Role of GLI2 Transcription Factor in Growth and Tumorigenicity of Prostate Cells

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Abstract

Aberrant activation of the Hedgehog (Hh) signaling pathway has been reported in various cancer types including prostate cancer. The GLI2 transcription factor is a primary mediator of Hh signaling. However, its relative contribution to development of prostate tumors is poorly understood. To establish the role of GLI2 in maintaining the tumorigenic properties of prostate cancer cells, we developed GLI2-specific small hairpin RNA. Knockdown of GLI2 in these cells resulted in significant down-regulation of the Hh signaling pathway, followed by inhibition of colony formation, anchorage-independent growth, and growth of xenografts in vivo. Conversely, ectopic expression of GLI2 in nontumorigenic prostate epithelial cells resulted in accelerated cell cycle progression, especially transition through G2-M, and augmented proliferation. Altogether, our findings suggest that GLI2 plays a critical role in the malignant phenotype of prostate cancer cells, and GLI2 may potentially become an attractive therapeutic target for the treatment of prostate cancer. [Cancer Res 2007;67(22):10642–6]

Introduction

The role of the Hedgehog (Hh) signaling pathway has been well documented in vertebrate embryonic development, stem cell maintenance, and tumorigenesis (reviewed in refs. 1, 2). Each of the three Hh genes (Sonic hedgehog, Desert hedgehog, and Indian hedgehog) encodes a secreted signaling peptide that binds to a membrane-bound 12-span transmembrane receptor called Patched (Ptc). Binding of Hh ligand to the Ptc receptor on the target cell alleviates Ptc-mediated suppression of a seven-span transmembrane protein called Smoothened (Smo). Smo activation initiates an intracellular signal transduction cascade that ultimately activates expression of Hh target genes.

Transcriptional activation of Hh target genes in mammals occurs through the actions of three related proteins, GLI1, GLI2, and GLI3 (3, 4). GLI3 acts primarily as a transcriptional repressor (5), whereas GLI2 is reported to be the primary activator of Hh signaling and GLI1 is a secondary target, downstream of GLI2, which also acts as a transcriptional activator (6). GLI1 is a transcriptional target of GLI2, and its up-regulation in response to the activation of the Hh signaling pathway depends on GLI2 protein stabilization. Therefore, GLI1 amplifies Hh-induced, GLI2-mediated transcription of GLI target genes.

The Gl2 transcription factor is indispensable for mouse development, as Gl2 knockout mice die prenatally and exhibit neural tube defects including a complete loss of the floor plate and a reduction in V3 interneurons (7, 8). Gl2 was also shown to be essential for normal prostate development (9). On the other hand, mice lacking Gl1 seem to be normal and fertile, suggesting that the Gl1 protein is not required for Hh signaling in mouse (10).

Recent studies have shown the importance of Hh signaling in human prostate cancer (11–14). Compelling data imply that elevated pathway activity is associated with high-grade and metastatic prostate cancer and show that pathway manipulation can modulate invasiveness and metastasis (12), but the role of ligand-dependent pathway activation in prostate cancer cells and the role of GLI1 in tumor cell behavior have been controversial (15). Although down-regulation of GL1 has been shown to inhibit prostate tumorigenesis (13), the major mode of GLI1 regulation is the induction of its transcription by GLI2 (16). Therefore, GLI1 amplifies GLI-dependent transcriptional outcome initially induced by GLI2. We have recently shown that stabilization of GLI2 protein could contribute to the overexpression of GLI2 protein in prostate cancer cells and primary tumors (16). In the present study, we have investigated the significance of GLI2 expression in prostate cell growth and tumorigenesis.

Materials and Methods

DNA constructs. pcDNA3.1-Flag-GLI2 was a kind gift of Drs. M. Grachtchouk and A. Dlugosz (University of Michigan, Ann Arbor, MI), pGL3-Bcl2 promoter luciferase was generously provided by Dr. F. ABerger (University of Salzburg, Salzburg, Austria; ref. 17), K17 luciferase was from Dr. P. Coulombe (Johns Hopkins University, Baltimore, MD), 8′-3′Gli BS-LucII (8 directly repeated copies of 3Gli binding site from HNF3β floor plate enhancer cloned into pS2LucII; ref. 18) was kindly provided by Dr. H. Sasaki (Osaka University, Osaka, Japan), and pSV40 β-gal was from Promega. The packaging plasmids pCMVΔR8.2 and pCMV-WS-VSV-G were obtained from Dr. Didier Trono (University of Geneva, Geneva, Switzerland) and Dr. Robert Weinberg (MIT, Cambridge, MA), respectively (Addgene plasmids 12263 and 8454). Construction of GLI2 small hairpin RNA (shRNA) was done according to Ambion recommendations. Briefly, siRNA sequences were selected using the siRNA Target Finder and Design Tool. The annealed shRNA insert was cloned into the modified pLTHM vector (19). The following GLI2 target sequence was used: 5′-GATCGAGACAGGATGACT-3′.

Tissue culture and transfections. 293T human embry kidney cells and prostate cancer cell lines 22R1, PC3, LnCaP, DU145, and RWPE1 were purchased from American Type Culture Collection. Prostate cancer cells and 293T were grown in DMEM-RPMI in the presence of 10% fetal bovine...
serum (FBS) and antibiotics at 37°C and 5% CO₂. RWPE1 cells were cultured in keratinocyte serum-free medium supplemented with bovine pituitary extract and epidermal growth factor from Invitrogen. RWPE1 stable clones were selected in complete medium containing G418 (Sigma-Aldrich) over 2 weeks. Transfections were done using the calcium phosphate procedure or lipofection with LipofectAMINE 2000 (Invitrogen).

**Lentiviral-mediated shRNA delivery.** For producing lentiviruses, 293T cells grown at 60% to 70% confluence were transfected with 10 μg of pLVTHM-Gli2 shRNA, 10 μg of pCMVΔR8.2, and 7.5 μg of pCMV-VSV-G plasmids by calcium phosphate method (20). The next day (day 1), complete medium without antibiotics was replaced to the cells. The virus was collected as media supernatant on days 2 and 3, passed through 0.45-μm filter, and stored at −80°C. At the time of transduction, 22Rv1 cells at 50% to 60% confluence were infected by adding 10 mL of media containing lentiviruses twice at 12-h interval, and then green fluorescent protein expression was analyzed 24 h after second infection.

**Figure 1.** shRNA-mediated inhibition of GLI2 inhibits GLI-dependent transcription in prostate cancer cells. A, immunoblot analysis of GLI2 protein isolated from 293T cells transfected with shRNA plasmids as indicated. Representative of two independent experiments. Prostate cancer cells were transfected with pGL3-Bcl2promoter luciferase (B), K17-luciferase (C), 8×3′Gli BS-LucII (D), pSV40 -gal (Promega), and shRNA expression plasmids as indicated. Luciferase activity was estimated using luciferase reporter assay reagent (Promega). β-Galactosidase was used for normalization and estimated using β-galactosidase assay reagent (Pierce). *, P < 0.01, compared with cells transfected with scrambled shRNA (Student’s t test).
Antibodies and Western blotting. Antibodies against GLI2 (G20), β-actin, and horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Immunoblotting procedures were done as described elsewhere (16).

Luciferase reporter assays. Prostate cells were transfected with 8×3Gli BS-Lacl reporter, K17 luciferase reporter plasmid, or pGL3-Bcl2promoter luciferase reporter plasmid, pSV40 β-gal (Promega), and respective shGL2 expression plasmids. Luciferase and β-galactosidase activities were done using Luciferase Reporter Assay Reagent (Promega) and β-gal assay reagent (Pierce Biotechnology), respectively, according to the manufacturers’ recommendations.

Colony formation assay. Scrambled and GL2 shRNAs were cotransfected with pTk-Puro in the indicated cell lines at a ratio of 19:1. The transfected cells were subcultured and selected with puromycin (Sigma; the final concentration of puromycin in the media varied for the cell lines from 1 to 3 µg/mL). Colonies that formed in 3 to 5 weeks were fixed with 10% formalin (Sigma) for 60 min, washed with PBS, stained with 2% Gentian violet (Rica Chemical Company) for 45 min, and air-dried.

Growth in soft agar. 22Rv1 prostate cancer cells were infected with lentiviruses encoding GL2 shRNA or scrambled shRNA. GL2 shRNA– and scrambled shRNA–expressing 22Rv1 cells were grown in six-well plates in complete media, trypsinized, washed with PBS, counted, and mixed at a concentration of 10,000 cells/2 mL of RPMI, 0.33% agar. The cells in 0.33% agar were plated over 2 mL of RPMI, 0.5% agar that had been allowed to harden in a six-well dish. Cells were fed 0.5 mL of RPMI twice a week and allowed to grow for 3 weeks. The cells were fixed with 10% formalin and stained with 2% Gentian violet. Colony formation was determined under low magnification (×10) in an inverted microscope.

Xenograft growth. For the tumor growth studies, transduced 22Rv1 cells infected with lentiviruses encoding GL2 shRNA or scrambled shRNA and BALB/c athymic (nude) male (NxGen Biosciences, Inc.) mice, 5 weeks old, were used. Cells (1 × 10⁶) in 0.1 mL of culture medium were mixed with 0.1 mL of Matrigel (BD Biosciences) and were injected s.c. into one flank of the mice. Tumors were measured and volume was calculated using the following formula: volume = 1/2 (π/3) (L₁/2) (L₂/2) (H) = 0.5238 L₁L₂H, where L₁ is the long diameter, L₂ is the short diameter, and H is the height. The time to a target mean tumor volume of 500 mm³ is defined as the elapsed time from the date of cell implantation to the date when a 500-mm³ target is reached or when the mouse was sacrificed. Of the 15 mice in the study, 10 reached a target tumor volume of 500 mm³ by day 72, at which point experiments were concluded. A Kaplan-Meier survival analysis with the corresponding log-rank analysis was done using S-plus Software (Insightful). A linear regression analysis was used to measure the rate of mean tumor volume growth as a function of time using S-plus Software (Insightful). P < 0.05 was considered to be statistically significant.

Proliferation assays. For measurement of proliferation, RWPE1 cells stably transfected with pcDNA3.1 or pcDNA3.1-Flag-Gli2 cells were plated at 2 × 10⁴ per well into 96-well flat-bottomed microtiter plates (Falcon) in triplicate. Twenty microliters of CellTiter 96 AQueous One Solution Reagent (Promega) were added to each well of the assay plate containing the cells in 100 µL of culture medium. The plate was incubated for 2 h at 37°C in a humidified 5% CO₂ chamber. The absorbance was recorded at 490 nm using an ELISA reader. The proliferation was assessed 24, 48, and 72 h after the initial reading.

Cell cycle analysis. Cell cycle analysis was carried out in stable RWPE1 clones of pcDNA3.1+ (control) and cells expressing Gli2 by propidium iodide staining. Cells were synchronized by starving for growth factors and medium supplements for 24 h and harvested at different time points, 0, 6, 12, and 24 h, after adding the complete media to the cells. For fixing, 95% ethanol was added to cell suspension, incubated for 45 min at 4°C, and then stored at −20°C overnight. Fixed cells were pelleted at 300 × g for 10 min, washed twice with PBS and once with staining buffer (PBS, 2% FBS, and 0.01% NaN₃), and treated with RNase A (100 µg/mL) for 30 min at 37°C. Propidium iodide (25 µg/mL; Molecular Probes) staining was carried out at 37°C for ≥30 min and stored at 4°C until flow analysis.

Results and Discussion

Different GL2-targeting shRNA constructs were designed and cloned in the vector that can be used for both transfection and lentiviral-mediated delivery. The construct most efficient in inhibiting GL2 expression was chosen for our experiments. It down-regulates endogenous GL2 expression in both transient transfection of 293T cells (Fig. 1A) and lentiviral delivery in 22Rv1 prostate cancer cells (Fig. 2C, inset). Prostate cancer cells exhibited high constitutive levels of Hh pathway activity as compared with nontumorigenic immortalized prostate epithelial cells (RWPE1), measured by three different Gli-dependent luciferase reporter

![Figure 2](image-url)
expressing GLI2-specific shRNA grew much slower than xenografts (Fig. 3). The linear regression analysis showed that tumors significantly inhibited the growth of 22R cells in vitro and suppressing tumorigenic properties of prostate cancer cells both in vivo.

To investigate the role of GLI2 in maintaining tumorigenic properties of prostate cancer cells, we used lentiviral-mediated delivery of GLI2 shRNA. 22Rv1 cells infected with lentiviruses encoding GLI2-specific shRNA exhibited 5-fold decrease in the number of colonies formed in soft agar as compared with cells infected with lentiviruses encoding scrambled shRNA (Fig. 2B and C). These data show that knockdown of GLI2 dramatically inhibits anchorage-independent growth of 22Rv1 prostate cancer cells.

We next investigated whether inhibition of GLI2 function affects the growth of prostate cancer cells in vivo. Inhibition of GLI2 expression by lentiviral-mediated introduction of GLI2 shRNA significantly inhibited the growth of 22Rv1 xenograft in nude mice (Fig. 3). The linear regression analysis showed that tumors expressing GLI2-specific shRNA grew much slower than xenografts expressing scrambled shRNA (average, 3.1 and 20.7 mm^3/d, respectively; Fig. 3A). The average time to a target tumor volume of 500 mm^3 was also significantly longer in GLI2-shRNA–expressing than in scrambled shRNA–expressing xenografts (64 and 44 days, respectively; Fig. 3B). The observed differences were statistically significant, with \( P < 0.05 \) according to a log-rank analysis (Fig. 3C).

Together, these data suggest that inhibition of GLI2 is efficient in suppressing tumorigenic properties of prostate cancer cells both in vitro and in vivo.

As discussed earlier, GLI2 protein is expressed at very low levels in RWPE1 cells (Fig. 4A, inset) and normal prostate epithelial cells (16). To assess the GLI2 function in these cells, the RWPE1 cells were stably transfected with pcDNA3.1 (as control) or pcDNA3.1-Flag-Gli2 plasmids. RWPE1 cells that stably express Gli2 proliferated at a much faster rate than control transfected cells (Fig. 4A). Interestingly, ectopic expression of Gli2 in RWPE1 cells resulted in both faster growth and higher saturation density (data not shown). Cell cycle analysis revealed accumulation of these cells in S phase (≈50% cells transfected with Gli2, as compared with ≈20% cells transfected with empty vector) and almost complete disappearance of cells in G2-M (Fig. 4B). These data suggest that ectopic expression of GLI2 accelerates cell cycle, especially transition through G2-M, and stimulates proliferation of nontumorigenic prostate epithelial cells.

We provided evidence that GLI2 transcription factor plays a critical role in the growth and tumorigenicity of prostate cells. Previous studies concentrated on the role of GLI1 oncogene in prostate tumorigenesis. Because GLI1 itself is a transcriptional target of the Hh pathway, GLI1 mRNA expression serves as a reliable indicator of activated Hh signaling, and elevated GLI1 expression was linked with cancer development and progression (12, 13). Our data showed that down-regulation of GLI2 alone is very efficient in inhibiting the transcriptional output of Hh signaling in prostate cancer cells. GLI2 transcription factor significantly contributes to malignant transformation of prostate cancer cells. GLI2 may become an attractive target for therapeutic intervention, especially for patients with high-grade and/or metastatic prostate cancer. The first small-molecule inhibitors of GLI
transcription factors efficient in inhibiting the growth of prostate cancer cells have recently been identified (21). These, as well as potential Gli2-specific inhibitors, may become powerful therapeutics for the management of prostate cancer and other malignancies characterized by constitutive activation of the Hh signaling pathway.

Future studies are needed to analyze the mechanisms responsible for overexpression of Gli2 in prostate cancer and the role of Gli2 activation in response to paracrine and autocrine Hh signaling in prostate cancer cells.

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References

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