* (Dressler *et al.* 1990), *Pax-8* (Plachov *et al.* 1990), *Wnt-11*, *c-ret*, *GDNF* (Majumdar *et al.* 2003), *E-cadherin* (Ringwald *et al.* 1987), and *BF-2* (Hatini *et al.* 1996) were also labeled with digoxigenin. The antisense and sense *ODC* probe was synthesized from the 749 bp *Hin*dIII-digest of the full-length mouse cDNA inserted into pBluescript SK- vector (IV).

4.7 Sex typing (III)

The sex of E11.5 and E12.5 embryos was determined by a PCR method. A 353-bp region from the last intron of the mouse Zfy gene was amplified as described previously (Chang *et al.* 1994).

4.8 Organ culture (IV)

Kidney rudiments were isolated from mouse embryos at E11.0 and placed on small pieces of Nucleopore filter supported by stainless steel grids in a medium consisting of DMEM with Glutamax-I supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) fetal bovine serum (Lin *et al.* 2001). Cultured kidneys were grown in 5% CO_2/95% air at 37 ˚C for 1, 2, 3, 4, 6 days, in the presence or absence of DFMO or together with DFMO and putrescine, the medium being changed every second day.

4.9 Cell proliferation assay (IV)

The cell proliferation assay was performed using cell proliferation kit (Amersham) following the manufacturer's instructions. E11.0 mouse kidneys were grown in culture for 3 days. After 1 hour of BrdU-labeling, kidney specimens were paraffin-embedded and cut into 5 µm sections. BrdU was incorporated into replicating DNA of proliferating cells and subsequently localized using specific monoclonal antibody. Detection of bound antibody was achieved using peroxidase conjugated antibody to mouse immunoglobulin and DAB as peroxidase substrate, giving blue-black staining at sites of BrdU incorporation.

4.10 Histology, β-galactosidase and antibody staining (IV)

Hematoxylin-eosin and β-galactosidase staining was performed using standard protocols (Hogan *et al.* 1994). For hematoxylin-eosin staining, kidney specimens were fixed in 4% paraformaldehyde, washed in PBS, dehydrated and embedded in paraffin. 5µm sections were cut and stained with hematoxylin and eosin.

For β-galactosidase staining, kidney specimens were fixed for 30 min at 4 ˚C in a solution containing 1% formaldehyde and 0.2% glutaraldehyde, washed at room temperature in PBS and stained overnight at 37˚C in PBS containing 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 2mM $MgCl_2$, 0.01% NaDeoxycholate, 0.02% NP-40 and 1mg/ml X-gal. After staining, specimens were washed in PBS, post-fixed in 4% paraformaldehyde and stored in 80% glycerol.

Methanol fixed organ culture samples were double-stained with Troma-1 against cytokeratin Endo A (Developmental Studies Hybridoma Bank, USA) and brushborder (BB) (Ekblom *et al.* 1980) antibodies. The secondary antibodies used were FITC-conjugated donkey anti-rat IgG (Jackson Immuno Research Laboratories, USA) and TRITC-conjugated donkey anti-rabbit IgG (Rockland Immunochemicals, USA) respectively.

4.11 Other methods (I, II)

The activity of S-AdoMetDC and ODC was measured as described earlier (Jänne and Williams-Ashman 1971b, 1971a) (I).

RT-PCR was used for the identification of the rat-specific S-AdoMetDC mRNA (I), the differential display analysis (II) and the determination of the half-life of S-AdoMetDC mRNA (II). In brief, total RNA (Chomczynski & Sacchi 1987) was reverse-transcribed with a sequence specific sense primer using an AMV (I) or a M-MLV (II) reverse transcriptase (Promega). After the first strand synthesis, antisense primer and a thermostable DNA polymerase were added, and PCR amplification was performed as described in the articles referenced above.

5 Results

5.1 Overexpression of S-AdoMetDC and ODC in transgenic mice does not dramatically affect tissue spermidine and spermine pools (I)

Cells have an efficient compensation machinery to correct disturbances in polyamine homeostasis. To study these mechanisms we have generated a mouse line overexpressing *S-AdoMetDC*. The UKU99 *S-AdoMetDC*-overexpressing transgenic mice have a 2-4 fold increase in S-AdoMetDC activity in all tissues tested. This increase in enzyme activity is not dramatic, but the expression of the transgene is presumably controlled by tissue-specific control elements located in the 3-kb-long 5'-flanking region of the transgene construct. Mice from another transgenic line, UKU2, generated by our collaborators (Halmekytö *et al.* 1991a) overexpress *ODC* 10-100-fold in their tissues, most markedly in the testis and brain. These mice have been generated with a shorter, 800bp, 5'-flanking region in the transgene construct, probably lacking the tissue-specific control elements (Halmekytö *et al.* 1991b).

In the *S-AdoMetDC*-overexpressing mice, tissue putrescine levels decreased but spermidine and spermine pools were practically unaltered. Nevertheless, the molar ratio of spermidine to spermine was lower in the transgenic animals, indicating a faster conversion of putrescine and spermidine into spermine in the presence of excess decarboxylated S-AdoMet. To study whether the decreased putrescine level is responsible for the unchanged spermidine and spermine pools, a hybrid UKU2/UKU99 transgenic mouse line was generated. In the hybrid mice overexpressing both *ODC* and *S-AdoMetDC*, the putrescine pool tended to normalize compared to the aberrantly high putrescine levels seen in UKU2 mice, whereas spermidine and spermine concentrations remained unchanged.

5.2 Transgenic mice compensate for increased S-AdoMetDC and ODC activities by acetylating and excreting excessive polyamines (I)

To study the mechanisms by which UKU2/UKU99 hybrid mice overexpressing both *ODC* and *S-AdoMetDC* maintain their polyamine homeostasis, we performed pulse-labeling of polyamine-flux in transgenic and non-transgenic early-passage embryonic fibroblasts. Pulse-labeling was carried-out using ^{14}C-labeled L-ornithine, the substrate of the rate limiting polyamine biosynthetic enzyme, ODC. Polyamine synthesis and secretion was monitored by measuring intracellular and extracellular concentrations of polyamines and their acetylated derivatives, as well as by following the incorporation of radioactivity into acetylated and non-acetylated polyamines.

We found a time-dependent polyamine concentration shift between hybrid and control fibroblasts. Intracellular putrescine levels were elevated in the transgenic fibroblasts 4 hours after pulse-labeling, but normalized after 24 hours. On the other hand, 24 hours after pulse-labeling, extracellular putrescine and N^1-acetylspermidine accumulated in the medium of the transgenic fibroblasts. By following the fate of radioactive ornithine, we have shown that polyamine biosynthesis is accelerated and polyamine acetylation is increased in the UKU2/UKU99 hybrid fibroblasts. These results indicate that a compensation mechanism, including acetylation and increased excretion, prevents the toxic accumulation of higher polyamines in cells.

5.3 Changes in gene expression patterns in response to polyamine depletion and activation of biosynthesis (II)

To further clarify the regulatory mechanisms involving polyamines, we applied a differential display approach for identifying mRNAs responsive to changes in polyamine levels. Rat-2 cells were treated for short-term (90 min) and for long-term (48 hours) with DFMO to block the function of ODC and consequently deplete cellular polyamines. The two different time periods served as a control to be able to distinguish between direct effects caused by polyamine depletion and secondary effects due to the cessation of cell growth. The concentration of higher polyamines, spermidine and spermine did not change after 90 min of DFMO treatment, but putrescine level decreased by about 30%. Long-term DFMO treatment almost completely depleted cellular putrescine, spermidine concentration decreased to 10% of the control level, whereas the spermine level remained unchanged.

Differential display resulted in 15 cDNA bands after 90 min and 44 cDNA bands after 48 hours with significant intensity differences. The majority of these cDNA bands (43) were more intense in the polyamine-depleted samples and only 16 of them indicated down-regulation after DFMO treatment. A repeated differential display analysis confirmed 35 cDNA bands of the 59 and these were subcloned, sequenced and 11 of them

subjected to Northern blot analysis to further confirm differential display results. Six clones, mitochondrial adenine nucleotide translocator, CarG-box binding factor, ribosomal proteins L3 and 3Sa, acetoacetyl-CoA thiolase (EC 2.3.1.9) as well as the mouse EST (MM97319), later characterized as cytosolic acetyl-CoA synthetase (EC 6.2.1.1) (see in chapters 5.6-5.7), showed results similar to those obtained from differential display analysis, the first two transcripts being present both after short-term and long-term DFMO treatment. Five of the six clones were upregulated and only one down-regulated in polyamine-depleted cells.

To study the compensation mechanisms under conditions where polyamine synthesis is enhanced, we performed differential display analysis with kidneys from UKU2/UKU99 hybrid mice overexpressing both *ODC* and *S-AdoMetDC* (I) and from non-transgenic mice. Following the confirmation procedure described previously, we have found only two cDNA clones having different gene-expression pattern in transgenic and non-transgenic kidneys, the ribosomal protein L3 and Tex261, both downregulated in transgenic animals. Table 1 shows the general features of the confirmed genes from both the cell culture and the transgenic approach.

Table 1. Gene-expression differences in cell culture in response to polyamine level alterations, function of genes and properties of their promoters

Gene	Effect of DFMO treatment	Status in transgenic mice	Function	Mammalian promoter properties
Cytosolic acetyl-CoA synthetase	down		Supplies acetyl-CoA for lipid biosynthesis	Lacks TATA box, CCAAT box, SRE, E-box, GC-box binding Sp1 and/or Sp3 (Ikeda *et al.* 2001)
Mitochondrial adenine nucleotide translocator	up		Mitochondrial ATP/ADP exchanger, part of the mitochondrial permeability transition pore complex, involved in apoptosis	TATA box, CCAT box, intronic Sp1 binding sites (Li *et al.* 1989)
CArG-box binding factor	up		Transcription regulator, enhances myogenic differentiation	Not published
Ribosomal protein L3 (rpL3)	up	down	Involved in the formation of the peptidyltransferase center	Not published
Ribosomal protein 3Sa	up		40S ribosomal subunit protein; involved in induction of apoptosis and malignant transformation	Lacks TATA and CAAT boxes, CpG-islands with potential Sp1 binding site, CREB/ATF binding sites (Nolte *et al.* 1996)
Mitochondrial acetoacetyl-CoA thiolase	up		Catalyses the conversion of acetyl-CoA to acetoacetyl-CoA in the cholesterol biosynthetic pathway	Lacks TATA box, CAAT boxes, Sp1, AP-2 and GTI binding sites (Kano *et al.* 1991)
Tex261		down	Potential transmembrane protein	High rate of CpG-islands (Lopez-Fernandez *et al.* 1998)

5.4 Increased GAPDH and β-actin expression in polyamine-depleted cells (II)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin probes are widely used as loading standards in Northern-blot analysis. Earlier studies have shown that β-actin levels increase upon DFMO treatment (Celano *et al.* 1988) and that was the case also under our experimental conditions. GAPDH responded similarly to polyamine depletion, with an increase in the intensity of the transcripts, whereas 28S and 18S rRNA levels remained unchanged. Hence we propose that hybridization or staining of 28S and 18S rRNA in Northern-blot analysis is a more reliable loading control than the use of β-actin or GAPDH.

5.5 Polyamine depletion results in selective stabilization of the mRNAs (II)

To clarify the mechanisms leading to an increase in mRNA levels in the majority of our polyamine-depleted samples, we studied whether mRNA accumulation is due to the stabilization of the message. Turnover of S-AdoMetDC, GAPDH and Tex261 was measured after actinomycin D treatment. All of the three transcripts were clearly stabilized after DFMO treatment with a constant mRNA level up to 6 hours for S-AdoMetDC, the same transcript having a half-life of 3 hours in control cells. These results indicate that polyamine depletion results in selective changes in the expression of a panel of genes, mostly by upregulation of transcripts, via stabilization of the message.

5.6 Cytosolic acetyl-CoA synthetase and polyamines

Cytosolic acetyl-CoA synthetase (*AceCS1*) was the only downregulated gene identified in polyamine-depleted cells using differential display analysis (II). Its response to polyamine concentration changes was similar to that of SSAT, the latter using acetyl-CoA to produce N^1-acetylspermidine and N^1-acetylspermine. Our hypothesis was that AceCS1might provide acetyl-CoA for polyamine acetylation by SSAT. We isolated the mouse cDNA clone (AF216873), used it for transfection of Rat-2 cells and measured polyamine concentrations. Although AceCS1 activity was two-three times higher in the transfected cells, there was no difference in the level of polyamines or acetylated polyamines relative to controls (unpublished results). At the same time we studied the tissue distribution of AceCS1 in adult mice and during embryonic development, to better understand the possible functions of this enzyme. Meanwhile Luong and co-workers (2000) published their results concerning the characterization of the homologous human AceCS1 gene, linking it to lipid biosynthesis. As by that date we did not have any

promising link between AceCS1 and polyamine catabolism, we decided to focus on the tissue distribution studies.

5.7 Expression of AceCS1 during mouse development (III)

Tissue distribution of the *AceCS1* mRNA in adult mice was determined with Northern-blot hybridization. A 3-kb-long single transcript was found in all the tissues studied with the highest intensity in the ovaries, testes and kidney.

To study *AceCS1* expression during embryonic development, we used whole-mount and tissue section *in situ* hybridization techniques. In the early embryonic stages (E9.5-E11.5) *AceCS1* transcripts were found in the cephalic region, with the strongest intensity in the forebrain. From E10.5 *AceCS1* was expressed also in the spinal cord and in the dorsal root ganglia and at E11.5 expression expanded to the liver as well. In later embryonic stages *AceCS1* transcripts were detected in the testes (from E12.5) and in the ovaries (from E13.5). Ovarian expression decreased gradually from E15.5, whereas in kidneys there was no detectable whole-mount staining at any of these stages. Section *in situ* hybridization of urogenital blocks from E14.5-E16.5 embryos and newborn mice revealed the distribution of *AceCS1* mRNA inside different tissues. In testes *AceCS1* transcripts were localized to the interstitial Leydig cells at all stages studied, while ovaries showed a transient expression, peaking at E14.5. In the kidneys *AceCS1* signals appeared at E16.5 in a subset of renal tubules and expression remained similar in newborns. These results indicate that expression of AceCS1 is developmentally regulated, suggesting a specific role during mouse embryogenesis in addition to the generally accepted function of providing acetyl-CoA for lipid biosynthesis.

5.8 Expression of ODC is developmentally regulated (IV)

For our studies on polyamine homeostasis, we applied a new approach in polyamine research, taking advantage of the various tools of developmental biology. We studied changes in morphogenesis and the expression of the developmental control genes in the embryonic mouse kidney in response to polyamine depletion, using kidney organ culture. Gritli-Linde and coworkers (2001) have previously systematically analyzed ODC expression during fetal development in the mouse, finding its expression mainly in the epithelium of the developing tooth, testis, kidney, gut and hair follicles, all of which are developmentally dependent on proper epithelial-mesenchymal interactions. We used section *in situ* hybridization to study the expression pattern of ODC in early renal organogenesis. We found that although at E14.5 and E18.5 *ODC* is expressed mainly in the epithelial tissues such as the ureteric bud and tubules (Gritli-Linde *et al.* 2001), at earlier developmental stages (at E11.0 and E12.5) it is present also in the surrounding condensing mesenchyme, suggesting that ODC plays an active role in both epithelia and mesenchyme during kidney organogenesis.

5.9 DFMO treatment reduces kidney size via reduction in cell proliferation (IV)

To explore the potential pathways through which polyamines act during kidney organogenesis, we adopted an organ culture approach. Kidney rudiments were isolated from mouse embryos at E11.0 and treated with DFMO to inhibit ODC and consequently reduce the polyamine pools. DFMO treatment caused a clear phenotype in the polyamine-depleted specimens including smaller size and reduction in the size and number of tubules. The morphological changes were at least partly reversed by the addition of putrescine. A cell proliferation assay indicated a 50% decrease in the number of actively proliferating cells in the DFMO-treated kidneys, with only a few of the tubular cells proliferating. These results show that polyamine homeostasis is essential for kidney development and that polyamines are involved in the control of cell proliferation.

5.10 DFMO causes reduced branching and a delay in renal tubule formation (IV)

Morphological changes caused by polyamine depletion were studied using Troma-I and brushborder (BB) double immunostaining. Troma-I stains the branching ureter and BB visualizes the tubules. Kidney rudiments from mouse embryos at E11.0 were cultured for different time periods and following immunostaining, the branches were counted. From the second day onwards the branching was continuously reduced in the DFMO-treated kidneys, reaching 56% of the controls after 3 days in culture. The addition of 10 μM putrescine to the growth medium partly reversed the reduction in branching to 70% of the control level. Tubule formation was delayed by one day in DFMO-treated kidneys, appearing only on day 4. Hence polyamine depletion leads to inhibition of ureteric branching and tubule formation.

5.11 Changes in gene expression patterns in response to polyamine depletion during kidney organogenesis (IV)

To explore the molecular mechanisms that could be responsible for the developmental dysmorphology that follows polyamine depletion, we studied gene expression patterns using whole-mount *in situ* hybridization. E11.0 mouse kidneys were grown for 2 days in culture in the presence or absence of DFMO. Table 2 shows the general features of the differently expressed genes from the kidney organ culture approach.

*Table 2. Gene-expression differences in kidney organ culture in response to polyamine depletion, function of genes and properties of their promoters. *: unchanged general but upregulated epithelial expression*

Gene	Effect of DFMO treatment	Function	Mammalian promoter properties
ODC	up	Decarboxylates L-ornithine to form putrescine	TATA box, CAAT box, Sp1, a cell-specific modulatory element and WT1 binding sites (Moshier *et al.* 1996, Palvimo *et al.* 1996)
C-ret	up	protooncogene, transmembrane receptor tyrosine kinase, crucial for neural and kidney development	Lacks TATA box, Sp1-binding sites (Sukumaran *et al.* 2001)
E-cadherin (uvomorulin)	up	Ca^{2+}-dependent cell adhesion molecule	Lacks TATA box, CCAAT box, Sp1, AP2, CP1, glucocorticoid receptor and progesterone receptor binding sites (Ringwald *et al.* 1991)
Wnt-11	unchanged	Secreted glycoprotein regulating ureteric branching	Not published
Pax-8	down	Transcription factor controlling mesenchymal-epithelial conversion throughout kidney development together with Pax-2	TATA box, CCAAT box, AP2, Sp1, PEA3, zeste and NF-κB binding sites (Okladnova *et al.* 1997)
Pax-2	unchanged/ up*	Transcription factor controlling mesenchymal-epithelial conversion throughout kidney development together with Pax-8	Lacks TATA and CCAAT boxes, WT1, NF-IL6, EGR-1, Sp1 and PEA3 binding sites (Stayner *et al.* 1998)
GDNF	unchanged	Neurotrophic factor signaling via c-ret	TATA box, CpG-islands, Sp1 binding sites (Tanaka *et al.* 2001)
BF-2 (Foxd1)	unchanged	Transcription factor, regulating the expression of the stroma-cell-derived signal in kidney	Not published

Polyamine depletion increased *ODC* mRNA expression and transcripts were found to accumulate in the ureter buds. Epithelial *c-ret* and *E-cadherin* expression increased as well, while the *Wnt-11* transcripts did not show any significant intensity changes but did indicate a decrease in tip numbers. From the mesenchymal genes tested, only *Pax-8* responded to polyamine depletion with a marked decline in mRNA level, whereas the *GDNF*, *BF-2* and *Pax-2* transcripts did not show significant differences between the controls and DFMO-treated samples. These results suggest that polyamine depletion has a selective regulatory effect on epithelial and mesenchymal gene expression during kidney organogenesis.

5.12 Effects of polyamine depletion on the Pax-2 promoter (IV)

Under normal conditions *Pax-2* is expressed in the ductal and mesenchymal components of the developing urogenital system, including the Wolffian duct, the ureter and the condensing mesenchyme. Whole-mount immunostaining visualizes both epithelial and mesenchymal *Pax-2* expression. Epithelial expression of *Pax-2* was studied in kidneys isolated from a *Pax-2/lacZ* mouse line (Kuschert *et al.* 2001), in which an 8.5kb *Pax-2* promoter drives β-galactosidase expression only in the epithelium of the ureter and the Wolffian duct, whereas the mesenchymal cells are not stained. Dissected E11.0 kidneys, cultured for 48 hours in the presence of DFMO, showed a strongly elevated epithelial *Pax-2* expression, whereas the addition of spermidine to the growth medium reduced this increase to close to the control level.

To study the mechanisms by which polyamine depletion results in increased *Pax-2* expression in the *Pax-2/lacZ* mice, we transiently transfected HEK 293 cells with a vector construct containing the minimal 4.1 kb *Pax-2* promoter fused to the luciferase gene (Patel & Dressler 2004). Polyamine depletion with DFMO caused a slight but significant increase in transcription from the *Pax-2* promoter. Thus we can conclude that polyamines participate in regulation of the *Pax-2* gene at the transcription level.

6 Discussion

6.1 Compensation mechanisms against increased S-AdoMetDC and ODC activities in transgenic mice

Polyamines are present in millimolar concentrations in cells and their homeostasis is tightly regulated by complex machinery including biosynthesis, catabolism and transport. One way to study this regulatory machinery is the generation of transgenic animals with enhanced polyamine biosynthesis. In this work a transgenic mouse line moderately overexpressing *S-AdoMetDC* was generated. Although in *S-AdoMetDC*-overexpressing cells (Kramer *et al.* 1995, Manni *et al.* 1995) polyamine profiles changed markedly, showing an increase in spermine and decrease in putrescine and spermidine levels, transgenic mice did not show any phenotypic alterations. A slight decrease in their tissue putrescine concentrations was observed, but spermidine and spermine levels remained practically invariable despite the two to four fold higher S-AdoMetDC activity. The fact that these animals endeavor to balance their polyamine pools is not surprising, as accumulation of spermidine and spermine is toxic for the cells (Poulin *et al.* 1995b). A transgenic mouse line generated earlier moderately overexpressing spermidine synthase (Kauppinen *et al.* 1993) showed similarly stable polyamine distribution, suggesting that accumulation of spermidine and spermine is possibly controlled by a more complex mechanism. In order to provide more putrescine as substrate for S-AdoMetDC, a hybrid mouse line overexpressing *S-AdoMetDC* and *ODC* was generated. In these hybrid mice the putrescine level was between those of the *S-AdoMetDC*-overexpressing and the *ODC*-overexpressing mice, still without an increase in spermidine and spermine tissue concentrations. Pulse-labeling studies with primary fetal fibroblasts revealed that in hybrid mice the polyamine flow was faster than in the nontransgenic ones and that accumulation of the higher polyamines is at least partly prevented by their acetylation and increased export from the cells. The fact that SSAT activity did not show any marked increase in these animals predicts new compensatory pathways and enzymes to be discovered in the regulation of polyamine homeostasis. A possible approach for future work could be the generation of a triple transgenic mouse line, overexpressing *S-AdoMetDC*, *spermidine synthase* and *ODC* together, possibly with higher S-AdoMetDC and spermidine synthase activities.

6.2 Genes responding to polyamine depletion and activation of polyamine biosynthesis

6.2.1 The differential display approach

We aimed to identify novel genes responsive to changes in polyamine synthesis. Differential display analysis resulted in seven candidate genes, of which only one was downregulated after polyamine deprivation (Table I). Among these candidate genes there were genes coding for ribosomal and mitochondrial proteins, proteins involved in lipid biosynthesis and apoptosis, a transmembrane protein and a transcription regulator. However, these genes did not share any obvious common functions and it was difficult to find a link between them and the various physiological processes in which polyamines are involved.

Polyamine depletion causes suppression of cell growth and inhibits DNA, RNA and protein synthesis (Oredsson *et al.* 1990). DFMO treatment resulted in both up- and downregulated genes indicating that these changes are not solely due to the generally decreased transcription rate during growth arrest. However most of the transcripts were upregulated suggesting a general phenomenon. In addition to polyamine depletion, similar prolongation in the half-lives of mRNAs has been observed by using protein synthesis inhibitors (Stimac *et al.* 1984, Altus & Nagamine 1991, Ichikawa *et al.* 2003). If polyamine depletion stabilizes mRNAs via inhibition of protein synthesis, a possible link between them could be the eukaryotic initiation factor 5A (eIF-5A). eIF-5A is a polyamine-dependent protein, necessary for cell proliferation, processed post-translationally by the synthesis of hypusine from spermidine and a lysine residue (Park *et al.* 1981). Spermidine depletion reduces the modified eIF-5A pool and suppresses cell growth (Tome & Gerner 1996). eIF-5A likely plays a role in mRNA turnover, since mRNAs are stabilized in yeast in which the *TIF51A* gene, encoding eIF-5A, is inactivated (Zuk & Jacobson 1998). Polyamine depletion was also reported to stabilize certain mRNAs containing an eIF-5A consensus binding sequence in their 3'-UTR (Parker & Gerner 2002). Stabilization of mRNAs contributes to strong and rapid induction of genes. mRNA stabilization with inhibition of translation might reflect a physiological process that occurs during mitosis (Ross 1997). Ross hypothesized that translational repression during mitosis inactivates a critical component of the mRNA degradation machinery to preserve the capacity of cells to synthesize essential proteins needed in the G_1 phase. If labile mRNAs were not stabilized during mitosis they would be depleted as the cell entered G_1. mRNA stabilization might serve a similar purpose during polyamine starvation or any other stress negatively effecting translation.

The only downregulated gene in polyamine-depleted cells was *acetyl-CoA synthetase* (*AceCS1*). AceCS1 was characterized as an enzyme regulated by sterol regulatory element-binding proteins (SREBPs) (Luong *et al.* 2000). SREBPs are transcription factors that coordinate the regulation of genes involved in the synthesis, transport and metabolism of lipids such as cholesterol and fatty acids. Lipogenic gene expression is upregulated by androgens, accompanied by an increase in the levels of transcriptionally

active SREBPs (Heemers *et al.* 2003), that could explain our finding of strong *AceCS1* expression in the testosterone-secreting Leydig cells in the developing mouse testis.

Similarly to AceCS1, polyamine depletion also reduces the mRNA level of SSAT, the rate-limiting enzyme in the degradation and interconversion of polyamines (Shappell *et al.* 1993). Protein synthesis inhibitors induce the accumulation the SSAT mRNA (Fogel-Petrovic *et al.* 1996) indicating two separate pathways. SSAT uses acetyl-CoA to produce N^1-acetylspermidine and N^1-acetylspermine. A recent study with TRAMP (transgenic adenocarcinoma of mouse prostate)/SSAT hybrid mice reported a markedly decreased acetyl-CoA pool in the bigenic animals relative to TRAMP mice (Kee *et al.* 2004). AceCS1 might provide acetyl-CoA to SSAT for polyamine acetylation, a reaction downregulated in polyamine-depleted cells, but we were not able to show it experimentally.

The fact that the expression of *AceCS1* is developmentally regulated suggests that its function might be more complex than providing acetyl-CoA for general lipid biosynthesis.

6.2.2 The kidney culture approach

The expression of *ODC* is developmentally regulated in the mouse. During early kidney organogenesis it is expressed in the ureteric bud and the surrounding mesenchyme, suggesting for it an active role in epithelial-mesenchymal interactions. Polyamine-depleted kidneys in organ culture have a severe phenotype, such as smaller size, reduced branching and retarded tubule formation, resulting at least partly from the decreased rate of cell proliferation.

Polyamine depletion results in a change in the expression of a panel of genes (Table 2). In addition to *ODC*, the epithelial markers *E-cadherin* and *c-ret* are upregulated, mesenchymal *Pax-8* downregulated, whereas *GDNF*, *Wnt-11*, *BF-2* and *Pax-2* transcripts remain unchanged. One possible reason for the increased *OCD*, *E-cadherin* and *c-ret* levels in the polyamine-depleted specimens might be mRNA accumulation as a result of the selective stabilization of the messages described earlier.

The Ret/GDNF signaling pathway is of great importance in the coordination of ureteric branching. GDNF released from the mesenchyme activates Ret in the adjacent epithelium and induces proliferation of epithelial cells, ureter growth and development (Durbec *et al.* 1996, Srinivas *et al.* 1999). Wnt-11 was reported to regulate mesenchymal GDNF expression, while ureteric Wnt-11 expression is reciprocally dependent upon Ret/GDNF signaling (Majumdar *et al.* 2003). Under our experimental conditions *c-ret* (the gene coding for Ret) mRNA levels increased but *GDNF* and *Wnt-11* expression did not show any changes after polyamine depletion. Srinivas (1999) reported that increased expression of *c-ret* in the ureteric bud results in the retardation of kidney development. Kidneys of the *c-ret* overexpressing mice had phenotype similar to the polyamine-depleted kidneys presented in this work, suggesting that polyamines might act directly on *c-ret* expression to induce cell proliferation without affecting *GDNF* or *Wnt-11* levels. The selective changes of various genes in the ureteric bud and

mesenchyme indicate a possible role for polyamines in the regulation of epithelial-mesenchymal interactions during mouse kidney development.

6.2.3 Common features of genes responding to polyamine depletion

Spermidine was shown to regulate protein synthesis by mRNAs containing a GC-rich 5'-untranslated region (Shimogori *et al.* 1996), with stimulation at low and inhibition at high spermidine levels. The common feature in all of the genes we found to be upregulated after polyamine depletion was a similar GC-rich 5' structure with one or more Sp1 binding sites (Tables 1 and 2). Transfection studies with the minimal *Pax-2* promoter revealed enhanced transcription in polyamine-depleted cells, although the detected difference in the Pax-2 mRNA level was much higher than expected from the promoter studies, suggesting a more complex mechanism. The lack of classical TATA or CCAAT boxes, an increased GC content with functional Sp1 site(s) in the proximal promoter region, and a CpG island close to the transcriptional initiation site are features typical of housekeeping genes (Rundlof *et al.* 2001), but a subclass of polymerase II promoters including developmental genes like *WT1* and *Pax-2* shows similar characteristics (Stayner *et al.* 1998). However, the *ODC* gene does have a TATA box, indicating that elevated transcription is not dependent on the existence of this core promoter element. Sp1 is considered to be a constitutively expressed transcription factor and has been implicated in the regulation of a wide variety of housekeeping genes, tissue-specific genes, and genes involved in the regulation of growth (Song *et al.* 2001). Sp1 can interact with other cellular transcription factors and activate expression of various genes with or without TATA boxes (Mitchell & Tjian 1989). Glycosylation and phosphorylation can affect the stability or activity of the Sp1 protein: a variety of cellular kinases are able to phosphorylate it (Leggett *et al.* 1995, Armstrong *et al.* 1997, Roos *et al.* 1997). One of the Sp1-phosphorylating kinases is casein kinase II (CK2), present in the cytoplasm and nucleus of eukaryotic organisms. Polyamines were shown to bind CK2, inducing conformational changes in the holoenzyme and stimulate CK2-mediated phosphorylation (Leroy *et al.* 1997). CK2-mediated phosphorylation decreases the DNA binding activity of Sp1, suggesting that CK2 may play a role in the regulation of this zinc finger transcription factor (Armstrong *et al.* 1997). In a different study (Chen *et al.* 1997) polyamines were shown to inhibit the binding of Sp1 to its cognate binding motif. One can hypothesize that depletion of polyamines might diminish Sp1 phosphorylation via CK2, and as a consequence Sp1 would be able to bind to the promoter and induce transcription of various genes (Figure 8).

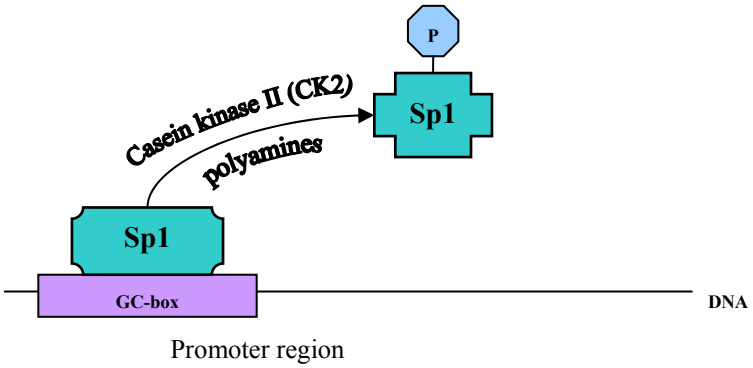


Fig. 8. Hypothesis of the effect of polyamines impeding transcription activation of Sp1. Detailed description in the text.

Interestingly, cell-type differential transcription was proposed to be regulated by Sp1 and Sp3, epithelial cells having higher Sp1 levels than fibroblasts (Apt *et al.* 1996). Based on the expression pattern of *ODC*, Gritli-Linde *et al.* (2001) have proposed that during early development of organs involving epithelial-mesenchymal interactions, polyamines are produced by the epithelia, secreted and then taken up by mesenchymal cells. ODC inhibitors were also the most effective in the inhibition of tumor formation in epithelial tissues (Meyskens & Gerner 1999) indicating cell-type specific mechanisms. Taken together with our results when epithelial *E-cadherin, c-ret* and *Pax-2* were upregulated after polyamine depletion, we propose that in addition to the selective stabilization of the mRNAs, polyamines might influence gene expression by regulation of transcription via the transcription factor Sp1. Naturally, regulation of a gene is not the function of a single parameter, as Sp1-binding GC-boxes also play a role in the gene expression of nonepithelial cells, and genes containing Sp1 binding sites in their promoters might respond differently to polyamine depletion, confirming that expression of any given gene depends on the simultaneous interaction of a specific combination of proteins.

7 Conclusions

In this work different approaches were used to elucidate the function of polyamines and specify mechanisms in which they are involved. Transgenic animals, overexpressing polyamine biosynthetic enzymes S-AdoMetDC and ODC were found to have an effective compensation mechanism, including acetylation and increased excretion, which prevents the toxic accumulation of higher polyamines in cells.

Polyamine depletion results in selective changes in the expression of a panel of genes, mostly by the upregulation of transcripts and by increasing the half-life of the mRNAs. The stabilization of mRNAs presumably occurs via inhibition of protein synthesis, resulting from the diminished pool of the eukaryotic initiation factor 5A (eIF-5A). Based on the promoter sequences of genes responding to changes in polyamine levels, it is possible that in addition to the selective stabilization of the mRNAs, polyamines might influence gene expression by regulation of transcription via the transcription factor Sp1. The only downregulated gene in polyamine-depleted cells, *acetyl-CoA synthetase* (*AceCS1*) is developmentally regulated. AceCS1 is involved in lipid biosynthesis, having among others a strong expression in the testosterone-secreting Leydig cells in the developing mouse testis.

The expression of *ODC* is also developmentally regulated, its transcripts being present in the ureteric bud and the surrounding mesenchyme during early renal organogenesis. Polyamine depletion results in clear alterations in kidney organogenesis in the mouse: ureteric branching is reduced and tubule formation retarded in DFMO-treated kidney specimens via a reduction in cell proliferation. Polyamine depletion results in a change in the expression pattern of epithelial and mesenchymal genes, indicating a possible role for polyamines in the regulation of mesenchymal-epithelial interactions during kidney development. Polyamines were shown to have an important role in proper kidney organogenesis and the kidney seems to serve as a suitable model for studying the detailed molecular mechanisms by which these cationic molecules are involved in inductive epithelial-mesenchymal tissue interactions.

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