Research Article

Cancer Prevention Research

Chemoprevention of B-Cell Lymphomas by Inhibition of the Myc Target Spermidine Synthase

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Abstract

The oncogenic transcription factor c-Myc (Myc) is frequently overexpressed in human cancers. Myc is known to induce or repress a large set of genes involved in cell growth and proliferation, explaining the selection for mutations in cancer that deregulate Myc expression. Inhibition of ornithine decarboxylase, an enzyme of the polyamine biosynthetic pathway and a Myc target, has been shown to be chemopreventive. In the present study, we have dissected the role of another enzyme in the polyamine biosynthetic pathway, spermidine synthase (Srm), in Myc-induced cancer. We find that Srm is encoded by a Myc target gene containing perfect E-boxes and that it is induced by Myc in a direct manner. RNA interference against *Srm* shows that it is important for Myc-induced proliferation of mouse fibroblasts but to a lesser extent for transformation. Using the compound *trans*-4-methylcyclohexylamine, we show that Srm inhibition can delay the onset of B-cell lymphoma development in λ-*Myc* transgenic mice. We therefore suggest that inhibition of Srm is an additional chemopreventive strategy that warrants further consideration. *Cancer Prev Res; 3(2); 140–7. ©2010 AACR.*

Introduction

Signaling pathways such as the Wnt, Hh, transforming growth factor-β, and receptor tyrosine kinases, all converge on the *MYC* gene family which encodes oncogenic transcription factors (1, 2). Emerging evidence suggests that these pathways are deregulated in most forms of cancer, indirectly resulting in elevated levels of Myc proteins. *MYC* (encoding c-Myc), *MYCN* (encoding N-Myc), and *MYCL* (encoding L-Myc) also become overexpressed by direct means due to genomic translocations and amplifications in B-cell malignancies, neuroblastoma, breast cancer, and lung cancer (1). These facts, taken together, suggest that Myc overexpression is a hallmark of cancer.

Myc and its obligatory dimerization partner Max bind to CACGTG E-boxes present in a large subset of genes (3). Myc:Max dimers will then activate transcription by recruiting complexes containing histone acetyltransferases and the general transcription machinery (4). Max can also bind the Mad/Mnt family of transrepressors and the levels (and possibly affinities) of Myc proteins or Mad/Mnt proteins decide whether a Max-containing dimer will stimulate or inactivate a gene. If a cell is stimulated to grow, Myc is produced and Myc:Max dimers can relieve transrepression by Mad/Mnt:Max dimers and activate transcription (5). Furthermore, some genes can also be repressed by Myc via interaction with proteins like Miz-1 (6).

Pre-genomics era transcriptional targets of Myc were difficult to identify and relied on biased assumptions based on Myc's functions to stimulate cell growth and proliferation. Some of the first identified, including ornithine decarboxylase (*Odc*), lactate dehydrogenase A (*LdhA*), and carbamoyl phosphate synthetase/aspartate *trans*-carbamoylase/dihydroorotase (*Cad*) fit with a function of Myc in stimulating cell growth, whereas cyclin-dependent kinase 4 (*Cdk4*) and cyclin D2 (*Ccnd2*) fit with a role of Myc in stimulating the cell cycle (7). Although these two groups of genes provided compelling evidence, they did not anticipate what was to come with the development of genomics tools. Serial analyses of gene expression, microarray analyses and genome-wide chromatin immunoprecipitation experiments have all indicated that the "Myc transcriptome" is very large and may comprise up to 15% of the genome (8). We are therefore faced with the possibility that Myc proteins are to be considered general transcription factors that stimulate the genes necessary for cell growth and proliferation. To lend further support of that view, Myc even transcriptionally induces the GCN5 histone acetyltransferase, which results in an "open" configuration of chromatin (4). Recently, Myc was also shown to cause a widespread repression of microRNAs, with the exception of a few, which instead had oncogenic capacities (9).

Despite the pleiotropic functions of Myc, inhibiting the products of a few downstream target genes may still be of use in chemoprevention or chemotherapy against cancer. A case for inhibiting *Odc* as a means to prevent the onset of tumors like colon cancer and lymphoma was established based on experimental evidence in mouse models

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(10, 11) and clinical trials (12) using the drug α-difluoromethylornithine (DFMO). Because the downstream products of Odc, polyamines, are involved in many aspects of cellular physiology, it is possible that targeting Odc also interferes with several of Myc's functions and that *Odc* thus may be a more crucial target gene of Myc's compared with most. It is therefore interesting to note that several genes of the polyamine biosynthetic pathway have been shown to be induced in B cells overexpressing Myc (11). In the present study, we are investigating the role of spermidine synthase (*Srm*), a Myc target gene encoding an enzyme which produces spermidine from the putrescine formed by Odc. Interestingly, we observe that Srm is more potently induced by Myc than Odc, and that inhibiting this enzyme also has a chemopreventive effect on lymphomagenesis. Srm has been ignored because it is considered constitutively active; however, these data suggest that it could be a promising future drug target in chemoprevention.

Materials and Methods

Mice

Eμ-Myc, *iMyc*^{Eμ}, and λ-*Myc* mice, all on a C57/BL6 background were obtained from The Jackson Laboratory, the National Cancer Institute mouse depository, and a kind gift from Dr. Georg Bornkamm (GSF, Munich, Germany), respectively. C57/BL6 mice (6 wk of age; Taconics, Denmark) were injected with 1 million lymphoma cells via the tail vein. λ-*Myc* mice and transplanted C57/BL6 mice were maintained in normal water (untreated) or treated with drinking water containing *trans*-4-methylcyclohexylamine (4MCHA, 0.025%; pH 4.0) and/or DFMO (1%; pH 4.0), starting 2 d post-injection. DFMO and 4MCHA-containing water was replaced every 3 to 4 d. All transgenic or transplanted mice were observed daily for signs of morbidity and tumor development. Sick mice were immediately sacrificed and tumors and lymphoid organs were analyzed by histology or fluorescence-activated cell sorting analysis to verify B-cell lymphoma.

Cell culture

NIH 3T3 fibroblasts and 293T human kidney cells were purchased from American Type Culture Collection and cultured in DMEM with 10% fetal bovine serum, 2 mmol/L of L-glutamine, 1 mmol/L of sodium pyruvate, and 1× antibiotic-antimycotic cocktail (penicillin/streptomycin/fungizone; Invitrogen). P493-6 cells expressing a tetracycline-regulated *MYC* oncogene (13) were a kind gift from Dr. Georg Bornkamm. B-cell lymphoma lines from Myc-transgenic mice were established by making single-cell suspensions from palpable lymphomas that originated in these mice. The lymphoma lines and P493-6 cells were seeded at a density of 2×10^5 cells/mL in RPMI 1640, 10% fetal bovine serum, 2 mmol/L of L-glutamine, 500 μmol/L of β-mercaptoethanol, and 1× antibiotic-antimycotic cocktail. Spermidine synthase inhibitor 4MCHA (TCI Europe) was added to cells to a final concentration of 100 or 250 μmol/L. Cells were grown in the presence of aminoguanidine at a final concentration of 1 mmol/L to inhibit serum amine oxidases.

Vectors

Retroviral vectors for RNA interference (RNAi) were created by PCR amplifying five fragments containing short hairpins directed against *Srm* using primers with *Eco*RI and *Xho*I ends. The templates were long synthesized oligonucleotides designed using the algorithm described for cloning into pSMc2 on the RNAi Central homepage.[1] After digestion of the PCR products with *Eco*RI and *Xho*I, the fragments were cloned into the MSCV-LMP vector (Open Biosystems), generating *Srm* RNAi-1 to RNAi-5. For constitutive or inducible Myc expression in cells, retroviral vectors pWZL-Blast-Myc (generously donated to Addgene by Dr. Robert Weinberg, Whitehead Institute, MIT, Cambridge, MA) or MSCV-MycER-IRES-puro (14) were used, respectively.

Retroviral infections of mouse NIH 3T3 fibroblasts and analyses thereof

Human kidney 293T cells were plated at a density of 200,000 cells in a 10 cm dish, then transfected with packaging plasmids and a retroviral construct using a standard calcium phosphate method. The virus produced by these cells supplemented with 8 μg/mL of polybrene (Sigma), was then used to infect NIH 3T3 cells plated at a density of 200,000 cells/10 cm dish. Infected cells were selected using medium containing 6 μg/mL of puromycin or 10 μg/mL of blasticidin-S (Invitrogen) until uninfected cells were dead. For RNAi, expression of spermidine synthase was analyzed using real-time quantitative reverse-transcriptase PCR (qRT-PCR). For Myc-ER experiments, puromycin-selected cells were grown in the presence or absence of cycloheximide and/or 4-hydroxytamoxifen (4-HT; both from Sigma) for 4 h, followed by monitoring of gene expression by qRT-PCR. For anchorage-independent growth of infected NIH 3T3 fibroblasts in soft agar, cells were suspended in 2.5 mL of 2× DMEM and 2.5 mL of 1.4% low melting point agarose (Sigma) and then plated in a 10 cm dish. They were incubated in a humidity chamber within a tissue culture incubator at 37°C with 5% CO_2 for 2 wk. Transformed cell colonies were visualized by light microscopy and compared with uninfected NIH 3T3 cells.

RNA and protein preparation and analysis

RNA from magnetically sorted B cells, cultured cells, or tumors from λ-*Myc* mice were isolated using the RNA II spin kit (Macherey-Nagel). For qRT-PCR, cDNA synthesis was done using the iScript first-strand synthesis kit (Bio-Rad) and the PCR was run using the SYBRgreen PCR Mastermix (Biotools) on an iQ Real-time PCR machine (Bio-Rad). Primers used to detect *Arf* expression were sense, 5′-TTTCTTGGTGAAGTTCGTGCGATCC-3′ and antisense, 5′-CTGCACCGTAGTTGAGCGAAGAG-3′), whereas those used to detect *Odc*, *Amd1*, *Srm*, and *ubiquitin* (*Ub*)

[1] http://katahdin.cshl.org:9331/RNAi_web/scripts/main2.pl

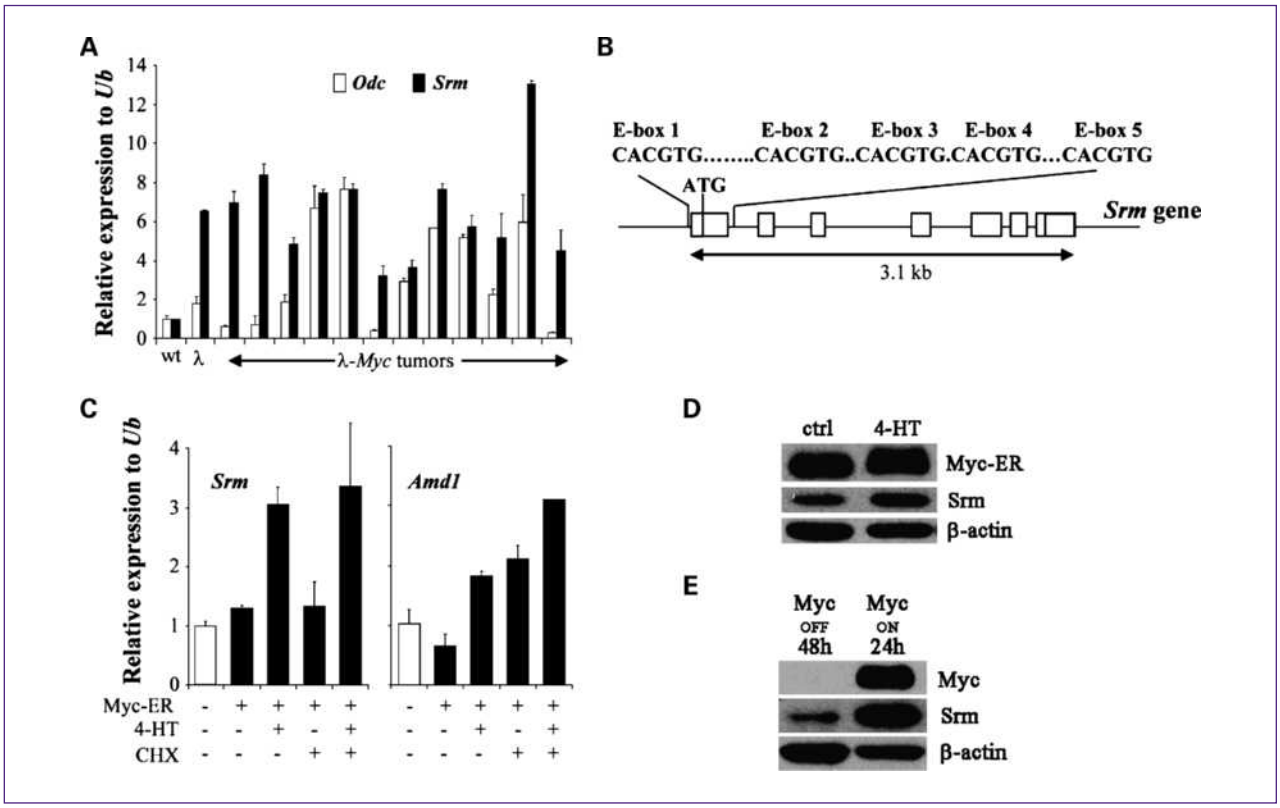

Fig. 1. *Srm* is a direct Myc target gene. A, qRT-PCR analysis of RNA from B cells of wild-type and *λ-Myc* mice and of tumors arising in the same mouse model. The mRNA levels of *Odc* and *Srm* were normalized to that of ubiquitin (*Ub*). B, genomic organization of the *Srm* gene showing schematically the position of five Myc-binding E-boxes. C, qRT-PCR analysis of RNA from NIH 3T3 cells infected with a Myc-ER–expressing retrovirus which had been treated with 4-HT to induce Myc nuclear translocation in the presence or absence of protein synthesis. Protein synthesis was blocked by the addition of cycloheximide (*CHX*). The mRNA levels of *Srm* and *Amd1* were normalized to those of *Ub*. D, NIH 3T3 cells infected with a Myc-ER–expressing retrovirus were treated with 4-HT for 24 h. Protein levels were analyzed by Western blot using an Srm antibody, and an ER-α antibody for the detection of the Myc-ER fusion protein. E, P493-6 lymphoblastoid B cells were treated with tetracycline to repress the *MYC* transgene. After 48 h, the tetracycline was washed away to induce Myc expression and, after 24 h, Myc and Srm levels were determined by Western blot analysis.

were as previously described (11). Relative mRNA levels were calculated using the ΔΔCT method, comparing expression of *Odc, Amd1, Arf,* and *Srm* with that of *Ub*.

Lysates were prepared from cultured cells, or tumors that originated in λ-*Myc* mice by pulverization in liquid nitrogen using a metal mortar followed by incubation in lysis buffer, vigorous vortexing, and sonication while it was kept on ice. Protein concentration was determined using the Bio-Rad protein determination kit. Fifty micrograms of protein per well was loaded on a Criterion 4% to 20% gradient Tris-HCl polyacrylamide gel and resolved by electrophoresis. Western blot was carried out according to standard methodologies and the antibodies used were from Sigma-Aldrich (β-actin, AC-15), Santa Cruz Biotechnology (p53, M-19; $p19^{Arf}$, 5-C3-1; c-Myc, C-19; ER-α, MC-20), and from Dr. Gregor Laube at the Institute of Integrative Anatomy CCM, Berlin, Germany (Srm; ref. 15).

Polyamine analysis

The reversed-phase high-performance liquid chromatography (HPLC) method was used to quantify cellular polyamine content (16). Cell pellets were sonicated in 0.2 mol/L of perchloric acid and centrifuged at 20,000 × *g* (10 min, 4°C). The supernatant was analyzed using the Varian Vista 5500 liquid chromatography system, equipped with a Model 9090 Autosampler, a Model 2010 HPLC pump, a Model 2050 variable-wavelength UV detector, and a Fluorichrom fluorescence detector. The software Dynamax HPLC Method Manager and MacIntegrator (Rainin Instruments) were used along with a Macintosh SE/30 for method editing, HPLC control, as well as data collection and analysis (peak identification and quantification).

Results

Srm is a Myc target gene and RNAi-mediated knockdown reduces Myc-induced proliferation of fibroblasts

Odc is one of the most established Myc target genes, but a previous report (11) and the Myc target gene database[2] suggest that other genes encoding polyamine biosynthetic

[2] http://www.myccancergene.org/

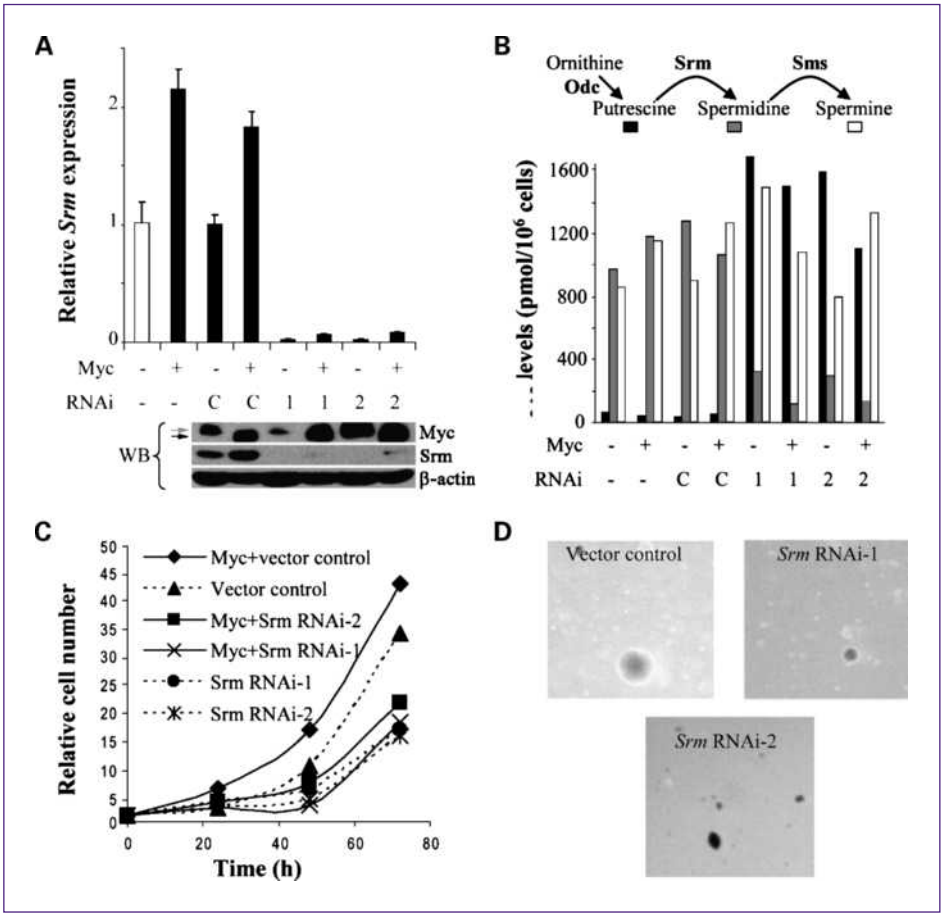

Fig. 2. *Srm* RNAi reduces Myc-induced proliferation. A, top, qRT-PCR analysis of RNA from NIH 3T3 cells infected first with either *Srm* RNAi-1, *Srm* RNAi-2, or a vector control retrovirus, and then with a Myc-expressing retrovirus (pWZL-Blast-Myc). The mRNA levels of *Srm* were determined by normalization to the expression of ubiquitin (*Ub*). Bottom, Western blot analysis of lysates from the same cells showing Myc and Srm protein levels. The faster-migrating Myc (black arrow) represents exogenous human Myc which represses the expression of the endogenous murine Myc (gray arrow). B, the levels of the polyamines (*PA*) putrescine, spermidine, and spermine in 10^6 of each of the cells described in A were measured by HPLC. Also shown here is where the enzymes Odc, Srm, and Sms fit in the polyamine biosynthetic pathway. C, the same cells as in A were grown and counted to follow the effect of *Srm* RNAi on Myc-induced proliferation. See Supplementary Fig. S1 for an additional experiment. D, cells overexpressing c-Myc were also seeded in soft agar to evaluate the effect of *Srm* RNAi on Myc-induced transformation. As seen, colonies still appeared but they were smaller (see Supplementary Fig. S1 for quantification).

enzymes may also be regulated by Myc, e.g., spermidine synthase (*Srm*) and *S*-adenosylmethionine decarboxylase (*Amd1*). In the present study, we have focused our attention on *Srm* (which forms spermidine from putrescine) and evaluated its regulation and importance in Myc overexpressing cells. To gain insight as to whether or not *Srm* might be involved in B-cell lymphomagenesis, we analyzed its mRNA levels by qRT-PCR in B cells from 6-week-old wild-type and λ-*Myc* mice, as well as in tumors that had arisen in the same mouse model. As previously reported for the *Eμ-Myc* mouse (11), *Odc* was induced by Myc in λ-*Myc* B cells before the onset of tumor development and was maintained at the same level or higher in most, but not all, tumors. Interestingly, *Srm* expression was elevated several-fold, and unlike *Odc*, there was not a single tumor expressing *Srm* to a lower or same degree as B cells from wild-type mice (Fig. 1A). This suggests that the *Srm* gene is as important for Myc-induced tumors as the *Odc* gene.

The fact that *Srm* is induced in B cells and tumors from λ-*Myc* mice does not reveal if the gene is a direct Myc target or if it is indirectly induced by, e.g., cell growth or a factor that is regulated by Myc. We therefore analyzed the mouse genome for the presence of E-boxes in the *Srm* regulatory region. As seen in Fig. 1B, *Srm* carries five canonical E-boxes (CACGTG), located just upstream and downstream of exon 1. To add additional formal evidence, we infected NIH 3T3 fibroblasts with a retrovirus expressing inducible Myc-ER. This construct expresses c-Myc fused to the ligand-binding domain of the estrogen receptor (ER), and the protein is therefore kept in the cytoplasm by heat shock proteins (17). Upon the addition of 4-HT, heat shock proteins could no longer bind the ER part, resulting in nuclear translocation and activation of Myc-induced gene transcription. Genes that are rapidly induced by 4-HT even in the presence of cycloheximide, a protein synthesis inhibitor, are dubbed true Myc targets. In this regard, *Odc, Amd1*, and *Srm* all qualify as such (Fig. 1C; refs. 18, 19). In addition, Myc activation also resulted in elevated protein levels of Srm in both fibroblasts expressing Myc-ER and in P493-6 lymphoblastoid cells expressing a tetracycline-regulated *MYC* oncogene (Fig. 1D and E).

Odc has been shown to be important for tumor development in mouse models of lymphoma, skin, prostate, breast, and colon cancer, and most recently, neuroblastoma (10, 11, 20–24). In the *Eμ-Myc* mouse, treatment with the suicide Odc enzyme inhibitor DFMO reduced rates of proliferation of B cells and delayed lymphomagenesis significantly (11). We wanted to examine if Srm operates in a similar way by using RNAi as a genetic tool

to assay the effect of the loss of *Srm* expression. We designed five different short hairpin RNAs against *Srm* and used retroviruses to deliver these into NIH 3T3 fibroblasts. Two short hairpin RNAs were very efficient in eliciting RNAi resulting in decreased *Srm* mRNA levels and protein as measured by qRT-PCR and Western blot analyses (Fig. 2A). Furthermore, measurements of polyamine levels by HPLC verified that loss of *Srm* expression indeed led to reduced enzyme activity. Expectedly, knockdown of *Srm* resulted in a substantial decrease in spermidine content of the cells and an accumulation of the precursor putrescine (Fig. 2B), which cannot be converted to spermidine in the absence of Srm.

With these RNAi tools in hand, we were able to determine the importance of Srm in Myc-overexpressing cells. We infected NIH 3T3 fibroblasts with control, *Srm* RNAi-1, or *Srm* RNAi-2–expressing retroviruses followed by retroviral infection with a Myc-expressing virus. Growth curves showed that *Srm* RNAi inhibited cell proliferation (Fig. 2C; Supplementary Fig. S1A). Because Myc can induce cellular transformation of murine fibroblasts with a defective Arf-p53 axis, e.g., NIH 3T3 cells which lack *Arf*, we were also able to study the effect of loss of *Srm* on Myc-induced transformation. Despite the potent effect of *Srm* loss on proliferation, the majority of Myc-overexpressing cells still became transformed cells in the absence of *Srm* but the colonies growing in soft agar were smaller (Fig. 2D; Supplementary Fig. S1B). Taken together, these data suggest that Srm contributes to Myc-induced proliferation and may therefore be an attractive target for inhibition in cancer cells that rely on Myc-induced proliferation.

Pharmacologic inhibition of Srm is chemopreventive but not chemotherapeutic *in vivo*

Human Burkitt lymphoma is characterized by a translocation of the *MYC* oncogene from its normal location on chromosome 8 to any of the three chromosomes (2, 14, or 22) carrying immunoglobulin light or heavy chains. The formal evidence that these translocations are oncogenic has come from the development of mouse models in which transgenic or knock-in mice have been created to mimic Burkitt lymphoma by placing *Myc* under the control of enhancers from IgM (Eμ) or IgL (λ) genes. In the present study, we used three different mouse models, the *Eμ-Myc* mouse, the $iMyc^{E\mu}$ mouse, and the λ-*Myc* mouse, which all develop Myc-induced B-cell lymphomas (Supplementary Fig. S2) of varying degrees of maturity and latency. Given the promising results with *Srm* RNAi in fibroblasts, we were interested in finding out if Srm inhibition would block Myc-driven B-cell lymphomas that had arisen in mouse models of Burkitt lymphoma. To that end, we first infected several B-cell lymphoma lines from Myc transgenic mice, exemplified here by Eμ#239 from the *Eμ-Myc* transgenic mouse, with control and *Srm*

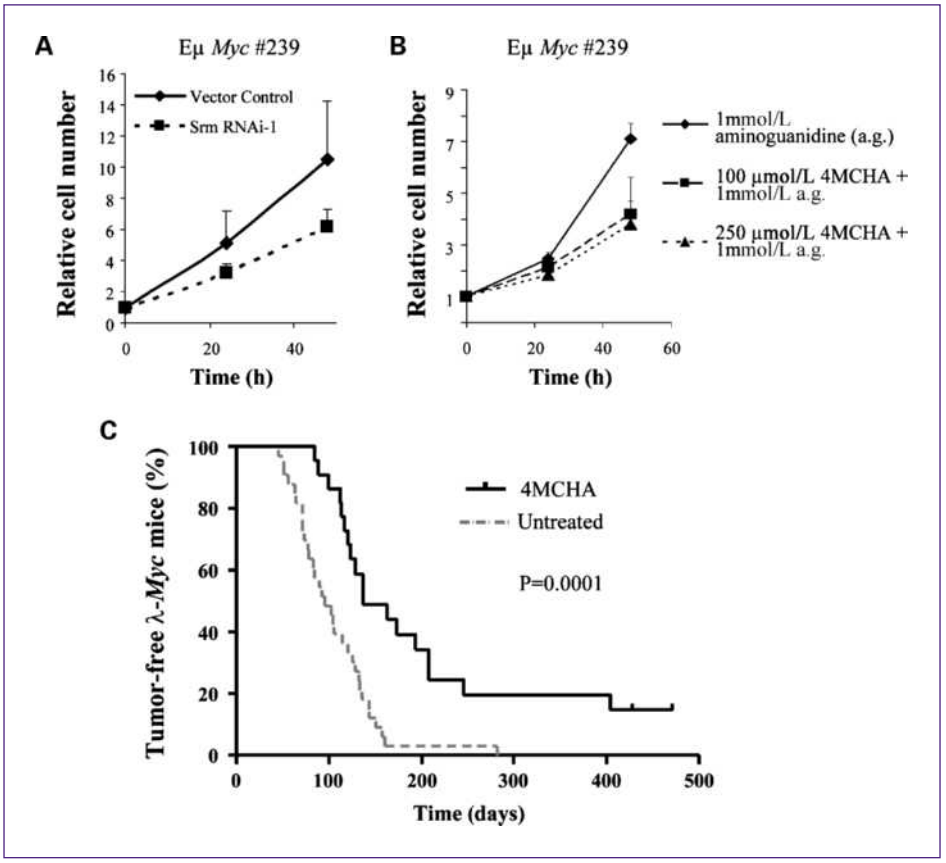

Fig. 3. Pharmacologic or genetic Srm inhibition reduces Myc-induced lymphoma cell growth *in vitro* and *in vivo*. A, primary lymphoma line Eμ#239 was established from a tumor that had developed in an *Eμ-Myc* transgenic mouse. Eμ#239 was infected with either a control retrovirus or *Srm* RNAi-1, and growth rate was determined by total cell count over 48 h. B, to determine the effect of pharmacologic inhibition of Srm on Eμ#239 proliferation, cells were cultured with 100 or 250 μmol/L of 4MCHA. To avoid toxic effects due to amine oxidase–mediated degradation of 4MCHA, cells were grown in the presence of 1 mmol/L of aminoguanidine (a.g.). C, the *in vivo* chemopreventive effect of pharmacologic inhibition of Srm was determined using λ-*Myc* transgenic mice. λ-*Myc* mice were maintained on normal drinking water (untreated) or drinking water containing 0.025% 4MCHA. The mice were followed for signs of morbidity and were sacrificed if unhealthy. Median survival time was 95 d for untreated mice, and 136 d for 4MCHA-treated mice ($P = 0.0001$).

RNAi-1–expressing retroviruses (Supplementary Fig. S3A) and assessed the effect on cell growth. Although there was a growth-suppressing effect *in vitro* (Fig. 3A), *Srm* RNAi-1 did not affect the ability of the tumor cells to engraft when transplanted into syngeneic recipients (Supplementary Fig. S3B), implying rescue by polyamine uptake. We also used a pharmacologic inhibitor of Srm, 4MCHA (25), to treat Eμ#239 cells. As seen in Fig. 3B, 4MCHA concentrations previously used in the literature (26) slightly reduced the growth rates of these cells on the second day of treatment. We therefore decided to analyze the effect of 4MCHA in primary tumor cells transplanted into recipient mice. These cells have never been in culture, allowing us to exclude overestimations or underestimations of the data due to cell culture artifacts. As seen in Supplementary Fig. S3C, transplantation via tail vein injections of tumor cells from $iMyc^{E\mu}$ mice and λ-*Myc* mice (data not shown) resulted in the reappearance of tumors in recipient C57/BL6 mice. However, administration of 0.025% 4MCHA in the drinking water did not delay tumor onset, again suggesting that uptake by tumor cells of polyamines in blood might compensate for Srm inhibition. We were not able to test higher concentrations because the mice would not tolerate higher doses, as previously described (25, 27, 28). We also tried the curative effect of 1% DFMO in the drinking water, alone or in combination with 4MCHA, with similar results (Supplementary Fig. S3D; data not shown). The lack of a curative effect of the polyamine synthesis inhibitors could not be attributed to the functional status of the p53 tumor suppressor because cells that had lost the upstream p53 regulator *Arf* or mutated *p53* all developed into tumors when injected into mice (Supplementary Fig. S3E).

Previous studies have shown that Odc inhibition could block tumor development in mouse models and patients and thus show promise for chemoprevention (rather than chemotherapy) of human cancer (29). To test if Srm would be an alternative target in chemoprevention, we treated λ-*Myc* mice with 4MCHA in their drinking water directly from weaning age onwards. As seen in Fig. 3C, 4MCHA effectively delayed tumor onset in λ-*Myc* mice, from a median survival time of 95 to 136 days. These *in vivo* data suggest that Srm is a promising target for chemoprevention against B-cell malignancies.

The most common proteins deregulated in B-cell lymphomagenesis in the *Eμ-Myc* mouse are the tumor suppressors Arf and p53 (30–32), which are inactivated by deletion or mutation, and the Bcl-2 family of antiapoptotic proteins which is activated by poorly defined mechanisms (33). Interestingly, previous studies using DFMO in this model suggested that the compound reduced cell proliferation rates and mutation spectra as a means of preventing lymphomagenesis (11). To analyze what the consequence of long-term treatment of λ-*Myc* mice with 4MCHA was on the mutation spectra, we performed Western blot and real-time PCR analyses on a panel of tumors from untreated and 4MCHA-treated animals. High levels or absent levels of p53 correlated with very strong expression of Arf, suggesting that the tumors contained nonfunctional p53 (and were therefore stable) or a deleted *p53* gene so that Arf no longer could be repressed [ref. 34; see tumor nos. 1930, 2019, and 3484 of the untreated mice; and no. 1422 (high *Arf* RNA) and no. 2529 of the 4MCHA-treated mice in Supplementary Fig. S4A]. Very low or absent levels of *Arf* RNA was used to determine which tumors had lost the *Arf* gene (Supplementary Fig. S4B). Taken together, the analysis suggests that the rates of p53 mutation and the deletion rates in 4MCHA-treated animals (4 out of 10) are similar to that found in tumors from untreated animals (4 out of 10). The rate of Arf deletion (6 out of 10), in 4MCHA-treated animals was also similar to that found in untreated animals (6 out of 10).

Discussion

The Myc-to-polyamine synthesis axis is an attractive target for chemoprevention that has produced very promising results in recent clinical trials on colon cancer using doses of DFMO that were free of side effect (12, 35). Because Myc overexpression is a hallmark of cancer, it is likely that chemoprevention of a large number of tumors could be employed by inhibiting this pathway. However, the use of polyamine synthesis inhibitors, such as DFMO, against established tumors has been a large disappointment. Several explanations for the poor success have been proposed including rescue via transport by the tumor cells of dietary polyamines from the blood, poor uptake of the drug, and metabolism thereof (36). Some of these issues, we could observe here as well when using the Srm inhibitor 4MCHA, which inhibited the growth of lymphoma cells *in vitro* but not *in vivo*. If these issues could be handled without increasing the level of side effects associated with polyamine depletion, a place for Odc and/or Srm inhibitors in the arsenal against established tumors might still be possible.

A case for Srm has been neglected because Srm is constitutively expressed and inhibition has been proven inefficient in blocking the proliferation of several cell lines (36). As shown here, *Srm* is induced in every Myc-induced tumor and to a higher relative level than *Odc*. Despite this, Srm inhibition with 4MCHA did not affect tumor growth *in vivo*, suggesting that it might complement rather than replace Odc as a target in the polyamine biosynthetic pathway. A concern about the specificity, efficacy, and bioavailability of 4MCHA could be raised but it seems sufficient to exert chemoprevention potential in λ-*Myc* mice, and original studies showed specificity of 4MCHA to Srm over the highly related spermine synthase (25). The mice only tolerated weak concentrations of the drug, due to the taste, so alternative routes of administration or new drugs should be developed. Novel Srm inhibitors are being developed and these may provide additional complementation to the chemoprevention exerted by DFMO. If a patient will require life-long treatment with a chemopreventive agent, it is likely that some mode of resistance could develop involving, e.g., amplification of the *Odc* gene (37), enhanced synthesis

of the protein (38), mutations in *Odc* or tumor suppressive pathways downstream of polyamine synthesis inhibition, or altered polyamine uptake. Changing from Odc to Srm inhibition in the midst of treatment may reduce the risk of resistance development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Nesbit CE, Tersak JM, Prochownik EV. MYC oncogenes and human neoplastic disease. Oncogene 1999;18:3004–16.
2. Nilsson JA, Cleveland JL. Myc pathways provoking cell suicide and cancer. Oncogene 2003;22:9007–21.
3. Eisenman RN. Deconstructing myc. Genes Dev 2001;15:2023–30.
4. Cowling VH, Cole MD. Mechanism of transcriptional activation by the Myc oncoproteins. Semin Cancer Biol 2006;16:242–52.
5. Hurlin PJ, Huang J. The MAX-interacting transcription factor network. Semin Cancer Biol 2006;16:265–74.
6. Kleine-Kohlbrecher D, Adhikary S, Eilers M. Mechanisms of transcriptional repression by Myc. Curr Top Microbiol Immunol 2006; 302:51–62.
7. Dang CV, O'Donnell KA, Zeller KI, Nguyen T, Osthus RC, Li F. The c-Myc target gene network. Semin Cancer Biol 2006;16: 253–64.
8. Patel JH, Loboda AP, Showe MK, Showe LC, McMahon SB. Analysis of genomic targets reveals complex functions of MYC. Nat Rev Cancer 2004;4:562–8.
9. Chang TC, Yu D, Lee YS, et al. Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet 2008;40: 43–50.
10. Jacoby RF, Cole CE, Tutsch K, et al. Chemopreventive efficacy of combined piroxicam and difluoromethylornithine treatment of Apc mutant Min mouse adenomas, and selective toxicity against Apc mutant embryos. Cancer Res 2000;60:1864–70.
11. Nilsson JA, Keller U, Baudino TA, et al. Targeting ornithine decarboxylase in Myc-induced lymphomagenesis prevents tumor formation. Cancer Cell 2005;7:433–44.
12. Meyskens FL, Jr., McLaren CE, Pelot D, et al. Difluoromethylornithine plus sulindac for the prevention of sporadic colorectal adenomas: a randomized placebo-controlled, double-blind trial. Cancer Prev Res 2008;1:32–8.
13. Pajic A, Spitkovsky D, Christoph B, et al. Cell cycle activation by c-myc in a Burkitt lymphoma model cell line. Int J Cancer 2000;87: 787–93.
14. Keller UB, Old JB, Dorsey FC, et al. Myc targets Cks1 to provoke the suppression of p27Kip1, proliferation and lymphomagenesis. EMBO J 2007;26:2562–74.
15. Krauss M, Langnaese K, Richter K, et al. Spermidine synthase is prominently expressed in the striatal patch compartment and in putative interneurones of the matrix compartment. J Neurochem 2006; 97:174–89.
16. Seiler N, Knodgen B. High-performance liquid chromatographic procedure for the simultaneous determination of the natural polyamines and their monoacetyl derivatives. J Chromatogr 1980;221: 227–35.
17. Littlewood TD, Hancock DC, Danielian PS, Parker MG, Evan GI. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res 1995;23:1686–90.
18. Bello-Fernandez C, Packham G, Cleveland JL. The ornithine decarboxylase gene is a transcriptional target of c-Myc. Proc Natl Acad Sci U S A 1993;90:7804–8.
19. Wagner AJ, Meyers C, Laimins LA, Hay N. c-Myc induces the expression and activity of ornithine decarboxylase. Cell Growth Differ 1993;4:879–83.
20. Li M, Ren S, Tilli MT, et al. Chemoprevention of mammary carcinogenesis in a transgenic mouse model by α-difluoromethylornithine (DFMO) in the diet is associated with decreased cyclin D1 activity. Oncogene 2003;22:2568–72.
21. Gupta S, Ahmad N, Marengo SR, MacLennan GT, Greenberg NM, Mukhtar H. Chemoprevention of prostate carcinogenesis by α-difluoromethylornithine in TRAMP mice. Cancer Res 2000;60: 5125–33.
22. Mitsunaga S, Clapper M, Litwin S, Watts P, Bauer B, Klein-Szanto AJ. Chemopreventive effect of difluoromethylornithine (DFMO) on mouse skin squamous cell carcinomas induced by benzo(a)pyrene. J Cell Biochem Suppl 1997;28–29:81–9.
23. Hogarty MD, Norris MD, Davis K, et al. ODC1 is a critical determinant of MYCN oncogenesis and a therapeutic target in neuroblastoma. Cancer Res 2008;68:9735–45.
24. Rounbehler RJ, Li W, Hall MA, Yang C, Fallahi M, Cleveland JL. Targeting ornithine decarboxylase impairs development of MYCN-amplified neuroblastoma. Cancer Res 2009;69:547–53.
25. Shirahata A, Takahashi N, Beppu T, Hosoda H, Samejima K. Effects of inhibitors of spermidine synthase and spermine synthase on polyamine synthesis in rat tissues. Biochem Pharmacol 1993;45: 1897–903.
26. Beppu T, Shirahata A, Takahashi N, Hosoda H, Samejima K. Specific depletion of spermidine and spermine in HTC cells treated with inhibitors of aminopropyltransferases. J Biochem (Tokyo) 1995;117: 339–45.
27. Kobayashi M, Watanabe T, Xu YJ, et al. Control of spermidine and spermine levels in rat tissues by trans-4-methylcyclohexylamine, a spermidine-synthase inhibitor. Biol Pharm Bull 2005;28:569–73.
28. He Y, Shimogori T, Kashiwagi K, Shirahata A, Igarashi K. Inhibition of cell growth by combination of α-difluoromethylornithine and an inhibitor of spermine synthase. J Biochem (Tokyo) 1995; 117:824–9.
29. Gerner EW, Meyskens FL, Jr., Goldschmid S, Lance P, Pelot D. Rationale for, and design of, a clinical trial targeting polyamine metabolism for colon cancer chemoprevention. Amino Acids 2007; 33:189–95.
30. Abrahams VM, Kamsteeg M, Mor G. The Fas/Fas ligand system and cancer: immune privilege and apoptosis. Mol Biotechnol 2003;25: 19–30.

31. Eischen CM, Weber JD, Roussel MF, Sherr CJ, Cleveland JL. Disruption of the ARF-Mdm2–53 tumor suppressor pathway in Myc-induced lymphomagenesis. Genes Dev 1999;13:2658–69.
32. Schmitt CA, McCurrach ME, de Stanchina E, Wallace-Brodeur RR, Lowe SW. INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. Genes Dev 1999;13: 2670–7.
33. Eischen CM, Woo D, Roussel MF, Cleveland JL. Apoptosis triggered by Myc-induced suppression of Bcl-X(L) or Bcl-2 is bypassed during lymphomagenesis. Mol Cell Biol 2001;21:5063–70.
34. Robertson KD, Jones PA. The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. Mol Cell Biol 1998;18:6457–73.
35. Sporn MB, Hong WK. Concomitant DFMO and sulindac chemoprevention of colorectal adenomas: a major clinical advance. Nat Clin Pract Oncol 2008;5:628–9.
36. Casero RA, Jr., Marton LJ. Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases. Nat Rev Drug Discov 2007;6:373–90.
37. Ask A, Persson L, Rehnholm A, Frostesjo L, Holm I, Heby O. Development of resistance to hydroxyurea during treatment of human myelogenous leukemia K562 cells with α-difluoromethylornithine as a result of coamplification of genes for ornithine decarboxylase and ribonucleotide reductase R2 subunit. Cancer Res 1993;53: 5262–8.
38. Das B, Vig M, Khurana KK, Madhubala R. Isolation and characterization of human breast adenocarcinoma cells made resistant to α-difluoromethylornithine. Cancer Invest 2000;18:115–22.