RNAi-Mediated Silencing of Myc Transcription Inhibits Stem-like Cell Maintenance and Tumorigenicity in Prostate Cancer

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Abstract
Several studies link disease progression, recurrence, and treatment failures to the cancer stem-like cell (CSC) subpopulation within the heterogeneous tumor cell population. Myc is a transcription factor having a central function in stem cell biology and in human cancers. Hence, Myc represents an attractive target to develop CSC-specific therapies. Recent findings suggest that Myc transcription can be silenced using an RNA interference (RNAi)-based strategy that targets noncoding promoter-associated RNA (paRNA) overlapping the transcription start site. In this study, we investigated the effects of silencing Myc transcription on prostate CSC in cell culture and xenograft models of human prostate cancer. Treatment with an effective promoter-targeting siRNA reduced the fraction of CSCs, leading to reduced self-renewal, tumor-initiating, and metastatic capability. Combined analysis of stem-like cells and senescence markers indicated that Myc silencing triggered a phenotypic shift and senescence in the CSC subpopulation. Notably, systemic delivery of the promoter-targeting siRNA in the xenograft model produced a striking suppression in the development of prostate tumors. Our results support a pivotal role for Myc in CSC maintenance and show that Myc targeting via RNAi-based transcriptional silencing can trigger CSC senescence and loss of their tumor-initiating capability. More generally, our findings demonstrate the efficacy of RNAi-based transcriptional strategies and the potential to target regulatory noncoding paRNAs for therapeutic applications. Cancer Res; 73(22): 6816–27. ©2013 AACR.

Introduction
Prostate cancer is the most common epithelial cancer and the third leading cause of cancer-related death in men in the western countries (1, 2). Considerable progress has been made in the last decade to understand the disease at the molecular and genetic level (3, 4). However, there has been limited improvement in the treatment of advanced, castration-resistant prostate cancer (CRPC; ref. 5). There is increasing evidence that most human cancers, including prostate cancer, are driven by a rare population of cancer cells that display stem cell-like properties, defined as cancer stem-like cells (CSC) or tumor-initiating cells (6). CSCs within primary tumors are likely the main cause of metastasis and disease recurrence (6, 7). CSCs contribute also to treatment failures by virtue of their intrinsic resistance to standard treatment, including chemotherapeutic drug and radiotherapy (8, 9). Indeed, the inability of the current treatments to affect CSCs may explain their limited efficacy. The development of new strategies for prostate cancer treatment must take into consideration this biologic complexity and cellular heterogeneity. Accordingly, agents that would effectively target and eliminate the CSC component in human tumors might have a considerable impact on treatment and patient outcome (8, 10).

CSCs are characterized by an imbalance between self-renewal and differentiation potential, which leads to the enhanced survival and expansion (6). The pathways controlling the enhanced self-renewal capability and the reduced differentiation potential of CSCs could provide ideal targets for development of CSC-specific treatment strategies (10). Myc is a transcription factor that is activated by multiple mitogenic signaling pathways (11). Myc is involved in many biologic processes, including transcription, replication, cell division, protein synthesis, and metabolism (11). Amplification, chromosomal translocations, and deregulated expression of Myc are among the most common alterations occurring in human cancers (11). Myc is upregulated in primary and metastatic prostate cancers and has been associated with progression to CRPC (12). Myc exerts multiple roles in tumorigenesis affecting cell proliferation, survival, metabolic adaptation, and angiogenesis (11, 13, 14). Myc has also a central role in stem cell biology controlling the equilibrium between self-renewal and differentiation of hematopoietic and epithelial stem cells (11). In normal prostate, Myc is detected mainly in basal epithelial...
cells, which are considered the main source of normal prostate stem cells (12, 15). Furthermore, Myc along with other stem cell genes, such as BMI-1 and POU5F1, is highly expressed in prostate cancer cells having the CD44+/CD24− phenotype, which is considered a hallmark of cancer progenitor/stem cells (16). Myc is overexpressed in the CSC subpopulation in other tumor types, such as glioblastoma (17) and Myc knockdown using short hairpin RNA (shRNA) in glioma stem cells decreases proliferation and induces cell-cycle arrest (17). Furthermore, Myc inactivation in conditional transgenic mouse models results in tumor regression associated with proliferation arrest, differentiation, senescence, or apoptosis depending on the cellular and genetic context (18–23). Notably, systemic inactivation of Myc in mice affects transiently normal proliferating tissues, but the effect is well tolerated and fully reversible, suggesting that Myc inhibition could be an effective and safe therapy for cancer (24). Together, these lines of evidence suggest that Myc has an important role in ensuring tumor development and maintenance of CSC component in human tumors and could be an attractive target for development of CSC-targeted therapies.

Like many other transcription factors, Myc is a difficult target to address directly with conventional small-molecule drugs (25). Targeting upstream regulators of Myc expression or downstream factors cooperating with Myc oncogenic functions is a valid and promising approach to explore (11, 26, 27). In addition, we showed recently that Myc could be epigenetically silenced with high efficiency by promoter-targeting siRNAs (28). Transcriptional silencing by promoter-targeting siRNAs could provide an effective means to modulate gene expression in addition to canonical posttranscriptional RNA interference (RNAi) directed to mRNAs (29). Our approach was based on the presence of a cis-acting noncoding promoter-associated RNA (paRNA) overlapping the Myc transcription start site (TSS) and positively regulating transcription initiation (28). Interfering with this paRNA inhibited transcription of Myc in a sequence- and gene-specific manner (28). The mechanism behind repression of Myc transcription by promoter-targeting siRNA involved the RNAi protein Argonaute 2 and interference with the formation of the preinitiation complex (PIC) at the Myc TSS. This strategy resulted in profound and prolonged repression of Myc transcription. Interestingly, a single transfection of prostate cancer cells with the promoter-targeting siRNA induced long-lasting effects on cell proliferation and colony formation, indicative of persistent loss of proliferative potential as a consequence of Myc silencing (28). On the contrary, there was no effect in normal human cells (28).

This remarkable reduction of the proliferative capacity of cancer cells led us to investigate the effects of this approach on prostate CSCs in cell cultures and tumor xenografts. In this study, we show that Myc silencing by promoter-targeting siRNA impairs prostate CSC maintenance and tumorigenicity and induces senescence in the prostate CSC subpopulation. These results are consistent with the role of Myc in the maintenance of CSCs in human tumors and identify induction of cell senescence as a primary mechanism of reduced self-renewal and loss of clonogenic, tumor-initiating, and metastatic capability in Myc-depleted prostate CSCs. These findings also demonstrate that RNAi-based targeting of regulatory noncoding paRNAs is an effective strategy to modulate transcription of genes involved in critical oncogenic pathways for therapeutic applications.

Materials and Methods

Detailed methods are included in the Supplementary Data.

Cell culture and transfection procedure

Human prostate cancer cell lines DU145 and PC3 were maintained in RPMI-1640 as described previously (30). PC3 cells constitutively expressing luciferase were a gift of Dr. M. Cecchini (University of Bern, Berne, Switzerland). Immortalized human prostate epithelial cells (PrEC) and PrECs stably expressing an ESE3-directed shRNAs were maintained as previously described (30). Prostastosphere cultures were established as previously described (30, 31). Myc13 and control GL3 siRNAs were purchased from Ambion and transfected at the concentration of 100 nmol/L with Lipofectamine 2000 as described previously (28).

Tumor xenografts

Mice were purchased from Harlan Laboratories. Animal experiments were approved by the Swiss Veterinary Authority (No. 5/2011) and performed according to national and international guidelines. Mice bearing subcutaneous PC3 xenografts were injected intraperitoneally with Myc13 and GL3 siRNA formulated with in viro-jetPEI (Polyplus Transfection) according to the manufacturer’s instructions using a N/P ratio of 5.

RNA extraction and quantitative reverse transcription PCR analysis

RNA was isolated using Trizol reagent (Invitrogen). mRNA levels were measured by quantitative reverse transcription (qRT)-PCR as described previously (28, 32). For Myc13 siRNA detection, total RNA was reverse-transcribed with custom-designed TaqMan Small RNA Assay (Applied Biosystems) and U6 small RNA (RNU6b) was used as control.

DNA extraction and PCR analysis

Lung metastases after intravenous injection of cancer cells were analyzed by quantitative PCR (qPCR) based on amplification of nontranscribed region in human and mouse genome as previously described (33). Results are expressed as percentage of human cancer cells relative to the total amount of mouse lung cells.

Gene expression data analysis

Prostate cancer gene expression datasets GSE14206 (34) and GSE21034 (35) were downloaded from Gene Expression Omnibus (GEO) database. Gene set enrichment analysis (GSEA; ref. 36) was performed using 13 predefined embryonic stem cell (ESC) gene sets derived from Ben-Porath and colleagues (37) to assess the enrichment of ESC-like gene signatures on the basis of Myc expression. The ESC gene sets represented four functional groups: ES expressed genes; NANOG, POU5F1, and SOX2 targets; MYC targets; and polycomb repressive complex 2 (PRC2) targets (37).
Statistical analysis

Differences between groups were assessed with an unpaired two-tailed t test and were considered statistically significant for **, P < 0.05.

Results

Myc is highly expressed in prostate cancer cells with stem-like and tumor-initiating properties

To establish the relevance of Myc as therapeutic target in prostate CSCs and identify adequate experimental systems, we examined the expression of Myc in PC3 and DU145 prostate cancer cells cultured either in adherent condition or as floating prostatospheres. These two cell lines are androgen receptor (AR)–independent and represent widely used models of CRPC. Despite the high level of Myc in monolayer cultures of PC3 and DU145 cells, prostatosphere-forming cells exhibited significantly higher expression of Myc compared with adherent growing cells as indicated by qRT-PCR, immunocytochemistry, and immunoblotting (Fig. 1A–C). Prostatosphere-forming cells had also higher expression of the stem cell markers NANOG, KLF4, POU5F1, SOX2, and BMI-1, consistent with enrichment of CSCs (Fig. 1D). Preferential expression of stem cell markers compared with monolayer adherent cultures was also reported in prostatospheres derived from DU145 cells (30). Myc has been linked previously to activation of an ESC-like transcriptional program, indicating that it could contribute to the stem cell–like phenotype displayed by many human cancers (11, 38). To establish whether Myc expression was also associated with CSC features in clinical human prostate cancer samples, we adopted a similar bioinformatic approach. To this end, we used predefined ESC-like gene sets (37) and performed GSEA in two prostate cancer gene expression datasets comprising 52 and 131 primary tumors (34, 35). In both datasets, Myc was significantly upregulated in tumors compared with normal prostate (Supplementary Fig. S1). In both cases, Myc-overexpressing tumors were highly enriched of ESC-like gene signatures, including targets of key ESC transcription factors POU5F1, NANOG, and SOX2 as well as Myc target genes (Supplementary Table S1). Conversely, Myc-overexpressing tumors displayed negative regulation of common PRC2 target genes. Thus, Myc expression was functionally associated with a CSC phenotype and ESC-like transcriptional features in human prostate cancer.
Prostatosphere-derived cells from PC3 (Fig. 1E) and DU145 (30) cells maintained intact their ability to form prostatospheres when passed in prostatosphere-forming conditions for multiple generations, which is indicative of their self-renewal capability. On the other hand, when prostatosphere-forming cells were dissociated and plated in adherent growth conditions in serum-supplemented medium, they generated monolayer cultures morphologically indistinguishable from those derived from adherent cells, indicating that prostatosphere cells retained the ability to generate daughter cells with a more differentiated phenotype. Notably, expression of Myc and POU5F1 was reduced when prostatosphere-forming cells were grown as monolayer (Fig. 1F). To further assess the properties of prostatosphere-forming cells, we compared the ability of prostatosphere-derived and adherent growing PC3 cells to form tumors in immunodeficient mice. To this end, we used PC3.Luc cells, which constitutively express the luciferase reporter gene and can be monitored by in vivo bioluminescence imaging. Prostatosphere-derived cells formed subcutaneous tumors with higher efficiency than adherent growing cells, consistent with an enrichment of the fraction of tumor-initiating cells. As low as 10^3 prostatosphere-derived cells were able to form subcutaneous tumors (Fig. 1G and Supplementary Fig. S2). On the contrary, adherent growing cells generated detectable tumors only when ≥10^5 were injected (i.e., > 100-fold difference).

The cell surface markers CD44 and CD24 have been used to identify cell subpopulations with different stemness capability within established human cell lines and primary cultures of normal and tumor-derived epithelial cells, with CD44^+/CD24^- cells generally having the highest CSC capability (16, 39). Consistently, we found that CD44^+/CD24^- cells sorted by fluorescence-activated cell sorting (FACS) from monolayer cultures of PC3 cells were able to generate tumors when injected subcutaneously in athymic nude mice, whereas the remaining CD44^-/CD24^+ fraction did not (Supplementary Fig. S3). Because primary prostatospheres are only partially enriched of stem-like cells (40, 41), we characterized the CD44 and CD24 expression of the prostatosphere-forming subpopulation in PC3 cells. About 95% of prostatosphere-forming cells were CD44^-/CD24^+ and 5% were CD44^-/CD24^- (Fig. 2D), confirming the heterogeneity of prostatosphere-forming cells. In addition, qRT-PCR confirmed that CD24 mRNA was lower in prostatospheres compared with monolayer cultures of PC3 and DU145 cells, whereas CD44 mRNA was slightly increased (Supplementary Fig. S4). Furthermore, when isolated by FACS from primary prostatospheres, CD44^-/CD24^- PC3 cells generated secondary prostatospheres with high efficiency, whereas CD44^+/CD24^- cells did not (Fig. 3A and B). Together, these data indicate that prostatosphere-forming cells and the CD44^-/CD24^- subpopulation derived from human prostate cancer cell lines have CSC properties and tumor-initiating capability and provide an appropriate system to address Myc as candidate for CSC-targeted therapies.

Myc silencing affects stem-like properties of prostatosphere-derived cells

We showed that Myc13 siRNA inhibited Myc transcription by interfering with PIC assembly on the promoter (Fig. 2A) and was effective in various prostate cancer cell lines independent...
of their AR status (28). To assess the effect of Myc silencing on human prostate CSCs, prostatospheres derived from PC3 and DU145 cells were dissociated, transduced with promoter-targeting (Myc13) and control (GL3) siRNA, and re-plated in prostatosphere-forming conditions. We used cyanine 3–tagged Myc13 (Cy3-Myc13) to ensure that siRNA uptake in single-cell suspensions of prostatosphere-forming or FACS-sorted cells was similar to that achieved in cells transfected as adherent monolayer (Supplementary Fig. S5). Transfection of Myc13 siRNA dramatically reduced prostatosphere formation in both cell lines (Fig. 2B). On the contrary, the control siRNA did not affect prostatosphere-forming ability. Notably, after treatment with Myc13 siRNA, isolated viable floating cells were still present in the culture dishes (Fig. 2C), indicating that Myc silencing arrested growth but did not induce apoptosis in prostatosphere-forming cells, in agreement with the previous results with adherent growing cells (28).

To determine whether phenotypic changes occurred in prostatosphere-forming cells after Myc silencing, we assessed CD44 and CD24 expression in prostatosphere cells after treatment with control and Myc13 siRNA. Control-treated prostatosphere cells contained 95% of CD44+/CD24– and no CD44+/CD24+ cells were present (Fig. 2D). After treatment with Myc13 siRNA, the number of CD44+/CD24– decreased (95% vs. 73%) and that of CD44+/CD24+ cells increased substantially (0% vs. 27%; Fig. 2D). Furthermore, using a flow-cytometric assay to detect senescent cells based on senescence-associated β-galactosidase (SA-β-gal) activity and the use of the fluorescent substrate fluorescein-di-β-D-galactopyranoside (FDG; ref. 42), we found that the CD44+/CD24+ subpopulation induced by Myc13 siRNA was phenotypically senescent (Fig. 2E). Essentially, 100% of FDG-positive cells in the Myc13-treated sample were CD44+/CD24–, indicating a link between Myc silencing, the CD44+/CD24+ phenotype and induction of cell senescence.

**Myc silencing reduces prostatosphere-forming ability of the CD44+/CD24– cell subpopulation**

To further investigate the function of Myc in prostate CSCs, we isolated the FACS two subpopulations present in prostatospheres derived from PC3 cells (i.e., CD44+/CD24– and CD44+/CD24+) and assessed their capacity to form secondary prostatospheres after transfection with control and Myc13 siRNA. CD44+/CD24– prostatosphere-derived cells transfected with control siRNA formed secondary prostatospheres (Fig. 3A). Remarkably, silencing of Myc in CD44+/CD24– prostatosphere-derived cells completely prevented formation of secondary prostatospheres. CD44+/CD24– cells derived from primary prostatospheres were unable to form secondary prostatospheres in control samples and Myc13 did not have any effect. As shown by phase contrast microscopy, while secondary prostatospheres formed in control CD44+/CD24– cultures, isolated viable cells were present after treatment with Myc13 and in CD44+/CD24– cultures (Fig. 3B). FACS analysis showed that control-transfected CD44+/CD24– cells reproduced the original CD44 and CD24 phenotype (~90% CD44+/CD24– and 10% CD44+/CD24+; Fig. 3C). CD44+/CD24– cells failed to regenerate the original phenotype of prostatosphere-forming cells and remained prevalently CD44+/CD24–.
Remarkably, treatment of CD44⁺/CD24⁻ cells with Myc13 siRNA reduced the CD44⁺/CD24⁻ subpopulation (~73%) and increased the fraction of CD44⁺/CD24⁺ cells (13%), confirming the phenotypic shift seen as result of Myc silencing in prostatosphere-derived cells. To define the mechanism causing the depletion of cells with stem-like properties by Myc13 siRNA, cells from primary prostatospheres were dissociated, sorted by FACS, and the CD44⁺/CD24⁻ fraction was transfected with Myc13 siRNA. Interestingly, after 10 days, we found that a relevant fraction of CD44⁺/CD24⁻ prostatosphere-derived cells (15%) was FDG-positive (Fig. 3D). Moreover, the majority of FDG-positive cells (80%) exhibited the CD44⁺/CD24⁺ phenotype (Fig. 3D). Therefore, Myc silencing in the CD44⁺/CD24⁻ subpopulation derived from primary prostatospheres was functionally linked to a shift toward the CD44⁺/CD24⁺ phenotype and cell senescence.

**Myc silencing reduces the CSC component and induces cell senescence in monolayer cultures of human prostate cancer cells**

To determine the ability of Myc transcriptional silencing to affect the fraction of CSCs within the heterogeneous cancer cell population of an established cell line, we transfected PC3 cells grown as monolayer cultures with control and Myc13 siRNA. As shown previously (28), transfection of adherent growing prostate cancer cell lines with Myc13 siRNA efficiently repressed Myc transcription (Fig. 4A). We reported previously that this was associated with morphologic changes (i.e., cells acquired a polygonal form and become larger and flatter), reduced proliferation, and induction of cell senescence (28). Using the flow-cytometric FDG assay we confirmed that, concomitant with Myc silencing, the percentage of senescent cells increased substantially in Myc13-treated PC3 cells (Fig. 4B).
4B). In parallel, we found that the ability of Myc13-treated cells to generate prostatospheres was significantly reduced as compared with control cells (Fig. 4C). Moreover, Myc silencing reduced the percentage of CD44+/CD24− cells (90% vs. 78%) and led to an increase of CD44+/CD24+ (9% vs. 18%) cells in the total cell population (Fig. 4D). This shift toward the CD44+/CD24+ phenotype is relevant as CD44+/CD24− cells showed impaired tumorigenicity in mice (Supplementary Fig. S3). Interestingly, in agreement with the data with CSC-enriched populations, FDG-positive cells induced upon Myc silencing in adherent PC3 cell cultures were almost exclusively CD44+/CD24− (Fig. 4E). Similar results, (i.e., increased number of CD44+/CD24− and concomitant FDG positivity) were observed upon transfection of adherent growing DU145 cells with Myc13 siRNA (Fig. 4F and G). We further validated these findings using immortalized normal PrECs and the transformed and tumorigenic derivative ESE3kd-PrECs generated by knocking down ESE3/EHF (30). Knockdown of this epithelial-specific transcription factor in PrECs resulted in acquisition of stem cell–like properties and tumorigenicity (30) and was associated with increased Myc expression (Supplementary Fig. S6). Transfection of Myc13 in ESE3kd-PrECs reduced Myc expression and concomitantly prostatosphere-forming ability (Fig. 4H and I). Moreover, Myc silencing was associated with increased cell senescence (Fig. 4j). Importantly, knockdown of Myc by Myc13 did not affect proliferation of normal PrECs (Supplementary Fig. S7).

Targeting Myc reduces CSCs and prevents tumor development in vivo

To determine whether Myc silencing and consequent depletion of CSCs would inhibit tumorigenicity of prostate cancer cells in vivo, we performed xenotransplantation studies with in vitro–transfected PC3 cells. In these studies, monolayer cultures of PC3 cells were transfected with control and Myc13 siRNA and, after 24 hours, equal numbers of viable control- and Myc13-treated cells were injected subcutaneously in immunodeficient mice. Growth of control PC3 cells was not affected and was comparable with non-transfected PC3 cells (Fig. 5A). On the contrary, injection of the same number of Myc13-treated PC3 cells resulted in significant delay of tumor growth. Remarkably, analysis of Myc mRNA in tumor tissues by qRT-PCR showed lower expression and concomitant FDG positivity (Fig. 5H). Furthermore, tumor xenografts from Myc13-treated cells exhibited increased expression of p21, a marker of growth arrest and senescence (2% vs. 8%) along with reduced Myc expression (50% vs. 0%; Fig. 5I). Collectively, these results indicated that Myc silencing reduced self-renewal capability and maintenance of prostate CSCs with a consequent decline of the CSC subpopulation and of the tumorigenic and metastatic potential in vivo.

The remarkable effect of the promoter-targeting siRNA on tumorigenicity and metastatic capability was consistent with inhibition of the CSC component in PC3 tumor xenografts as result of the repression of Myc transcription. To verify this hypothesis, tumors were explanted and single-cell suspensions were obtained from control- and Myc13-treated xenografts and analyzed by FACS. Notably, Myc13-treated tumor xenografts had reduced content of CD44+/CD24− cells (27% vs. 11%) and concomitant increase of CD44+/CD24+ cells (51% vs. 66%), indicative of a reduction of CSC component analogous to the in vitro experiments (Fig. 5F). To further assess the effects on the CSC compartment in tumor xenografts, single cells derived from tumors generated from in vitro–transfected PC3 cells were re-plated in vitro in prostatosphere-forming conditions (Fig. 5G). Notably, prostatosphere-forming capacity of Myc13-treated and control xenografts was similar at the first prostatospheres generation, likely due to transient expansion of ex vivo–generated primary prostatospheres. However, there was a sharp decline of secondary prostatospheres from Myc13-treated xenografts, consistent with reduced self-renewal capability. Prostatosphere formation from the control xenografts at the second generation was unaffected. To assess in vivo the CSC capability of the cells derived from control- and Myc13-treated xenografts, tumor-derived cells were reimplanted subcutaneously in athymic nude mice. Strikingly, cells derived from control xenografts produced second-generation tumors with 100% efficiency (Supplementary Fig. S8A). On the contrary, cells derived from Myc13-treated xenografts produced second-generation tumors with lower efficiency (Supplementary Fig. S8B and S8C). In addition, second-generation tumors from Myc13-transfected cells grew less than control tumors, indicating that the proliferative and tumor-initiating potential was persistently impaired in Myc13-treated cells (Supplementary Fig. S8D). Concomitant with the depletion of CSC capability, we found that a higher fraction of FDG-positive senescent cells in the primary Myc13-treated tumor xenografts compared with control xenografts (81% vs. 57%; Fig. 5H). Strikingly, as seen in the in vitro experiments, FDG-positive senescent cells derived from Myc13 tumor xenografts were mainly CD44+/CD24+ (Fig. 5H). Furthermore, tumor xenografts from Myc13-treated cells exhibited increased expression of p21, a marker of growth arrest and senescence (2% vs. 8%) along with reduced Myc expression (50% vs. 0%; Fig. 5I). Collectively, these results indicated that Myc silencing reduced self-renewal capability and maintenance of prostate CSCs with a consequent decline of the CSC subpopulation and of the tumorigenic and metastatic potential in vivo.

Systemic delivery of Myc promoter-targeting siRNA inhibits tumor development in mice

Both in vitro and in vivo experiments showed consistent and long-term effects on proliferative and tumorigenic potential of prostate cancer cells as result of transcriptional silencing of Myc. We next investigated whether systemic delivery of Myc13 siRNA could affect Myc expression and tumor growth in mouse xenografts. Systemic administration of siRNAs is challenging due to their poor stability and biodistribution (43, 44). For delivery of Myc13 siRNA, we used in vivo-jetPEI, a cationic
Figure 5. Reduction of Myc in PC3 cells prevents tumor growth and promotes cellular senescence. A, PC3 cells were transfected in vitro with GL3 and Myc13 siRNA and injected subcutaneously into nude mice \((n = 5)\) in both flanks \((4.0 \times 10^6\) cells/injection). Tumor growth was monitored weekly for 4 weeks. Statistical significance of the differences was evaluated at the end of experiment \((P < 0.05)\). B, Myc mRNA level in tumor xenografts generated as indicated in above-determined by qRT-PCR. Results are normalized to B2M. C, PC3.Luc cells transfected in vitro with GL3 and Myc13 siRNA were injected subcutaneously as in A and tumor growth monitored after 4 weeks by in vivo imaging. D, PC3 cells transfected in vitro with GL3 and Myc13 siRNA were injected intravenously and lung metastasis assessed by qPCR of human and mouse genomic DNA. E, representative images of lung tissue of mice injected intravenously with PC3 cells transfected in vitro with GL3 and Myc13 siRNA. F, CD44 and CD24 levels in xenografts generated from PC3 cells transfected in vitro with GL3 and Myc13 siRNA were assessed by flow cytometry. G, sphere-forming capacity was estimated in cells derived from xenografts generated by PC3 cells transfected in vitro with GL3 (black bars) and Myc13 (white bars) siRNA at the first- and second-generation generation. H, SA-β-gal levels in cells derived from xenografts generated as above and CD44/CD24 levels in the FDG-positive cell subpopulation. I, Myc and p21 protein levels determined by immunohistochemistry in subcutaneous tumor xenografts generated by PC3 cells transfected in vitro with GL3 and Myc13 siRNA. **, \(P < 0.05\).
polymer preparation successfully used in previous studies (45). In cell culture experiments, this reagent was as effective as Lipofectamine in transfecting siRNA and inducing Myc silencing (~80% gene knockdown). To accurately assess the delivery of Myc13 siRNA in tumor xenografts, we used a qRT-PCR method with Myc13-specific primers and U6 small RNA as control. Previous tests showed the specificity and accuracy of this assay to quantify the amount of Myc13 siRNA taken up by cells in cell culture experiments (data not shown). Furthermore, the amount detected by the qRT-PCR assay reflected intact Myc13 siRNA accumulated within cells or tissues. To determine dose and time dependence of siRNA uptake, mice received a single intraperitoneal injection of increasing doses of siRNA (0.5, 2, and 5 mg/kg) and were sacrificed after 2 days. Myc13 siRNA uptake increased with the dose, although the difference between 2.0 and 5.0 mg/kg was minimal, probably reflecting saturation of the uptake in the tissue (Fig. 6A). Furthermore, in mice receiving a single injection of 2 mg/kg, the amount of siRNA in tumors was similar after 1 and 3 days from the injection and then declined at 7 days (Fig. 6B). In parallel, we measured in these same samples the level of human Myc mRNA and human β-2-microglobulin as reference gene. After 2 days from a single injection of 2 and 5 mg/kg of Myc13 siRNA, Myc mRNA was reduced by about 40%, whereas no effect was seen at 0.5 mg/kg (Fig. 6C). The effect on Myc mRNA level after a single injection of 2 mg/kg of siRNA was similar at 1 and 3 days, whereas the effect was lost after 7 days, in line with the kinetics of siRNA uptake (Fig. 6D). Therefore, detectable and biologically active amounts of Myc13 siRNA were delivered by intraperitoneal injection at doses >2.0 mg/kg. Remarkably, an effect on Myc mRNA was seen after a single administration of siRNA and the effect persisted for up to 3 days.

On the basis of the kinetics of siRNA uptake and pharmacodynamic data, mice with subcutaneous tumor xenografts were treated with repeated intraperitoneal injections twice a week. There were no signs of toxicity (i.e., loss of body weight, behavioral changes, etc.) associated with repeated administration of control and Myc13 siRNA. For accurate measurement of tumor size, groups of control and Myc13 siRNA–treated mice were sacrificed to determine tumor weight. The effect on tumor growth was evident after the last cycle of treatment, when tumors in the control group expanded and those in Myc13-treated group were almost completely arrested (P < 0.005; Fig. 6E). At this time, immunohistochemistry showed a clear reduction of Myc protein and of the

Figure 6. Systemic treatment with Myc13 siRNA inhibits tumor development. A and B, accumulation of Myc13 siRNA in tumor xenograft tissues. Mice (n = 5/group) bearing subcutaneous PC3 xenografts received a single intraperitoneal injection of GL3 or Myc13 siRNA and tumor tissues were collected 2 days after treatment at the indicated doses (A) or 1, 3, and 7 days after treatment with a dose of 2.0 mg/kg (B). The level of Myc13 siRNA was measured by qRT-PCR in total RNA extracted from frozen tumor tissues and normalized to the level of U6 small RNA. C and D, Myc downregulation in tumor xenografts. Myc mRNA level was determined by qRT-PCR in tumor tissues from mice treated with GL3 or Myc13 siRNA as indicated in A and B and normalized to the level of the human B2M. E, tumor-bearing mice were treated systemically with GL3 and Myc13 siRNA by intraperitoneal injections at the dose of 2.0 mg/kg twice a week. Mice (n = 5/group) were sacrificed after the first, fourth, and seventh injection to assess tumor growth. F, Myc and Ki67 levels assessed by immunohistochemistry in tumor tissues from mice treated as indicated in E and collected at the end of the experiment. **, P < 0.01.
proliferation marker Ki67 in Myc13-treated tumors as compared with the control xenografts (Fig. 6F).

Discussion

In this study, we show that RNAi-based transcriptional silencing of Myc is an effective strategy to block maintenance and propagation of prostate CSCs in cell cultures and tumor xenografts. Myc silencing was associated with the induction of phenotypic changes and senescence in the prostate CSC subpopulation, leading to reduced self-renewal and tumor-initiating capability. In vivo, Myc silencing resulted in remarkable suppression of subcutaneous tumors and lung metastasis, consistent with the impairment of essential tumor-initiating functions and depletion of CSCs. This study also demonstrates the efficacy of the RNAi-based strategy for targeting regulatory noncoding paRNAs and modulating transcription of genes involved in critical oncogenic pathways.

Current evidence suggests that tumor initiation, progression, and metastasis in human cancers are supported by a specific subpopulation of cancer cells with stem cell–like properties (9). CSCs are operationally defined as cells that have high tumor-initiating capability in vivo, are capable of self-renewal, and have partial differentiation potential (6). Human prostate CSCs with these characteristics have been described by various groups using specific cell surface markers and a combination of in vitro and in vivo assays (8, 16, 41). Experimental systems that allow to reproducibly isolate, quantify, and functionally characterize cells with stem-like features are essential to investigate the pathways involved in self-renewal and identify CSC-specific therapeutic targets (6, 16, 41). In this study, we show that prostatosphere-forming cells derived from prostate cancer cell lines had high self-renewal capability, maintained their characteristics through multiple passages in stem cell selective conditions and, nevertheless, retained the capacity to reconstitute the heterogeneity of the original cell population when re-plated in serum-supplemented medium. Prostatospheres derived from PC3 and DU145 cell cultures expressed high levels of Myc and other genes important for preserving stem cell functions (Fig. 1). Furthermore, prostatospheres were highly enriched of CD44+/CD24− cells, a hallmark of progenitor/stem cells in many epithelial tissues (16). Consistently, CD44+/CD24− cells isolated from prostatospheres had the ability to form secondary prostatospheres in vitro and to reproduce the original phenotypic heterogeneity. Moreover, prostatosphere-forming cells and the CD44+/CD24− cell subpopulation derived from human prostate cancer cell lines were able to form tumors in mice with higher efficiency than the bulk cancer cell population, indicating that they behaved as bona fide CSCs and tumor-initiating cells.

Myc is overexpressed at early and late stages of development of many human cancers, including prostate cancer (11, 12, 15). Among its many functions, Myc has an important role in normal and neoplastic stem cells (11). Consistently, we found that Myc overexpression was associated with enrichment of ESC-like gene signatures in human prostate cancers (Supplementary Table S1). Myc is also implicated in the inhibition of oncogene-induced senescence (46). Myc inactivation in Myc-driven transgenic mouse models induces tumor regression and senescence, although the genetic and epigenetic context can influence type and reversibility of the response (22, 46). We described previously that transcriptional silencing of Myc reduced proliferation and clonogenic capacity of prostate cancer cells with high Myc expression, whereas growth of nontransformed human cells was not affected (28). In the present study, we investigated the consequences of Myc silencing on the CSC subpopulation. We used prostatosphere-forming cells and the CD44+/CD24− subpopulation derived from human prostate cancer cell lines as model systems to analyze the effects of Myc silencing on the CSC component in human prostate tumors. Transfection of Myc13 siRNA in prostatosphere cells led to almost complete suppression of in vitro prostatosphere-forming and self-renewal capacity (Fig. 2). Interestingly, many cells in Myc13-treated samples were still viable in prostatosphere-forming conditions, whereas the fraction of CD44+/CD24− cells was reduced with concomitant appearance of double-positive CD44+/CD24+ cells (Fig. 2). A relevant portion of cells treated with Myc13 siRNA was senescent and double-positive for CD44 and CD24 (Fig. 2). Notably, after continuous growth in prostatosphere-forming conditions, Myc13-treated cells did not reacquire prostatosphere-forming ability, indicating that they had irreversibly lost their stem-like properties. Similarly, Myc silencing in CD44+/CD24− cells blocked formation of secondary prostatospheres, reduced the fraction of cells having the CD44+/CD24− phenotype, and increased the fraction of CD44+/CD24+ and senescent cells (Fig. 3). Interestingly, this was observed also in prostate cancer cell lines and transformed PrECs cultured in adherent conditions (Fig. 4), underlying the ability of this approach to affect the CSC compartment within the heterogeneous bulk cancer cell population.

We showed previously that RNAi-based transcriptional silencing produced a prolonged suppression of Myc expression and function in vitro even after a single administration, resulting in persistent effects on the proliferative and clonogenic capacity of prostate cancer cells (28). To investigate the possibility to impair tumor growth and metastasis in vivo with promoter-targeting siRNA, in vitro–transfected prostate cancer cells were implanted subcutaneously or injected intravenously in mice. Myc silencing had profound effect on both subcutaneous tumors and lung metastasis (Fig. 5). Notably, tumors derived from xenografts generated by Myc13-treated cells had a reduced fraction of CD44+/CD24− cells and increased content of CD44+/CD24+ cells. Consistent with a depletion of CSCs, cells from Myc13-treated xenografts had reduced ability to propagate ex vivo in prostatosphere-forming conditions and to form secondary tumors when re-implanted in mice. Strikingly, cells derived from Myc13-treated xenografts had also a higher percentage of senescent cells compared with control-treated xenografts and these cells were mainly CD44+/CD24−, consistent with a phenotypic switch and increased senescence. Furthermore, we found that systemic administration of Myc13 siRNA reduced Myc expression and led to profound inhibition of the development of prostate tumor xenografts (Fig. 6). Thus, depletion of CSC in response to Myc
silencing was associated with activation of a latent senescence program and reduced tumorigenic and metastatic potential. These findings are in agreement with the hypothesis that, although perhaps less able to induce tumor regression, CSC-targeted therapies could be particularly advantageous in the adjuvant setting or in combination with drugs directed to the bulk tumor cell population to reduce disease recurrence and metastasis (10). Moreover, our data suggest that this Myc-targeting strategy might be equally effective in AR-dependent and -independent prostate cancers and as well in other tumor types with high Myc expression. Indeed, Myc expression, alone or in combination with other CSC markers, could serve to identify patients that might benefit specifically from this approach. Although many concerns still remain of the clinical applicability of RNAi-based therapeutics (43, 44), the feasibility of this approach has been demonstrated preclinically in mice and non-human primates and, more recently, in human clinical trials (47–50). Notably, the doses used in these preclinical and clinical trials are similar to those tested here for systemic administration of Myc13 siRNA to mice. Collectively, our findings therefore provide evidence of the efficacy of this approach to silence genes, like Myc, essential for prostate CSC maintenance and of the feasibility of delivering effective doses of Myc promoter-targeting siRNA to impair CSC functions and tumor development in vivo.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: G. Civenni, A. Malek, C.V. Catapano
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Civenni, A. Malek, S. Di Marco, S. Pinton
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Civenni, D. Albino, R. Garcia-Escudero, M. Sarti, G.M. Carbone, C.V. Catapano
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Civenni, S. Pinton
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