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# CpG Hypermethylation of the *C-myc* Promoter by dsRNA Results in Growth Suppression

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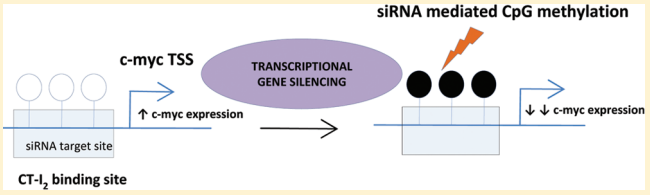
Supporting Information

**ABSTRACT:** Deregulation of the *c-myc* proto-oncogene plays an important role in carcinogenesis. It is, therefore, commonly found to be overexpressed in various types of tumors. Down-regulation of *c-myc* expression assumes great importance in tumor therapy because of its ability to promote and maintain cancer stem cells. Apart from post-transcriptional gene silencing (PTGS), siRNAs have also been shown to cause transcriptional gene silencing (TGS) through epigenetic modifications of a gene locus. This approach can potentially be used to silence genes for longer periods and at a much lesser dosage than PTGS. In this study, we have examined the effect of transfection of a novel siRNA directed against a CpG island encompassing the CT-$I_2$ region in the P2 promoter of *c-myc* in U87MG and other cell lines. Transient transfection with this siRNA resulted in *c-myc* promoter CpG hypermethylation and decreased expression of *c-myc* (both mRNA and protein) and its downstream targets. A decrease was also observed in the expression of some stemness markers (*oct-4* and *nanog*). Stable transfection also confirmed the promoter CpG hypermethylation and reduced *c-myc* expression along with reduced cell proliferation and an increase in apoptosis and senescence. A significant decrease in *c-myc* levels was also observed in three other cancer cell lines after transient transfection under similar conditions. Thus this novel siRNA has the capability of becoming an effective therapeutic tool in malignancies with overexpression of *c-myc* and may be of particular use in the eradication of recalcitrant cancer stem cells.




**KEYWORDS:** transcriptional gene silencing, siRNA, *c-myc*, DNA methylation, cancer stem cells

## INTRODUCTION

Deregulation of the proto-oncogene *c-myc* occurs in many types of cancers. In normal cells, it plays a pivotal role in the regulation of a variety of cellular processes including cell growth and proliferation, progression of the cell cycle, motility, metabolism and senescence.[1] It has been implicated in all aspects of tumor biology like apoptosis, metastasis, angiogenesis and changes in the microenvironment of the tumor.[1] *C-myc* is also required for the maintenance of cancer stem cells.[1] These cancer stem cells are refractory to available therapies because of their slow rate of proliferation, proficient DNA damage repair and drug efflux mechanisms.[2] Consequently, comprehensive regimens for the therapy of cancer should be able to target all cancer cells, including cancer stem cells.[3] Hence, *c-myc* is a potential therapeutic target for combating cancer and the recalcitrant cancer stem cells in particular.

The *c-myc* promoter is frequently hypomethylated in cancer cells.[4] A CT-$I_2$ site (corresponding to ME1a1 in mouse) has been described between the P1 and P2 promoters of *c-myc* in the human genome (Figure 1A, Supplementary Figures 1 and 2 in the Supporting Information), and this site is required for transcription through the P2 promoter.[1]

siRNAs are the key molecules of the RNA interference (RNAi) pathway involved in sequence-specific degradation of mRNAs: a process known as PTGS.[5] TGS was initially described in plants[6] and more recently in mammalian systems.[7] It is mediated by siRNAs targeting the promoter region of a gene resulting in DNA methylation of CpG islands and/or histone (H3K9, H3K27) methylation of the targeted locus[7] resulting in down-regulation of gene expression. We have previously demonstrated dsRNA induced transcriptional gene silencing at the human papillomavirus-16 E6/E7 enhancer NF-1 binding region in HPV-16 integrated cell lines which resulted in H3K9 methylation and heterochromatization at the targeted locus.[8] This strategy provides a means by which long-term reduction can be achieved in

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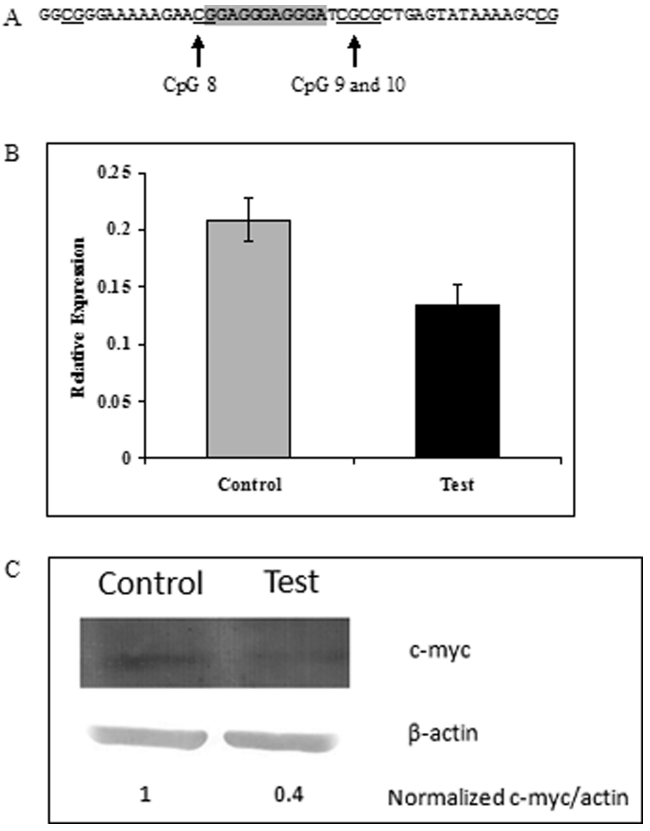


**Figure 1.** (A) Target sequence of test siRNA (Also mentioned in Supplementary Table 1 in the Supporting Information) and the CT-$I_2$ site (shaded) of P2 promoter region of c-myc. It includes three CpG sites designated as CpG 8, 9 and 10. (B) Relative expression of *c-myc* ($\beta$ actin as a reference gene) 48 h after three transfections with test or control siRNA ($p < 0.05$). Error bars represent SD. (C) Western Blot of c-myc in U87MG cell line 48hrs after three transfections with test/control siRNA.

the expression of the target gene, more than what can be achieved by PTGS.[7]

In our present study, we have attempted to trancriptionally silence *c-myc* by an siRNA directed against the CT-$I_2$ site of the *c-myc* promoter in a human glioma cell line and demonstrate that silencing is a result of CpG island methylation which leads to decreased proliferation and stemness while causing increased apoptosis and senescence.

## MATERIALS AND METHODS

**Cell Culture.** The cell lines U87MG, MCF7, SiHa and Caco-2 used in this study were procured from ATCC (American Type Culture Collection, Manassas, VA, USA). ATCC uses STR (short tandem repeat) typing to characterize cell lines.

All cell lines were maintained in DMEM (Sigma-Aldrich, Germany) supplemented with 10% FCS (Sigma-Aldrich) and 5% $CO_2$ at 37 °C. Photomicrographs were taken using an inverted phase contrast microscope (Nikon TMS, Japan).

**siRNA Designing, Synthesis and Cloning.** The target site for the test siRNA was homologous to the CT-$I_2$ site in the *c-myc* promoter (Figure 1A) which has three CpG sites. siRNAa and siRNAb also targeted the same region, but they encompassed only 2 CpG sites (Supplementary Figures 5A and 5B in the Supporting Information). The scrambled sequence of the test siRNA which had no homology to any known human mRNA sequence was used as control siRNA.

For stable transfection, complementary deoxyoligonucleotides were synthesized, annealed and cloned in an siRNA expression vector (pSilencer 2.1-U6 neo (Ambion, Austin, TX, USA)) as per the protocol shown in Supplementary Figure 7 in the Supporting Information. Briefly, deoxyoligonucleotides corresponding to the shRNA were designed according to the siRNA target using the "Insert Design Tool for the pSilencer Vectors" software available at www.ambion.com. The complementary oligos were annealed at their 3′ end and extended using Taq polymerase. They were amplified using primers containing restriction sites. The extended DNA fragment and the pSilencer vector backbone were digested using *Bam*HI and *Hin*dIII enzymes (both from MBI Fermentas, USA). The DNA fragment and the vector backbone were then ligated using T4 DNA ligase (MBI Fermentas, USA). This ligated vector was used for stable transfection experiments.

A negative control plasmid (Ambion) transcribing a short hairpin dsRNA which also had no homology to any known human mRNA sequence was used as a control for stable transfection.

The sequences of the synthetic siRNAs used for transient transfections are given in Supplementary Table 1 in the Supporting Information. The synthesis of siRNA was done by using an in vitro transcription kit (MBI Fermentas, Hanover, MD, USA) as described previously.[9] siRNAs were checked for their purity by running on a 4% agarose gel and then quantified using RiboGreen RNA binding dye (Invitrogen, CA, USA) .

**Transient Transfections.** $10^5$ cells were plated per 25 $cm^2$ flask (Corning, NY, USA) on day 0. siRNAs were transfected at 20 nM concentration using Lipofectamine 2000 (Invitrogen) as per the manufacturer's protocol. Transfections were done on day 1, day 4 and day 7. RNA and DNA were extracted 48 h after day 7.

**Stable Transfection.** $10^5$ cells per 25 $cm^2$ flask were plated on day 0. Transfection was done using 6 $\mu$L of Transfast reagent (Promega, WI, USA) per $\mu$g of plasmid DNA per flask along with appropriate controls. G418 (Sigma-Aldrich) was added at a concentration of 800 $\mu$g/mL during medium replenishment starting from the day after transfection. The colonies were isolated using cloning cylinders (Corning) and expanded.

**DNA Methyl Transferase and Histone Deacetylase Inhibition.** For DNMT and HDAC inhibition 5 $\mu$M 5-aza 2-deoxycytidine (Aza) and 300 nM Trichostatin (TSA) dissolved in DMSO[10] were added in respective culture plates during change of medium every 3 days. It was observed that under these conditions there was no significant change in cell death or proliferation. In control cells an equivalent amount of DMSO alone was used. Transfection of test/control siRNA was done after 48 h of treatment of U87MG cells with Aza/TSA, and RNA was isolated after 6 days of siRNA transfection.

**Cell Proliferation Assay.** Untransfected cells and cells stably transfected with negative control plasmid and test plasmid were plated in 96 well plates (Corning) at a density of $5 \times 10^3$ cells per well in quadruplicate. Viable cells were counted on the second, fourth, sixth and eighth days using trypan blue staining.

**MTT Assay.** Cells transfected with test and control siRNA expression vector as well as untransfected cells were replated in 96-well plates ($5 \times 10^3$ cells/well) in sextuplets. MTT (Sigma-Aldrich) assay was performed on days 3, 6, and 9. Color developed was read in an ELISA reader (Anthos Hill, Austria) at 550 nm.

**Flow Cytometry.** Stably transfected cells (test, control and normal) were grown to 50% confluency, fixed with 70% ethanol and stained with propidium iodide (Sigma-Aldrich) before analysis

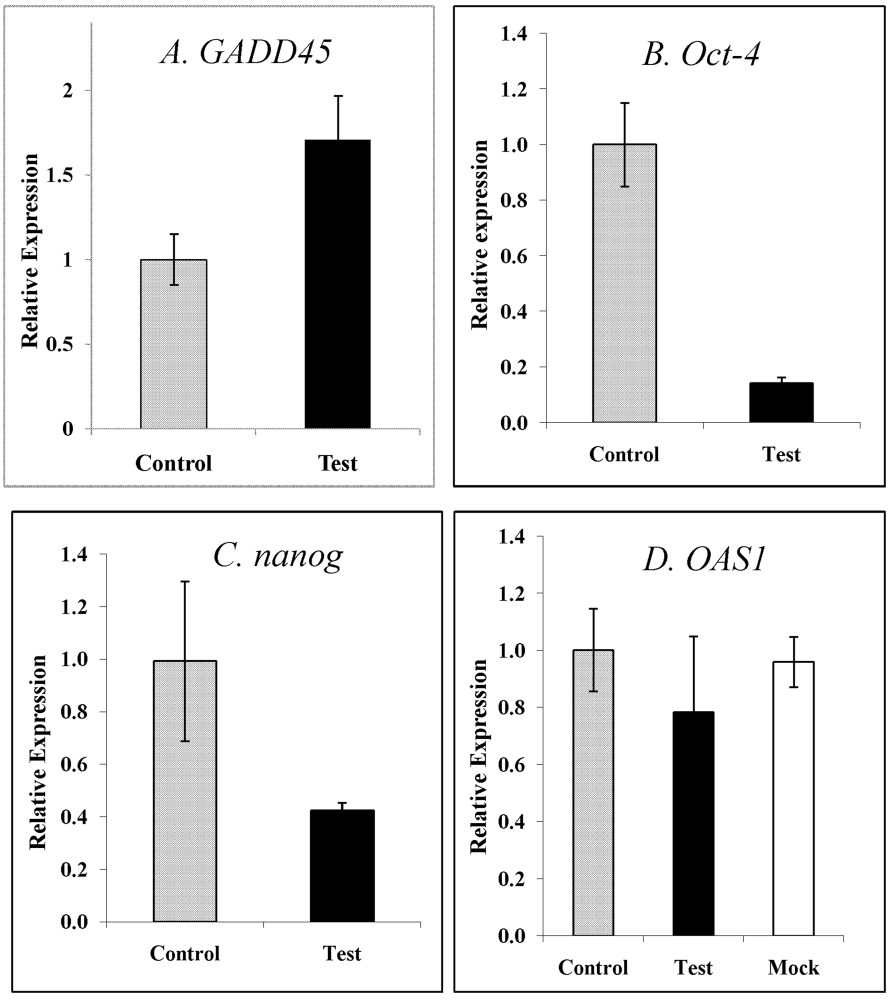


**Figure 2.** Relative expression of (A) *GADD45*, (B) *Oct-4*, (C) *nanog* ($p < 0.05$ in all cases) and (D) *OAS1* ($p = 0.41$) 48 h after three transfections with test or control siRNA. Error bars represent SD.

in a Coulter Epics XL Flow Cytometer (Beckman Coulter, CA, USA). The results were then evaluated using WinMDI software.[11]

**Senescence Associated β Galactosidase Assay.** Cytochemical staining for senescence associated β galactosidase assay was carried out according to a previously published protocol.[12]

**Primers.** All the primers used in the study for expression analysis by Real Time PCR and methylation analysis by bisulfite-PCR-sequencing are given in Supplementary Table 2 in the Supporting Information.

**DNA Methylation Analysis by Bisulfite Treatment and PCR Sequencing.** Genomic DNA was isolated using GenElute Mammalian genomic DNA Miniprep kit (Sigma-Aldrich) and quantified using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, CA, USA). Nested PCR was carried out with 2 μg of genomic DNA after digestion with *Hin*dIII (MBI Fermentas) and bisulfite treatment using EpiTect bisulfite kit (Qiagen, Germany). The inner primers were M13 universal primer-tagged, and sequencing was done using M13 primers. The methylation status was analyzed at the CpG sites mentioned in Supplementary Figure 1A in the Supporting Information from the height of the peaks of the A (corresponding to unmethylated C in complementary strand) and G (methylated C in complementary strand) at every CG site in the amplicon using BioEdit software.

**Real Time PCR.** RNA was isolated from cells at appropriate time points by TRIzol reagent (Sigma-Aldrich), DNase (MBI Fermentas) treated and quantified using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA (500−1000 ng) was converted to cDNA using random decamers as primers and MMLV-RT (MBI Fermentas). Realtime PCR was done on a RotorGene 6000 real-time PCR machine (Corbett Research, Australia). The real-time PCR products were run on an agarose gel to check for the specific product formed. The analysis of data was carried out using delta−delta Ct method.

**Western Blot.** After three transfections, cells (U87MG and MCF7) were lysed in triple detergent buffer containing protease inhibitors. Equal amounts of protein lysate (as quantified by using bicinchoninic acid protein assay, BCA) were electrophoresed in 5−12% SDS−PAGE and electroblotted on a nitrocellulose membrane. Blocking was done with 4% bovine serum albumin (BSA) in TBS at room temperature for two hours. Immunoblotting antibodies used were anti c-myc (BD Biosciences, USA) and anti β actin (Cell Signaling Technology, USA). Detection was done using an alkaline phosphatase labeled secondary antibody and BCIP/NBT (Promega, USA) as the substrate. Integrated density values (IDV) were measured (Alpha-Imager), and the relative expression was calculated with reference to β actin.

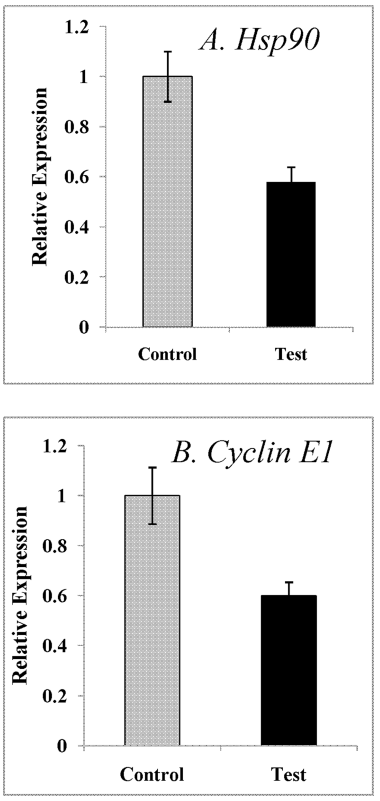


**Figure 3.** Relative expression of (A) HSPCAL3 (Hsp90) and (B) CCNE1 (cyclin E1) in cells transfected with control siRNA and test siRNA ($p < 0.05$ for both genes). RNA for expression profiling was extracted 48 h after three transfections (*18S* as reference gene). Error bars represent SD.

**Immunofluorescence Studies.** Control and test siRNA transfected cells were trypsinized and cytocentrifuged onto glass slides. They were then air-dried and then fixed with 4% formaldehyde. The slides were then washed with PBS, permeabilized using 0.3% Triton-X, washed with PBS and then incubated overnight with anti-p21 antibody (Catalog No. 2947, 1:500 dilution) (Cell Signaling Technology, USA) at 4 °C with shaking. The slides were then washed again with PBS and incubated with Alexa Fluor 488 labeled anti-rabbit secondary antibody (1:2000 dilution) for one hour at room temperature. The slides were washed thrice with PBS and visualized using a Nikon Eclipse fluorescence microscope (Nikon, Japan).

**Statistical Analysis.** Significance in differences between means of untreated and differently treated cells was calculated using the unpaired Student's *t*-test. All the experiments were repeated at least twice (biological replicates). All the Real Time PCR experiments were done in triplicate, and the rest of the experiments were done in duplicate. Figures show the representative results, and the error bars in the bar charts represent the standard deviation (SD). *P* values less than 0.05 were taken as significant, and those less than 0.001 were taken to be highly significant.

## RESULTS

**Transient Transfections with a Specific siRNA Targeted against the CT-$I_2$ Site of *c-myc* Promoter Resulted in Downregulation of the *c-myc* Gene and Modulation of Expression of Other *c-myc* Dependent Genes and Stemness Markers.** The *c-myc* gene is mainly transcribed from two promoters: the P1 and P2 promoters. The P1 promoter is upstream of exon 1 while the P2 promoter is present at the 5′ end of exon 1 (Supplementary Figure 1 in the Supporting Information). However the P2 promoter is stronger than the P1 promoter and the ratio of P2 promoter driven transcripts to P1 promoter driven transcripts varies around 5:1 to 10:1.[1] We have also quantified the P1 and P2 promoter derived transcripts using the primers depicted in Supplementary Figure 1 in the Supporting Information and given in Supplementary Table 2 in the Supporting Information in three cell lines: U87MG, U87MG with dominant negative p53 and siHa. Our data also corroborates previous reports showing that around 90% of *c-myc* transcripts are expressed from the P2 promoter (Supplementary Figure 3 in the Supporting Information).

After U87MG cells were transfected three times with the test siRNA targeted against the CT-$I_2$ site in P2 promoter of *c-myc* (Figure 1A), there was a 40% decrease in the expression of *c-myc* mRNA ($p < 0.05$) in comparison with the cells transfected with negative control siRNA (Figure 1B). This decrease was significant with respect to all the reference genes studied (*β actin, GAPDH, PPIA* and *18S rRNA*).

At the protein level, the decrease in *c-myc* expression was around 60% (Figure 1C). The *c-myc* protein directly binds to the p21 promoter and represses its expression.[1] U87MG cells with knockdown of *c-myc* by test siRNA showed an increase in the level of p21 protein expression as seen by immunofluorescence studies using anti p21 antibody and Alexa 488 labeled secondary antibody (Supplementary Figure 4 in the Supporting Information).

Also post-transfection, there was a significant increase ($p < 0.05$) in *gadd45* mRNA level, which is normally downregulated by *c-myc*[13] (Figure 2A). Moreover, the expression of two markers for stemness, *oct4* and *nanog*,[14] was also significantly reduced ($p < 0.05$) (Figures 2B and 2C).There was a significant downregulation ($p < 0.05$) of two other *myc*-responsive genes, HSPCAL3 (HSP90) and CCNE1 (cyclinE1) (Figures 3A and 3B), as well.

U87MG cells were also transfected with two other siRNAs (siRNAa and siRNAb), each targeting 2−3 bases upstream or downstream of the target site of the test siRNA but targeting only two of the three CpG sites in the original target region (Supplementary Figures 5A and 5B in the Supporting Information). There was no significant change in the expression of *c-myc* after transfection with these siRNAs (Supplementary Figures 5C and 5D in the Supporting Information).

**No Interferon Response Was Elicited by siRNA Transfection.** Double stranded RNA often causes nonspecific cell death by initiating an interferon response as seen by an increase in 2′-5′-oligoadenylate synthetase (*OAS1*) mRNA, a recognized interferon response marker.[15] We studied the expression of *OAS1* in U87MG cell line after mock, control siRNA and test siRNA transfection. No significant increase was observed in the OAS1 mRNA levels after test siRNA transfection ($p = 0.41$) (Figure 2D), implying that there was no interferon response.

**Transient Transfection of Test siRNA Resulted in Hypermethylation of the *c-myc* Promoter.** There was hypermethylation of CpG sequences in the targeted locus of the *c-myc* promoter of U87MG cells (Figure 4A) after transient transfection with test siRNA as demonstrated by bisulfite-PCR sequencing (Supplementary Figure 1B in the Supporting Information). This

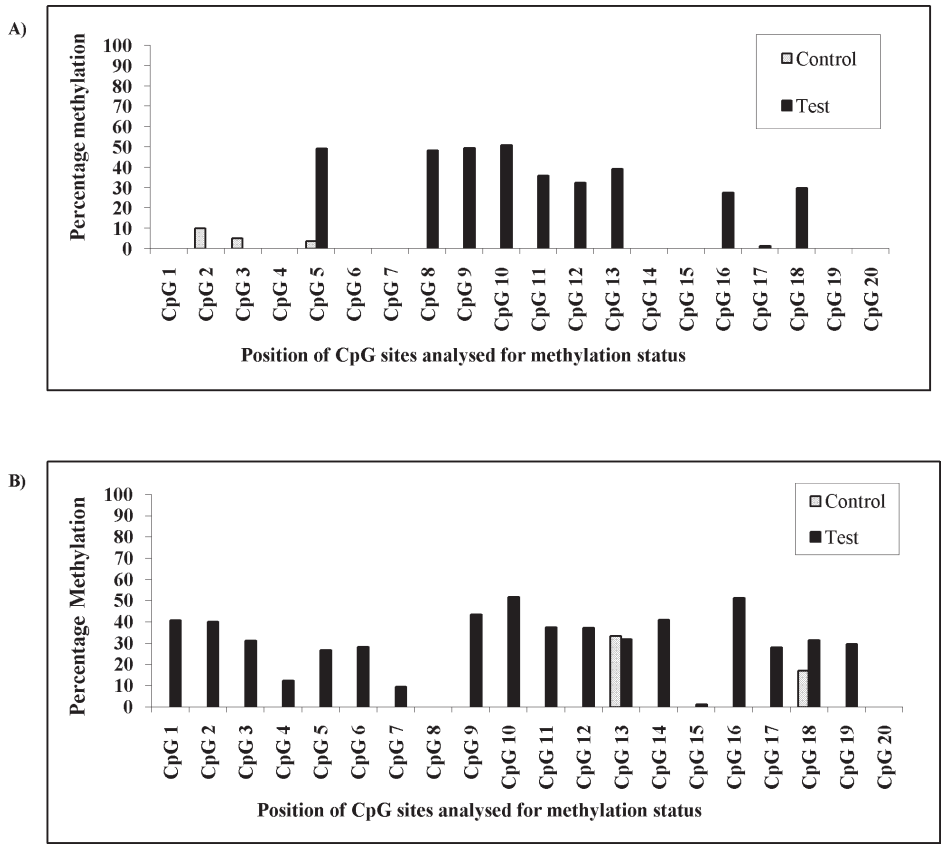


**Figure 4.** Analysis of bisulfite-sequencing chromatograms illustrating methylation status of the *c-myc* locus in cells transfected with test or control siRNA after (A) transient and (B) stable transfections. Percentage methylation was calculated at the CpG sites present in *c-myc* promoter. CpG site are numbered in 5′ to 3′ orientation. Sites 8, 9, and 10 are present in the target region located in P2 promoter of *c-myc*.

occurred at the CpG sites present in the target as well as in the upstream and downstream regions.

**Transcriptional Gene Silencing of the *c-myc* Promoter Is DNMT Dependent.** Treatment of U87MG cells with 5 $\mu$M 5-aza 2-deoxycytidine (Aza: DNMT inhibitor) for 48 h followed by *c-myc* siRNA transfection resulted in significant increase in *c-myc* expression ($p < 0.001$) compared to *c-myc* siRNA alone (Supplementary Figure 7 in the Supporting Information). However, treatment of U87MG cells with Aza alone caused a ~45% fall in *c-myc* mRNA levels, similar to a published report,[16] and adding *c-myc* siRNA after Aza treatment resulted in no significant change in *c-myc* expression ($p = 0.36$). When cells were treated with 300 nM trichostatin (HDAC inhibitor) for 48 h followed by test siRNA transfection, U87MG cells still showed a significant fall ($p < 0.001$) in *c-myc* mRNA levels (Supplementary Figure 7 in the Supporting Information). This fall was not significantly different from what was observed after treatment with *c-myc* siRNA alone ($p = 0.1$). This demonstrates that the transcriptional gene silencing of *c-myc* by test siRNA is dependent on DNMT activity but not on HDAC activity.

**Stable Transfection of Test siRNA Resulted in a Decrease in the Proliferation Rate as Well as an Increase in Apoptosis and Senescence.** We next studied the proliferation rate, apoptosis and senescence in U87MG cells stably transfected with pSilencer 2.1 U6 neo, an siRNA expression vector, containing an insert that codes for shRNA which is processed into the test siRNA inside the cell after transcription. The test shRNA expression vector was designed as mentioned in

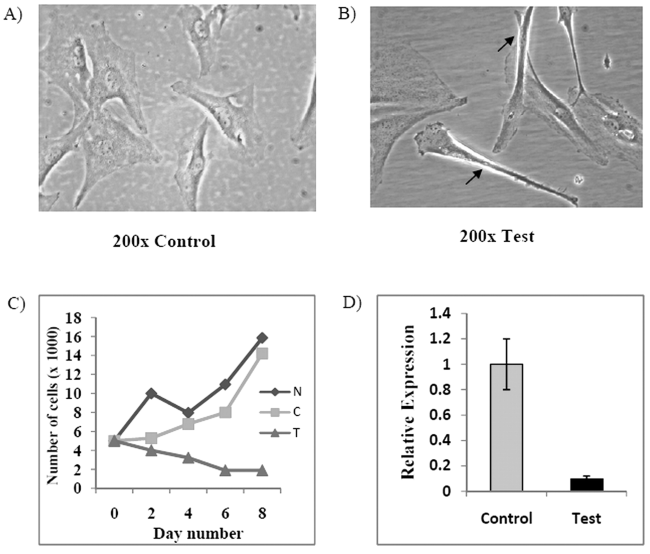


**Figure 5.** (A, B) Photomicrographs of the cells stably transfected with control and test siRNA respectively, three months post-transfection. Arrows denote typical morphological changes (elongated and spindle shaped) observed in test siRNA transfected U87MG cells. (C) Cell proliferation assay of untransfected normal (N) cells and cells stably transfected with test (T) or control (C) siRNA after selection. Viable cells were counted on days 2, 4, 6 and 8 (test vs normal $p < 0.05$; test vs control $p < 0.05$). Error bars represent SD. (D) Relative expression of *c-myc* in the cells stably transfected with vector coding for test or control siRNA (*GAPDH* as reference gene $p < 0.001$). Error bars represent SD.

Materials and Methods (Supplementary Figure 8 in the Supporting Information).

After G418 selection, only two colonies were obtained from the cells transfected with test shRNA as compared to 20 colonies obtained with control shRNA. We hypothesized that this may be the result of loss of clones due to the effect of the siRNA against *c-myc* itself. When the transfected cells were maintained further with G418, morphological changes were apparent when test cells became elongated and spindle shaped. The control cells maintained the normal morphology of U87MG cells (Figures 5A and 5B and Supplementary Figure 9 in the Supporting Information). Cell counting demonstrated that the rate of proliferation was significantly lower in test cells as compared to control ($p < 0.05$) or normal ($p < 0.05$) cells (Figure 5C). The results of MTT assay corroborated with the findings of the cell proliferation assay (data not shown).

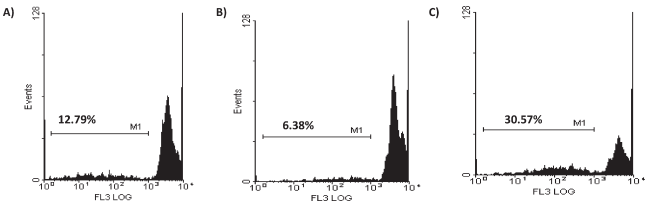


**Figure 6.** Flow cytometry of U87MG cells after propidium iodide staining: (A) normal, (B) control shRNA transfected and (C) test shRNA transfected cells ($p < 0.001$ in both test vs normal and test vs control). The M1 gate indicates hypoploid cells (sub-G1 phase) i.e. the cells undergoing apoptosis.

Flow cytometric analysis of the stable transfectants after fixation and propidium iodide staining illustrated that there was an increase in the percentage of cells undergoing apoptosis in test cells in comparison with control and normal cells ($p < 0.001$) (Figure 6).

The role of *c-myc* in causing a decrease in senescence has previously been demonstrated.[1] Senescence associated $\beta$ galactosidase (SA $\beta$ gal) assay demonstrated an increase in $\beta$ galactosidase activity in test cells in comparison with control and normal cells. Staining was evident in test cells within three hours, with no evidence of staining in control and normal cells. Staining became more intense and frequent after five hours in the test cells while only a few cells were stained in control and none of the untransfected cells were stained (Supplementary Figure 10 in the Supporting Information). This indicates that there was an increase in senescence in test cells as a result of transcriptional gene silencing of *c-myc*.

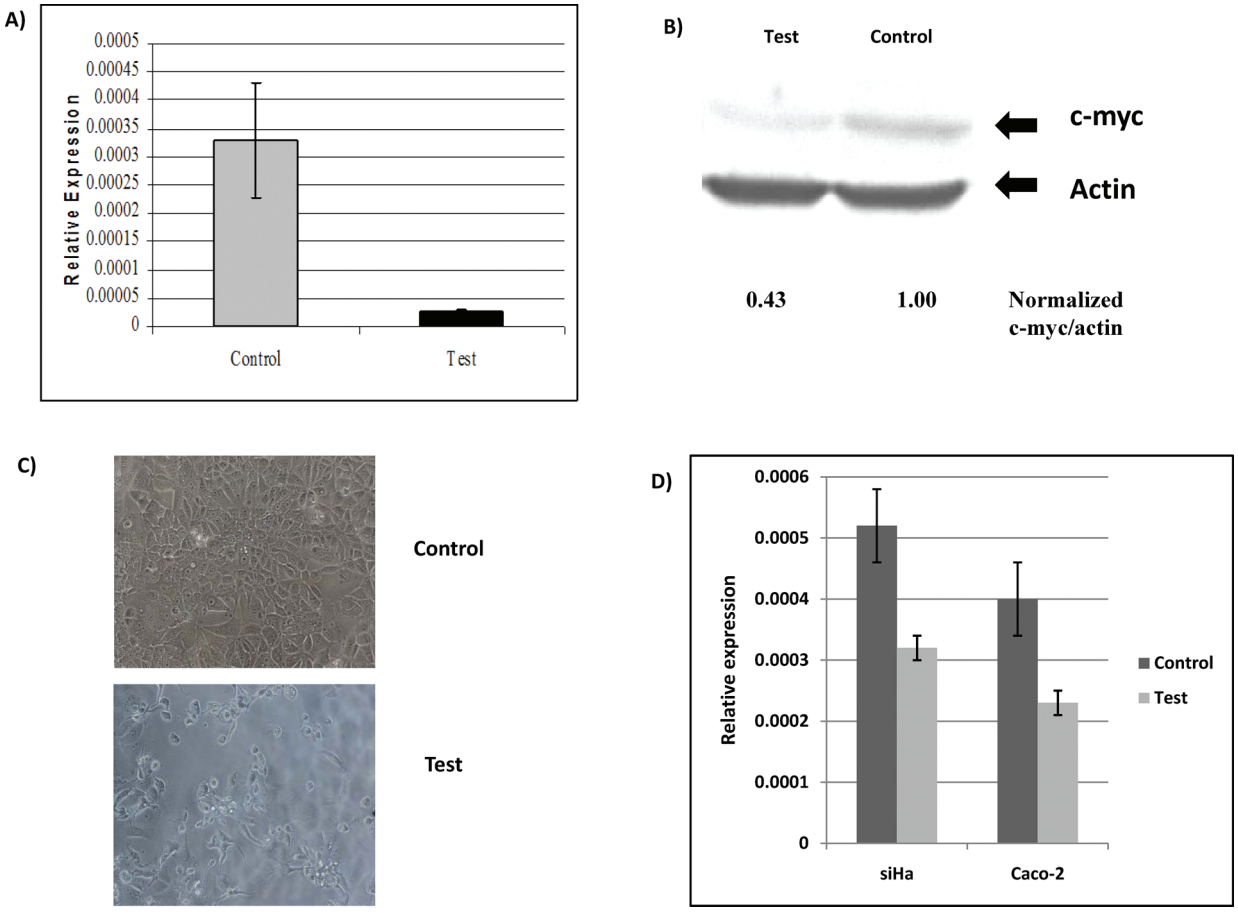


**Figure 7.** (A) Relative expression of *c-myc* in MCF7 cells transfected with control and test siRNA. RNA for expression profiling was extracted 48 h after three transfections (*18S* as a reference gene $p < 0.001$). (B) Western blot of *c-myc* in MCF7 cell line. $\beta$ *actin* was used as a reference. (C) Photomicrographs (100× magnification) of MCF-7 cells 48 h after 3 transfections with test or control siRNA. Transfection of test siRNA in MCF-7 cells causes a marked reduction in cell number and apoptotic morphology. (D) Relative expression of *c-myc* in SiHa and Caco-2 cells transfected with control and test siRNA. RNA for expression profiling was extracted 48 h after three transfections (*18S* as a reference gene $p < 0.05$ in both cases). Error bars represent SD.


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**Stable Transfections Also Resulted in a Decrease in *c-myc* Expression as Well as an Increase in Promoter Methylation.** Real time PCR data revealed a considerable decrease ($p < 0.001$) in the expression of *c-myc* in test cells in comparison with control cells (Figure 5D). CpG methylation status of the targeted locus was then assessed in both test and control cells by bisulfite sequencing. It was found to be increased in test cells, similar to the results obtained from the transient transfection experiments (Figure 4B and Supplementary Figure 11 in the Supporting Information).

**Other Cell Lines Similarly Treated with the Same siRNA Also Showed Reduced Expression of *c-myc*.** On transient transfection of three other cell lines (MCF7 ($p < 0.001$), SiHa and Caco-2 ($p < 0.05$ in both cases)) with the test siRNA, *c-myc* mRNA expression declined by 40 to 90% (Figures 7A and 7D). In MCF7 cell line, which showed the maximum decrease in *c-myc* mRNA expression after transient transfection, *c-myc* protein level fell by ~60% when compared with the control (Figure 7B). MCF-7 cells transfected with the test siRNA also showed a decrease in cell number and apoptotic morphology (Figure 7C).

## DISCUSSION

*c-myc* plays an important role in the behavior of tumors by affecting almost every aspect of tumor biology, for example, cell proliferation, senescence, metastasis, apoptosis, angiogenesis and maintenance of stemness. The altered expression of MYC is often an early step in multistage transformation[17] and has been described to have an addictive effect on cancer cells and is, therefore, an Achilles heel for them.[18] Interventions that reduce *c-myc* expression are more advantageous than the more conventional approach in which the protein is inactivated or inhibited, because inactivated/inhibited MYC can still mediate some of the protein−protein interactions of the wild-type protein.[18] Targeting *c-myc* at the transcriptional or post-transcriptional level in a tumor cell where it is upregulated, therefore, not only is likely to rob the tumor cells of the benefits of the proliferative and antisenescence effects of *c-myc* but also may lead to a decline in the number of cancer stem cells as *c-myc* is an important factor in increasing the stemness of a cancer cell.[1] While siRNAs are more active than antisense approaches[19] and siRNAs targeting the *myc* mRNA (PTGS) have been known to effectively reduce *c-myc* levels in MCF7 cells,[20] siRNA guided TGS by CpG methylation may give a much longer duration of suppression and thus have better efficacy.[7,8]

Our data indicates that this novel siRNA, directed against a specific region of the *c-myc* promoter, can induce CpG methylation and thus downregulate its expression at the mRNA as well as protein level. This could be reversed by inhibiting DNMT by 5-aza 2-deoxycytidine but not by trichostatin, a HDAC inhibitor. However, it is also apparent that the selection of the target is very decisive as a minor divergence from the target led to a loss of this silencing effect. The same siRNA was effective in reducing the *c-myc* levels in the other cell lines tested (MCF7, SiHa and Caco-2). However, the degree of this knockdown varied from cell line to cell line. This was most likely the result of difference in transfection efficiency and varying amounts of transcription enhancers at the *c-myc* locus in the different cell lines, which may prevent the siRNA−protein complex from exerting its full effect at the target within the limited time period of transient transfection.

In our study, the decrease in *c-myc* protein in U87MG cell line was associated with a significant modulation of some of the downstream effector molecules of the *c-myc* pathway (*gadd45*, HSP90 and cyclinE1). This also resulted in decreased proliferation and stemness while causing increased apoptosis and senescence of the cells.

It has been recently demonstrated that siRNA, directed against the *c-myc* transcription start site, could suppress transcription with subsequent effects on cell growth characteristics.[21] The study concluded that downregulation of *c-myc* mRNA occurred as a result of direct transcriptional interference by the transfected siRNA. However, in the same study no inheritable epigenetic alterations like CpG methylation or histone methylation were observed, which might affect the duration of the response.

In conclusion, our study indicates that silencing by CpG hypermethylation is also an important mechanism of gene silencing by dsRNA leading to marked downstream effects on cell growth characteristics. An inheritable, epigenetic mark on the target raises the possibility of long-term gene silencing. This siRNA can thus be used as an antitumor therapy after the development of cell specific delivery systems which opens up a window of opportunity in its use as a therapeutic option in malignancies where *c-myc* is deregulated and the cancer cells are "addicted" to it.

## ASSOCIATED CONTENT

**Supporting Information.** Additional figures and tables as discussed in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

‡Both authors contributed equally to the work.

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## REFERENCES

(1) Wierstra, I.; Alves, J. The c-myc promoter: still MysterY and Challenge. *Adv. Cancer Res.* **2008**, *99*, 113–333.

(2) Rich, J. N. Cancer stem cells in radiation resistance. *Cancer Res.* **2007**, *67*, 8980–8984.

(3) Wicha, M. S.; Liu, S.; Dontu, G. Cancer Stem Cells: An Old Idea—A Paradigm Shift. *Cancer Res.* **2006**, *66*, 1883–1890.

(4) Fang, J. Y.; Xiao, S. D. Alteration in DNA methylation in gastrointestinal carcinogenesis. *J. Gastroenterol. Hepatol.* **2001**, *16*, 960–968.

(5) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **1998**, *391*, 806–811.

(6) Mette, M. F.; Aufsatz, W.; Winden, J. V. D.; Matzke, M. A.; Matzke, A. J. M. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J.* **2000**, *19*, 5194–5201.

(7) Morris, K. V. RNA-Mediated Transcriptional Gene Silencing in Human Cells. *Curr. Top. Microbiol. Immunol.* **2008**, *320*, 211–24.

(8) Palanichamy, J. K.; Mehndiratta, M.; Bhagat, M.; Ramalingam, P.; Das, B.; Das, P.; Sinha, S.; Chattopadhyay, P. Silencing of integrated human papillomavirus-16 oncogenes by small interfering RNA-mediated heterochromatization. *Mol. Cancer Ther.* **2010**, *9*, 2114–22.

(9) Donze, O.; Picard, D. RNA interference in mammalian cells using siRNAs synthesised with T7 RNA polymerase. *Nucleic Acids Res.* **2002**, *30*, e46.

(10) Vallot, C.; Stransky, N.; Bernard-Pierrot, I.; Hérault, A.; Zucman-Rossi, J.; Chapeaublanc, E.; Vordos, D.; Laplanche, A.; Benhamou, S.; Lebret, T.; Southgate, J; Allory, Y.; Radvanyi, F. A novel epigenetic phenotype associated with the most aggressive pathway of bladder tumor progression. *J. Natl. Cancer Inst.* **2011**, *103*, 47–60.

(11) Salk Institute for Biological Studies, La Jolla, CA. Available at http://pingu.salk.edu/software.html.

(12) Gary, R. K.; Kindell, S. M. Quantitative assay of senescence-associated beta-galactosidase activity in mammalian cell extracts. *Anal. Biochem.* **2005**, *343*, 329–34.

(13) Bush, A.; Mateyak, M.; Dugan, K. c-myc null cells misregulate cad and gadd45 but not other proposed c-myc targets. *Genes Dev.* **1998**, *12*, 3797–3802.

(14) Clement, V.; Sanchez, P.; Tribolet, N.; Radovanovic, I.; Altaba, A. R. HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal and tumorigenicity. *Curr. Biol.* **2007**, *17*, 165–172.

(15) Fish, R. J.; Kruithof, E. K. O. Short-term cytotoxic effects and long-term instability of RNAi delivered using lentiviral vectors. *BMC Mol. Biol.* **2004**, *5*, 9.

(16) Valdez, B. C.; Li, Y.; Murray, D.; Corn, P.; Champlin, R. E.; Andersson, B. S. 5-Aza-2′-deoxycytidine sensitizes busulfan-resistant myeloid leukemia cells by regulating expression of genes involved in cell cycle checkpoint and apoptosis. *Leuk. Res.* **2010**, *34*, 364–72.

(17) Weinstein, I. B.; Joe, A. Oncogene addiction. *Cancer Res.* **2008**, *68*, 3077–3080.

(18) Brooks, T. A.; Hurley, L. H. The role of supercoiling in transcriptional control of MYC and its importance in molecular therapeutics. *Nat. Rev. Cancer* **2009**, *9*, 849–861.

(19) Bertrand, J. R.; Pottier, M.; Vekris, A.; Opolon, P.; Maksimenko, A.; Malvy, C. Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 1000–1004.

(20) Wang, Y.; Liu, S.; Zhang, G.; Zhou, C. Q.; Zhu, H. X.; Zhou, X. B.; Quan, L. P.; Bai, J. F.; Xu, N. Z. Knockdown of c-Myc expression by RNAi inhibits MCF-7 breast tumor cells growth in vitro and in vivo. *Breast Cancer Res.* **2005**, *7*, R220–R228.

(21) Napoli, S.; Pastori, C.; Magistri, M.; Carbone, G. M.; Catapano, C. V. Promoter-specific transcriptional interference and c-myc gene silencing by siRNAs in human cells. *EMBO J.* **2009**, *28*, 1708–19.