Depletion of cellular polyamines, spermidine and spermine, causes a total arrest in translation and growth in mammalian cells

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The polyamines, putrescine, spermidine, and spermine, are essential polycations, intimately involved in the regulation of cellular proliferation. Although polyamines exert dynamic effects on the conformation of nucleic acids and macromolecular synthesis in vitro, their specific functions in vivo are poorly understood. We investigated the cellular function of polyamines by overexpression of a key catabolic enzyme, spermidine/spermine N^1-acetyltransferase 1 (SAT1) in mammalian cells. Transient cotransfection of HeLa cells with GFP and SAT1 vectors suppressed GFP protein expression without lowering its mRNA level, an indication that the block in GFP expression was not at transcription, but at translation. Fluorescence single-cell imaging also revealed specific inhibition of endogenous protein synthesis in the SAT1 overexpressing cells, without any inhibition of synthesis of DNA or RNA. Overexpression of SAT1 using a SAT1 adenovirus led to rapid depletion of cellular spermidine and spermine, total inhibition of protein synthesis, and growth arrest within 24 h. The SAT1 effect is most likely due to depletion of spermidine and spermine, because stable polyamine analogs that are not substrates for SAT1 restored GFP and endogenous protein synthesis. Loss of polysomes with increased 80S monosomes in the polyamine-depleted cells suggests a direct role for polyamines in translation initiation. Our data provide strong evidence for a primary function of polyamines, spermidine and spermine, in translation in mammalian cells.

polyamine depletion | translational regulation | cell proliferation | RNA-FISH

The polyamines, putrescine [$NH_2(CH_2)_4NH_2$], spermidine [$NH_2(CH_2)_4NH(CH_2)_3NH_2$], and spermine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$], are ubiquitous in living organisms and are essential for eukaryotic cell proliferation (1–3). Because their primary and secondary amino groups are protonated at physiological pH in cells, these polycations interact with negatively charged molecules such as DNA, RNA, proteins, and phospholipids (4). Polyamines have been implicated in diverse biological processes, including replication, transcription, translation, post-translational modification, ion channel gating, and membrane stability (4), and they regulate cellular proliferation, transformation, differentiation, apoptosis, and tumorigenesis (3, 5). Dysregulation of cellular polyamines is associated with various pathological conditions, including cancer, and polyamine pathways have been explored as targets for cancer chemotherapy and chemoprevention (5, 6). However, the precise physiological functions of polycationic polyamines in vivo and the mechanism of their actions in mammalian cell proliferation have remained largely obscure.

One known critical function of polyamines in eukaryotes is the role of spermidine for the covalent modification of one cellular protein, eukaryotic initiation factor 5A (eIF5A), resulting in an unusual amino acid, hypusine [N^{ε}-(4-amino-2-hydroxybutyl) lysine] (7, 8). eIF5A and hypusine modification are absolutely required for the viability and growth of mammalian cells (9–12).

The functions of polyamines in mammalian cells have been investigated using polyamine auxotrophic mutants defective in their biosynthesis (13, 14) or inhibitors of polyamine biosynthesis such as α-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC) (15, 16). Although DFMO treatment usually causes a rapid depletion of putrescine and spermidine, the arrest in growth appears somewhat delayed, suggesting potential mediators or secondary effects (17). Because cellular spermine was not depleted in the DFMO studies, the combined function of the two polyamines could not be properly addressed and their specific molecular mechanisms could not be deduced.

Spermidine/spermine N^1-acetyltransferase 1 (SAT1), the key regulatory enzyme in the catabolism of polyamines (18), catalyzes acetylation of spermidine or spermine to generate N^1-acetyl spermidine or N^1-acetyl spermine, and N^1, N^{12}-diacetylspermine. The acetylated polyamines, in turn, are oxidatively degraded by N^1-acetylpolyamine oxidase (APAO) to *N*-acetylaminopropanal and a lower polyamine. Thus, the induction of SAT1 leads to a decrease in cellular spermidine and spermine with an increase mainly in putrescine and N^1-acetylspermidine. The cellular level of SAT1 is normally extremely low, but it is induced rapidly by a variety of stimuli, including polyamines, polyamine analogs, toxic chemicals, certain drugs, and growth factors (18). SAT1 is regulated at the level of transcription, mRNA splicing, translation, and protein stability (18).

We previously reported a suppression of GFP protein expression in HeLa cells upon transient cotransfection with GFP and SAT1 vectors (19). The inhibition of GFP expression was dependent on the SAT1 activity and the effect was observed for SAT1 itself and any cotransfected genes. Degradation of plasmid DNA, RNA, or the protein did not appear to be involved in the suppression of GFP expression, as a nuclease inhibitor and various inhibitors of proteases, proteasome, autophagy, and lysosomes did not restore GFP expression.

In the present study, we have investigated the mechanism of the SAT1 effects to delineate at which step of transcription or translation GFP expression was blocked. Real-time PCR, RNA fluorescence in situ hybridization (FISH), and single-cell imaging of the endogenous synthesis of DNA, RNA, and protein revealed that protein synthesis was selectively blocked in the SAT1 overexpressing cells. By using a SAT1 adenovirus (AdSAT1), we

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examined the temporal effects of SAT1 overexpression on polyamine content, macromolecular syntheses, and polysome profiles. Our data demonstrate that depletion of spermidine and spermine causes an arrest in cell growth specifically by inhibition of translation.

Results

SAT1 Overexpression Blocks GFP Protein Expression Without Reducing Its mRNA. Transient transfection of HeLa cells with a FLAG-SAT1 vector suppresses expression of a cotransfected gene (19). To investigate the mechanism, we first measured the mRNA levels in transiently transfected HeLa culture by real-time PCR (Fig. 1*A*). At 24 h after transfection with the FLAG-SAT1 vector, the SAT1 mRNA level was increased by ~700-fold over its endogenous level in untransfected, or the empty vector (pEV) transfected culture. However, no reduction in the GFP mRNA occurred upon SAT1 cotransfection (Fig. 1*A*), suggesting that SAT1 overexpression does not inhibit transcription or alter mRNA stability. We also examined the mRNA levels by the Stellaris RNA FISH method (20) using Quasar 670-labeled SAT1 probes and Quasar 570-labeled GFP probes. Whereas endogenous SAT1 mRNA was very low (Fig. 1*B*), a high level of exogenous SAT1 mRNA was detected in cytoplasm of the SAT1-transfected cells (Fig. 1 *B* and *C*). RNA FISH imaging confirmed that the GFP mRNA level was not reduced in the cells overexpressing SAT1 mRNA (Fig. 1*B*) and suggested a block specifically in translation, not in transcription. Furthermore, SAT1 overexpression did not affect the mRNA levels of two endogenous genes, namely *GAPDH* and *EIF5A* (Fig. 1*C*), indicating sustained transcription of endogenous genes in the SAT1 overexpressing cells.

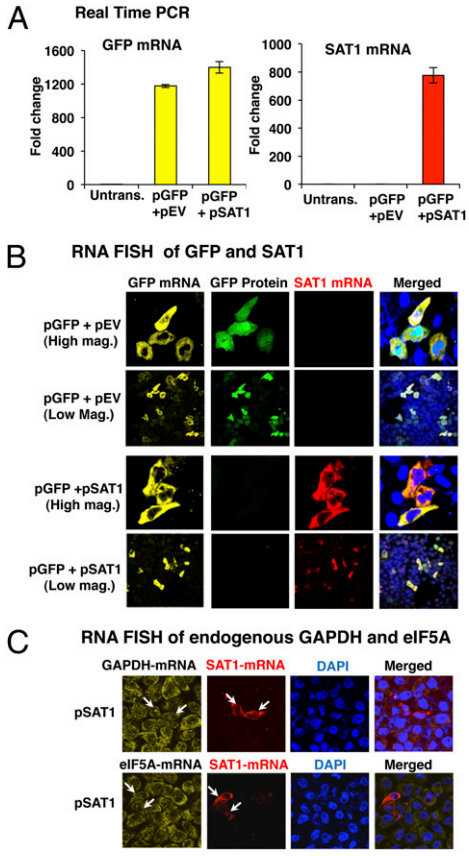

Fig. 1. Lack of effect of SAT1 overexpression on mRNA levels of GFP and two endogenous genes. (*A*) Fold change in GFP and SAT1 mRNA levels compared with untransfected control shown by real-time PCR. Total RNA was isolated from HeLa cells using the RNeasy kit and reverse transcription was performed using ThermoScript reverse transcription-PCR kit (Invitrogen) in a 20-μL reaction mixture containing 500 ng purified RNA, according to the manufacturer's instructions. PCR was performed in triplicates using the IQ SYBR Green Super mix (Bio-Rad) and the Bio-Rad Q-cycler machine, as follows: 50 °C for 2 min and 95 °C for 10 s followed by 40 cycles at 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 20 s. The generation of specific PCR products was confirmed by melting curve analysis. Error bars refer to SD for three independent experiments done in triplicate. GFP and SAT1 mRNA levels were normalized using GAPDH as an internal control. (*B*) RNA FISH image showing GFP mRNA (Q570, yellow) and SAT1 mRNA (Q670, red) in high and low magnifications. HeLa cells cultured on glass coverslips were washed with PBS, fixed in 3.7% (vol/vol) formaldehyde for 10 min at room temperature, washed twice with PBS, and permeabilized at 4 °C in 70% (vol/vol) EtOH for 1 h. A total of 1 μL of respective probe stock (12.5 μM) in 100 μL of hybridization solution was added and cells were incubated in a dark chamber at 37 °C overnight. After washing with the wash buffer [2× SSC with 10% (vol/vol) formamide], DAPI nuclear stain in PBS (5 ng/mL) was added to counterstain the nuclei. (*C*) RNA FISH of endogenous GAPDH and eIF5A-1 mRNAs (Q570, yellow) and SAT1 mRNA (Q670, red) in cotransfected HeLa cells. Images were obtained using a Zeiss LSM510 META inverted confocal system. pGFP, pCEFL/GFP; pEV, pCMV7.1. 3xFLAG empty vector; and pSAT1, pCMV7.1.3xFLAG/SAT1.

Single-cell imaging of mRNAs by RNA FISH offered unique insights into our transient transfection experiments. Interestingly, SAT1 was not overexpressed evenly in the transfected HeLa cell culture: Instead, only a small fraction (<20%) of cells was expressing high levels of SAT1 mRNA at 24 h of transfection (Fig. 1 *B* and *C*). The percentage of SAT1 overexpressing cells declined with time to <20% by 24 h of transfection probably because SAT1 transfected cells stopped growth and division (see Fig. 4), whereas untransfected cells continued to divide.

Endogenous Synthesis of Proteins Is Specifically Inhibited in the SAT1 Overexpressing Cells. Because only a minor fraction of cells overexpressed SAT1 in the transfected culture, we directly measured new synthesis of endogenous proteins, RNA, and DNA, at the single-cell level by incorporating analogs for fluorescent labeling. An anti-FLAG antibody was used to counterimmunostain the cells that overexpressed FLAG-SAT1. In HeLa cell culture transfected with an empty vector, endogenous protein synthesis (green fluorescence) was detected in all cells (Fig. 2*A*). In contrast, upon transient transfection with a SAT1 vector, no green fluorescence was observed in the SAT1 overexpressing cells (marked with white arrows), indicating lack of endogenous protein synthesis (Fig. 2*A*). These data are consistent with the data in Fig. 1 and substantiate the conclusion that SAT1 overexpression causes inhibition of the synthesis of endogenous cellular proteins as well as transfected GFP.

We also examined the synthesis of RNA (Fig. 2*B*) and DNA (Fig. 2*C*) by single-cell imaging after fluorescent labeling of newly synthesized RNA and DNA. Synthesis of RNA and DNA was observed in all cells regardless of SAT1 overexpression. These data illustrate that SAT1 overexpression selectively blocks global translation, without any inhibition of replication and transcription.

AdSAT1 Transduction Causes a Rapid Depletion of Cellular Spermidine and Spermine and an Arrest in Protein Synthesis and Cell Growth in HEK293 Cells. Because biochemical parameters cannot be determined using the heterogeneous, transiently transfected culture containing only a minor fraction of cells overexpressing SAT1, we used adenoviral vectors to achieve transduction of all cells and high expression of target genes (21). Upon transduction of HEK293 with AdGFP or AdSAT1 virus, robust expression of GFP mRNA or SAT1 mRNA was observed in all cells (Fig. 3*A*). Cotransduction with AdGFP and AdSAT1 viruses totally blocked expression of GFP protein without reducing its mRNA level. An increase in the SAT1 activity (Fig. 3*B*) and the protein (Fig. 3*C*) was detected by 8 h of AdSAT1 transduction, both peaking at 12–24 h and declining to less than 5% of the peak levels by 48 h. The levels of SAT1 activity at 12–24 h were estimated to be >300-fold over that in untransduced or AdGFP-

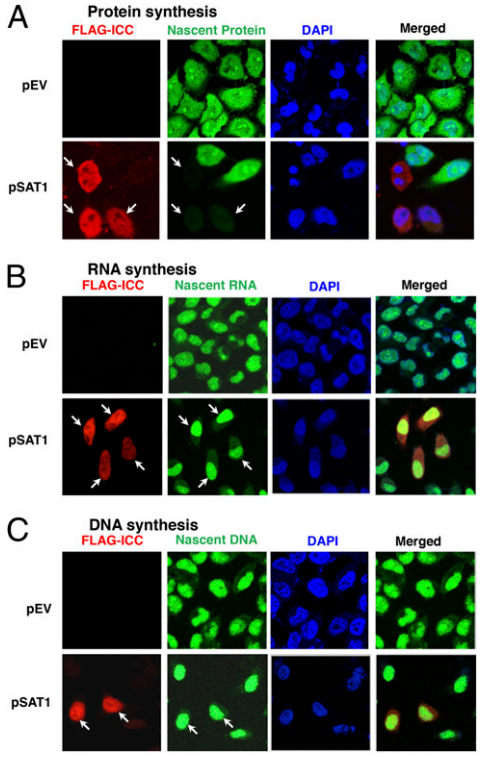

Fig. 2. Fluorescence imaging of nascent synthesis of protein, RNA, and DNA in HeLa cells. HeLa cells were transfected with the empty vector (pEV) (*Upper*) or the FLAG-SAT1 vector (*Lower*). After 24 h of transfection, new syntheses of protein (*A*), RNA (*B*), and DNA (*C*) were imaged using three respective Click-IT assay kits (Invitrogen) by incubation with L-azidohomoalanine, 5-ethylene uridine, and 5-ethynyl-2′-deoxyuridine, respectively, for 30 min at 37 °C. They were fixed in 3.7% (vol/vol) formaldehyde for 15 min, permeabilized with 0.5% Triton X-100 in PBS for 15 min, and fluorescent labeling was performed by addition of Click-IT reaction mixtures. SAT1 overexpressing cells were visualized by counterimmunostaining with anti-FLAG antibody and are indicated by white arrows.

transduced cells. The steep decline in the enzyme activity and protein after 24 h is probably due to the shortening of half-life of SAT1 as cellular polyamines were depleted and inactivation of protein synthesis machinery upon SAT1 overexpression, shutting down its own new synthesis.

Next we measured the temporal effects of SAT1 overexpression on growth (Fig. 4*A*), protein synthesis (Fig. 4*B*), and polyamine levels (Fig. 4*C*). There was little or no change in growth, protein synthesis, or polyamine content in AdGFP-transduced cells compared with untransduced cells. Upon AdSAT1 transduction, a reduction in spermidine and spermine with a significant increase in putrescine and N^1-acetylspermidine was observed by 8 h when the enzyme was significantly induced (Fig. 3). Protein synthesis was not affected up to 8 h, but declined to ~30% of control level by 12 h, when spermidine and spermine were reduced to ~10% and 30% of normal levels, respectively. Total inhibition of protein synthesis and cell growth occurred by 24 h of AdSAT1 transduction, when both cellular spermidine and spermine were virtually depleted.

When we analyzed newly synthesized proteins by SDS/PAGE, similar patterns of radiolabeled proteins were observed in untransduced and AdGFP-transduced cells (Fig. 4*B*). In contrast, labeling was abolished in the AdSAT1-transduced cells, consistent with the lack of trichloroacetic acid (TCA) precipitable radioactivity and of fluorescently labeled nascent proteins (Fig. 2*A*). Only a few, faintly radiolabeled bands were detected in the AdSAT1-transduced cells and these bands were identified as adenoviral proteins by Western blotting using antiadenovirus type 5 antibody. The level of adenoviral proteins synthesized was estimated to be less than 5% of total cellular proteins synthesized in the AdGFP-transduced cells at 24 h of viral transduction.

Inhibition of Translation in the SAT1 Overexpressing Cells Is Due to Depletion of Cellular Spermidine and Spermine. The close correlation between depletion of polyamines (spermidine and spermine) and inhibition of protein synthesis (Fig. 4) suggests that polyamine deprivation is the cause for the cessation in protein synthesis and cell growth. However, the arrest could also be attributable to other metabolic changes associated with SAT1 overexpression, such as the pronounced increase in cellular N^1-acetylspermidine and putrescine (Fig. 4*C*). N^1-acetylspermidine peaked at 12–24 h after AdSAT1 transduction and decreased afterward, correlating with the temporal pattern of SAT1 activity (Fig. 3*B*). The rise in cellular putrescine lagged behind N^1-acetylspermidine, peaking at 24–48 h, consistent with it being the final product of the catabolic pathway. However, exogenously added putrescine and N^1-acetylspermidine (0.5–5.0 mM) caused no inhibition in GFP expression in cells (Fig. S1), excluding a significant involvement of these amines in the translational block in the SAT1 overexpressing cells.

SAT1 overexpression (22–24) is often accompanied by a compensatory induction of ODC and S-adenosylmethionine decarboxylase (AdoMetDC) resulting in an accelerated metabolic

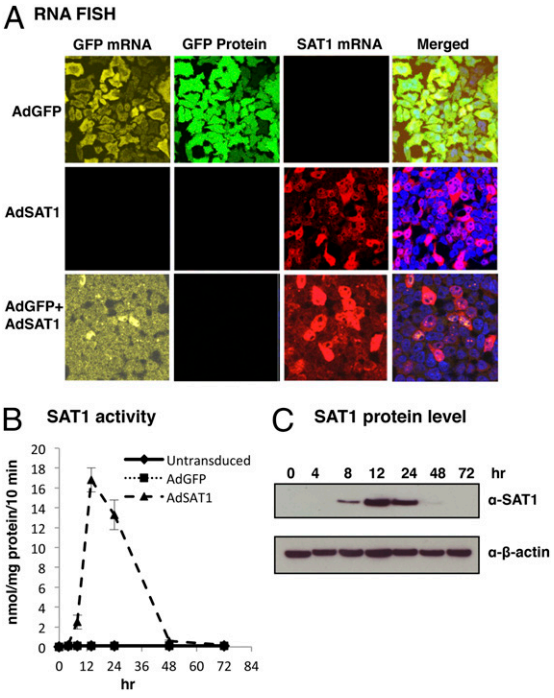

Fig. 3. Effect of AdSAT1 and AdGFP cotransduction and the time course of overexpression of SAT1 upon AdSAT1 transduction. (*A*) GFP fluorescence and RNA FISH of GFP and SAT1 mRNAs. HEK293 cells were transduced with AdGFP alone or cotransduced with AdSAT1 and RNA FISH was performed at 24 h. (*B*) Time course of induction of SAT1 activity upon AdSAT1 transduction. SAT1 activity assays were performed as described under *SI Materials and Methods* using different amounts of cell lysate proteins (0.3–3 μg), 0.5 μCi [^{3}H]AcCoA (2.53 Ci/mmol, 8 μM), and 1 mM spermidine. (*C*) Western blotting of lysates (50 μg proteins) of AdSAT1-transduced cells using SAT1 antibody (Santa Cruz) at the indicated time after transduction. β-Actin was used as a loading control. AdGFP-transduced and untransduced cell lysates showed no SAT1 signals at any time points under the same condition.

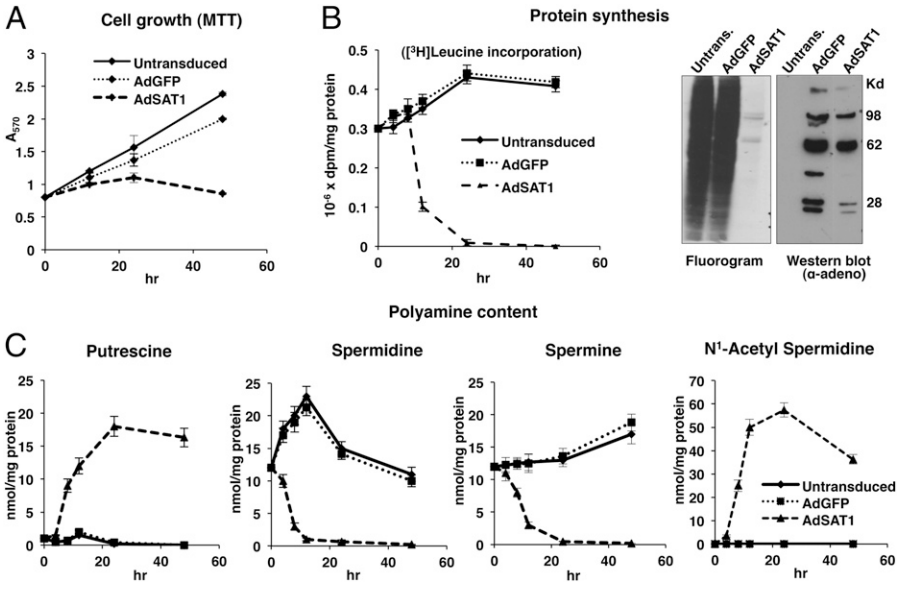

Fig. 4. The temporal effects of AdGFP or AdSAT1 transduction on cell growth, protein synthesis, and polyamine content. HEK293 cells were transduced with AdGFP or AdSAT1 viruses. (*A*) Cell growth was measured by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in triplicates. (*B*) Protein synthesis was measured in cells in 24-well dishes at indicated time points by measurement of radioactivity incorporated into TCA precipitable material after 2 h incubation in 0.5 mL medium containing 5 μCi of [^{3}H]leucine (*Left* graph). Newly synthesized proteins of cells radiolabeled by incubation in leucine-free DMEM containing 10% (vol/vol) FBS and 20 μCi/mL of [^{3}H]leucine for 1 h at 24 h of transduction was visualized by fluorography (*Left*) after SDS/PAGE. Immunoblotting of the same samples with antiadnovirus type 5 antibody is shown on the *Right*. (*C*) Cellular content of putrescine, spermidine, spermine, and N^1-acetylspermidine was measured as described in *SI Materials and Methods*. The experiments were carried out in duplicate and repeated two or three times with similar results. Representative data are shown.

cycling of polyamine biosynthesis and catabolism (25), an increased oxidative stress from hydrogen peroxide generated by the APAO reaction and the consumption of acetyl CoA. However, two inhibitors of APAO, MDL72521 (N^1-methyl-N^2-(2,3-butadienyl)-1,4-diaminobutane) and MDL72527 (N^1,N^4-bis(2,3-butadienyl)-1,4-diaminobutane), did not restore GFP expression in cells overexpressing SAT1 (Fig. 5*A*, *Left*), suggesting that the inhibition of translation and cell growth is neither due to increased oxidative stress nor due to accumulation of putrescine.

We further tested various polyamine analogs for their ability to restore GFP expression in the SAT1 overexpressing cells (Fig. 5*A*) and as substrates for SAT1 in vitro (Fig. 5*B*). The natural polyamines, spermidine and spermine, and analogs (ω-methylspermidine (MeSpd), caldine, *cis*-unsaturated spermidine) that are good substrates for SAT1, did not restore GFP synthesis, probably because they are rapidly acetylated and degraded in the SAT1 overexpressing cells. Only those stable polyamine analogs, e.g., N^1, N^{11}-Bis-ethylnorspermine (BENSpm), α-methylspermidine (α-MeSpd), and 1,12-dimethylspermine (Me_2Spm) and homospermidine (hSpd), that are not substrates for SAT1 supported GFP expression in the SAT1 cotransfected cells. These analogs restored GFP expression without enhancing cellular spermidine and spermine, suggesting that they substitute for the function of natural polyamines in promoting translation. Neither the diamine putrescine nor N^1-monoguanyl-1,7-diaminoheptane (GC7) (not substrates for SAT1) rescued GFP expression, suggesting a requirement for a triamine and/or tetraamine structure resembling spermidine and spermine for the protein synthesis machinery.

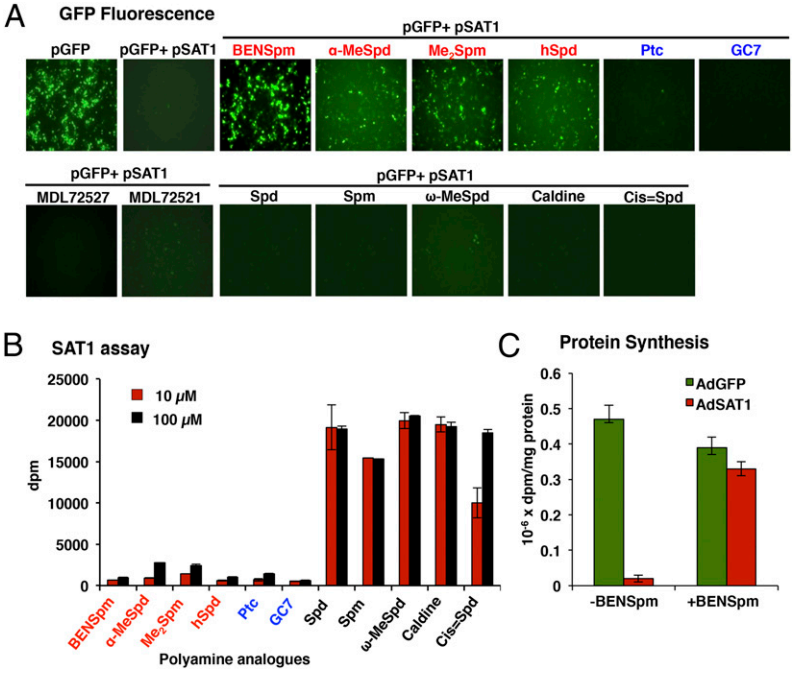

Fig. 5. The effects of polyamine analogs and APAO inhibitors on GFP expression or protein synthesis. (*A*) GFP expression in HeLa cells transfected with GFP vector alone or cotransfected with SAT1 vector. The APAO inhibitors, MDL72527 and MDL72521 (200 μM each), or polyamine analogs (10 μM) were added at the time of transfection and images were taken at 24 h. (*B*) Polyamines and their analogs (10 and 100 μM) were tested as substrates of SAT1 in a reaction mixture (25 μL containing 50 mM Tris·HCl, pH 8.0, 1 mM DTT, 10% (vol/vol) glycerol, 0.1 mM EDTA, 0.5 μCi [^{3}H]AcCoA (3.62 Ci/mmol, 5.5 μM), and 20 ng of recombinant human SAT1). After incubation for 10 min at 30 °C, 10 μL of reaction mixture was spotted in duplicates on phosphocellulose P81 filter disk (Whatman; 2.5 cm diameter), and the filters were washed three times in water and the radioactivity on filters was measured. (*C*) Protein synthesis was measured as in Fig. 4 in HEK293 cells after 24 h of adenovirus transduction and incubation with or without 10 μM BENSpm. Spd, spermidine; Spm, spermine; Ptc, putrescine; MeSpd, methylspermidine; hSpd, symhomospermidine; Me_2Spm, 1,12-dimethyl spermine; *cis*, Spd, *N*-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene; GC7, N^1-monoguanyl-1,7-diaminoheptane; and BENSpm, N^1, N^{11}-bis(ethyl)norspermine.

BENSpm restored endogenous protein synthesis in the AdSAT1-transduced HEK293 cells (Fig. 5*C*). In view of the fact that BENSpm restored GFP expression, while highly inducing the SAT1 enzyme and depleting cellular polyamines, the availability of polyamines (or the analogs), and not the induced level of SAT1 enzyme per se, appears to be the critical factor dictating translational activity. Taken together, these findings support the conclusion that lack of spermidine and spermine is the underlying cause for the arrest in translation and cell growth in the SAT1 overexpressing cells.

Loss of Polysomes in AdSAT1-Transduced Cells Suggests a Role for Polyamines in Translation Initiation. We analyzed polysome profiles of AdSAT1-transduced, vs. AdGFP-transduced, vs. untransduced HEK293 cells at 12 and 24 h, to investigate the mechanism of polyamine action in translation. The relative absorbance of 40S, 60S, 80S, and polysome peaks in AdGFP-transduced cells (12 and 24 h) was comparable to those of untransduced cells (Fig. 6), consistent with similar rates in protein synthesis measured by [^{3}H]leucine incorporation (Fig. 4*B*). In contrast, polysomes were markedly reduced at 12 h after transduction with AdSAT1, with an increase in the 80S peak, consistent with the inhibition (by 70%) of protein synthesis at this time point. After 24 h of AdSAT1 transduction, no polysome peaks were detectable, suggesting a total arrest in translation in line with the total loss of [^{3}H]leucine incorporation (Fig. 4*B*). The loss of polysomes with an increase in 80S monosomes suggests a block in translation initiation and underscores an important role for cellular spermidine and spermine in the regulation of translation in mammalian cells.

Discussion

The in vivo physiological functions of cellular polyamines have remained elusive even though their essential nature is firmly established. In this study we show that depletion of cellular spermidine and spermine by overexpression of a polyamine catabolic enzyme, SAT1, causes a rapid arrest in protein synthesis and cell growth. Single-cell imaging of mRNA levels by RNA FISH and of the endogenous synthesis of protein, RNA, and DNA revealed a block specifically in translation in the SAT1 overexpressing cells, with no inhibition of replication or transcription. Furthermore, the loss of polysomes with an increase in 80S monosomes observed in the AdSAT1-transduced cells suggests a block at the initiation step of translation in mammalian cells. Our data demonstrate a primary function of the polycationic polyamines, spermidine and spermine, in the regulation of translation in mammalian cells. An extensive depletion of spermidine and spermine achieved here by use of a potent AdSAT1 virus offers a unique paradigm for future studies addressing the cellular mechanisms of polyamine actions in and beyond translation and suggests a potential application of an AdSAT1 virus in tumor-targeted gene therapy.

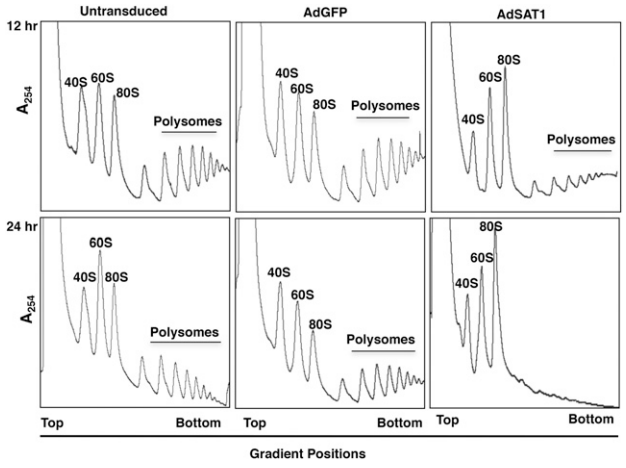

Fig. 6. Polysome profiles of HEK293 cells untransduced or transduced with AdGFP or AdSAT1 viruses. HEK293 cell lysates were prepared at 12 and 24 h after transduction and polysome profiles were obtained as described under *SI Materials and Methods* using 0.1 mm/s speed and Bio-Rad EM-1 flow cell at 254 nm. Representative set of profiles from three independent experiments is shown.

In this study, we reexamined two suppositions made in our previous paper regarding the effects of SAT1 overexpression on GFP expression (19). We found no significant inhibition of total protein synthesis (when measured by [^{3}H]leucine incorporation) in HeLa cell culture after 42 h of transient transfection with the SAT1 vector and this finding led us to suggest that SAT1 overexpression only suppressed expression of exogenous genes, but not of endogenous genes. It is now evident from the single-cell imaging (RNA FISH images, Fig. 1 *B* and *C*, low magnifications), that the inhibition of protein synthesis was obscured because only a small fraction of the heterogeneous transiently transfected culture overexpressed SAT1. This exposes the difficulty in detecting loss of function when transfecting with a toxic (growth deleterious) gene and underscores the advantage of single-cell imaging used in the current study. We also suggested that decreases in polyamine levels were not involved, because changes in cellular polyamine levels were relatively small and because treatment with DFMO did not inhibit GFP expression (19). However, pretreatment with DFMO before GFP transfection did not appear to block GFP expression and caused a relatively small inhibition (<30% at 24–72 h) of endogenous protein synthesis (Fig. S2), suggesting that spermine alone can support translation in the time period (24–72 h). Cellular functions that can be fulfilled by either spermidine or spermine would not be blocked using DFMO treatment that depletes only spermidine. Even though polycationic polyamines were suggested to play a role in translation initiation from studies involving DFMO-treated NIH 3T3 cells (26), our data display a clear distinction in biological responses between cells depleted of putrescine and spermidine (DFMO treatment) and those depleted of spermidine and spermine.

Two major independent roles of polyamines in mammalian cell proliferation have emerged in previous studies and are further supported by our data. One well-defined function is the role for spermidine as a substrate for the covalent modification of eIF5A to form hypusine. In addition, cationic polyamines also appear to be required for mammalian cell growth, because inhibition of cell growth was observed upon reduction of polyamines before a significant decrease in hypusinated eIF5A (27). Due to the narrow specificity of deoxyhypusine synthase, spermidine and only a couple of closely related analogs can be incorporated into eIF5A and support long-term cell growth in the absence of spermidine (28). On the other hand, various analogs of spermidine and spermine can support growth of DFMO-treated cells in the short term (28, 29), suggesting that the structural requirement for the polycationic function is not as strict as that for hypusine modification. It is interesting to note that BENSpm can act as a polyamine agonist in supporting translation in the short term (Fig. 5), even though it cannot substitute for the natural polyamine, spermidine, in supporting growth in the long term, as it is not a substrate for deoxyhypusine synthase.

We considered the possibility of acetylation of eIF5A (30) or another target protein by SAT1 leading to an arrest in translation. However, only a small fraction (<10%) of eIF5A was acetylated at hypusine in AdSAT1-transduced cells (Fig. S3), suggesting no significant reduction in active eIF5A. The polysome profiles (Fig. 6) suggest a block in translation initiation, not a block in translation elongation, as would be expected in case of deficiency of hypusinated, active eIF5A (31, 32). Furthermore, there are no translation initiation factors known to be regulated by lysine acetylation. These findings together with data in Fig. 5

provide strong evidence that the arrest in translation in SAT1 overexpressing cells results from depletion of polycationic polyamines.

The effects of overexpression of SAT1 were previously reported by others using cell lines overexpressing SAT1 stably or in an inducible manner (22–24, 33), SAT1 transgenic mice (34–36), and a SAT1 adenovirus (37, 38). Although retardation of growth and decreases in spermidine and spermine were consistently associated with SAT1 overexpression, the degree of growth inhibition and polyamine depletion were much lower than those reported in this study. This may be attributable to differences in the promoters or in cellular compensatory responses to the SAT1-mediated polyamine depletion. Our data display early cellular events following SAT1 superinduction before stable adaptation and point to a specific mechanism leading to growth arrest in SAT1 overexpressing cells. Our AdSAT1 virus exhibits potent effects i.e., complete depletion of spermidine and spermine and total arrest in protein synthesis and cell growth ensuing with apoptotic cell death. Such a strong SAT1 adenovirus holds great promise for targeted inhibition of tumor growth.

It is quite remarkable that protein synthesis was totally inhibited in the SAT1 overexpressing cells (Figs. 2 and 4*B*). Polyamines have been proposed to play an important role in the regulation of global translation (26) and through induction of polyamine-responsive modulons (4). Most cellular polyamines are known to exist as complexes with DNA, RNA, and ATP, with the largest portion as polyamine-RNA complexes (4). Polyamine binding to RNA displays specificity in inducing RNA structural changes (4). Our data are totally consistent with independent in vitro studies that demonstrated the requirement for polyamines in the translation initiation step (39) and thus substantiate an important role of spermidine and spermine in translation.

The disappearance of polysomes with an increase in 80S monosomes in AdSAT1-transduced cells (Fig. 6) suggests a block in translation initiation. It is not known precisely which step in the translation initiation is inhibited in the polyamine-depleted cells. Efforts are underway to develop a reconstituted cell-free translation system from SAT1 overexpressing cells, to identify defective steps and molecular components of translation in polyamine-depleted cells and to determine the specific role of polyamines and their analogs in the translation machinery.

Materials and Methods

Materials are described in *SI Materials and Methods*. Detailed methods on real-time PCR, RNA FISH, confocal microscopy, single-cell fluorescence imaging of endogenous synthesis of protein, RNA and DNA, and transfection are included in *SI Materials and Methods*. Adenoviral transduction, determination of cell growth, protein synthesis and polyamine content, SAT1 assay, and polysome analyses are described in *SI Materials and Methods*.

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