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14. ABSTRACT

The project studies the relationship between Hedgehog Signaling and hyperactive androgen signaling in androgen independent prostate cancer and tests the idea that Hedgehog inhibitors can block hyperactive androgen signaling leading to suppression of castration resistant tumor growth. Progress during this first annual period has established a relationship between the expression and/or activity of Hedgehog signaling proteins (Smoothened, Gli1 and Gli2) and increased androgen signaling and androgen independent (AI) growth of prostate cancer cells. Other work has linked Hedgehog signaling activity in prostate cells to the biosynthesis of testosterone. These data supports the idea that Hedgehog activity in an androgen deprived tumor both sensitizes the tumor cell to low levels of androgen and increases the local production of androgen in the tumor microenvironment and both have the potential to increase tumor growth in androgen deprived conditions. The outcomes are consistent with the idea that Hedgehog inhibitory drugs have a significant potential for treatment of advanced prostate cancer.

15. SUBJECT TERMS
Prostate Cancer, Castration Resistant Disease, Hedgehog Signaling, Smoothened, Gli, Cyclopamine, Androgen Signaling, Androgen Biosynthesis, Androgen Receptor

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Introduction

Deaths from prostate cancer are the consequence of castration resistant (CR) disease that grows despite the low level of circulating androgens in patients treated by androgen deprivation therapy. The androgen growth independent (AI) prostate cancer cells in CR disease have acquired the ability to "reactivate" their endogenous androgen signaling pathway. A tolerable drug that is able to target and block reactivated signaling pathway in AI cells offers an opportunity to increase the lifespan of patients with CR disease and to decrease the death rate from prostate cancer. We proposed that Hedgehog signaling is increased by exposure of prostate cancer cells to androgen-depleted conditions and that this signaling pathway has an important role in in reactivating androgen signaling in androgen deprived prostate cancer cells and in enabling AI growth of prostate cancer cells. Our hypothesis was based upon our preliminary evidence that aspects of Hedgehog (Hh) signaling are awakened in prostate cancer cells switched to an androgen deprived environment and that an inhibitor of Hh signaling (cyclopamine) was able to suppress reactivated androgen signaling in androgen-deprived cancer cells. Furthermore, we proposed that drugs that inhibit Hh signaling might be useful in the treatment of advanced/CR prostate cancer. Our experimental Aims were to test whether Hedgehog signaling proteins (Smoothened, Gli1 or Gli2) were involved in regulating androgen-dependent gene expression and androgen-independent growth of prostate cancer cells and to confirm that the suppressive effects of cyclopamine on androgen-dependent gene expression and cancer growth were related to its actions in suppressing Hh signaling in the prostate cancer cell. Other work was designed to test the hypothesis that Hedgehog signaling promotes the local synthesis of androgens in the microenvironment of a prostate tumor. Finally, we sought to test whether the mechanism of cyclopamine action involved effects on β-catenin modification (phosphorylation) and on its ability to interact with the androgen receptor proteins. During the past year, we completed all tasks associated with Aim1 and many of the other tasks associated with the other 3 Aims and this work is described below. While this work represents significant accomplishment, we were greatly affected by Institutional bankruptcy that occurred near the end of the first year but have now found the means to continue this innovative and exciting work at a different institution.

Body
(Progress During the First Year)

1. *Identification of key roles for Hedgehog signaling proteins (Smoothened, Gli1 and Gli2) in androgen signaling support and AI growth of prostate cancer (**Specific Aim 1, Task 1 and 2)*.

   Our proposal was driven by our preliminary evidence that the Hedgehog (Hh) inhibitor drug, cyclopamine, specifically suppressed the expression of androgen-regulated genes in prostate cancer cells and that this effect was linked to suppression of AI growth. The work in Aim 1 was intended to confirm that the action of cyclopamine in this regards was a consequence of its effects on critical Hh signaling gene products that included Smoothened (Smo), Gli1 and Gli2. The tasks in Aim 1 involved manipulating the expression of these key Hh regulators and then measure how this manipulation affected androgen signaling and AI growth in androgen-deprived prostate cancer cells. The manipulations involved increasing the expression of these 3 gene products through transduction of prostate cancer cells with gene expression vectors or decreasing their expression with siRNAs followed by tests on androgen signaling and androgen independent growth of the cancer cells. To this end, we obtained a cDNA expression vector encoding activated Smo as a gift from Dr F. de Sauvage and Genentech, Inc and we separately cloned Gli1 and Gli2 cDNA into tagged expression vectors. Transfection (transient or stable) of each of the vectors into LNCaP cells significantly increased expression of the corresponding mRNAs encoding these genes. While the Gli1/Gli2 vectors likewise induced expression of tagged polypeptides of appropriate molecular weight, the Smo expression vector resulted in the appearance of a super-high molecular weight protein band on Western blots (detected by an anti-Smo antibody) that was strikingly inconsistent with the known molecular weight of human Smo. Likewise, transfection of either Gli1 or Gli2 into androgen deprived LNCaP cells significantly increased the expression the androgen dependent genes and enabled AI growth, but transfection of activated Smo did not. At this time, we postulate that the dysregulated expression of exogenous Smo from the vector resulted in aggregation of this extremely hydrophobic protein in our cancer cells and that the aggregated Smo was dysfunctional. This is supported by our ability to affect androgen signaling through Smo knockdown, described below. However, the evidence that overexpression of active

Gli1 or Gli2 was associated with effects on expression of androgen dependent genes and AI growth strongly supports the idea that active Gli proteins interact with the androgen signaling system to increase its activity in a low-androgen condition as we postulated. This effect was also confirmed by a gene knockdown approach. Here, we showed that Smo, Gli1 or Gli2 siRNA was each able to suppress the expression of androgen regulated genes in prostate cancer cells grown in an androgen-free medium. Since the funding decisions for this grant were made relatively late and all tasks associated with Aim 1 were completed prior to project funding, the work was submitted for publication and was rapidly published (1) before project funding was received. For the purposes of this grant, however, we then developed a new Aim 1 that sought to determine the mechanism through which active Gli proteins might promote androgen signaling and AI growth in androgen deprived prostate cancer cells. The tasks in the modified Aim were designed to test whether Gli proteins might directly interact with the AR and act as a co-activator of AR function under low androgen conditions. Indeed, during the first year, we have accumulated significant reproducible data showing that each of the 3 human Gli proteins (Gli1, Gli2 and Gli3) directly interact with AR using a co-immunoprecipitation approach that involved immunoprecipitating one the proteins (Gli or AR) from cell extracts and testing whether the other protein was co-precipitated after Western Blotting (Figure 1). The outcome identifies the potential for a direct interaction between each of the individual Gli proteins and the AR protein in prostate cancer cells. Furthermore, by developing a series of partially deleted Gli2 and AR expression vectors, we have mapped the interaction sites of these two proteins (Figure 2). At the current time, our data clearly shows that Gli2 interaction with AR requires the presence of a specific region within the C-terminal domain of Gli2 and that the ability of Gli2 to increase AR activity requires the activation domain of Gli2 that also lies within the Gli2 activation domain. With regards to the relative domain on AR needed for interaction with Gli2 protein, we have shown that C-terminal truncated AR still binds effectively to Gli2 and is co-activated by the presence of Gli2. At the current time, our mapping of the AR interaction site is incomplete, but we have already developed a series of AR deletion vectors that will allow us to fine map the Gli2 interaction domain on AR and this should be accomplished within the next two months. With this data on hand, we will submit this work for publication. Please be aware that we have prepared a modified Statement of Work that addresses our essential completion of Aim 1 prior to the funding of the grant and the addition of a new Aim1 involving the work just described that keeps with the theme of the project. Finally, we have published an article citing the support of this grant in which we showed that the Gli-specific inhibitor drug, GANT-61, also suppresses expression of androgen dependent genes under low androgen conditions (2) and this work further substantiates that the Gli proteins, that lie at the end point of the Hh signaling pathway, are involved in the AR reactivation that is associated with long-term androgen deprivation of prostate cancer in conjunction with Hh signaling.

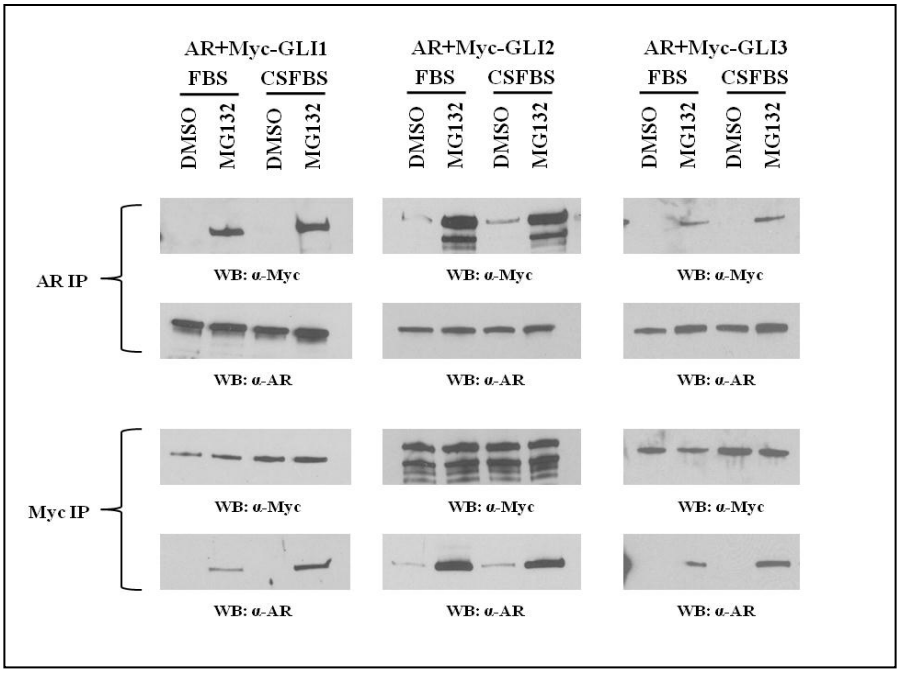
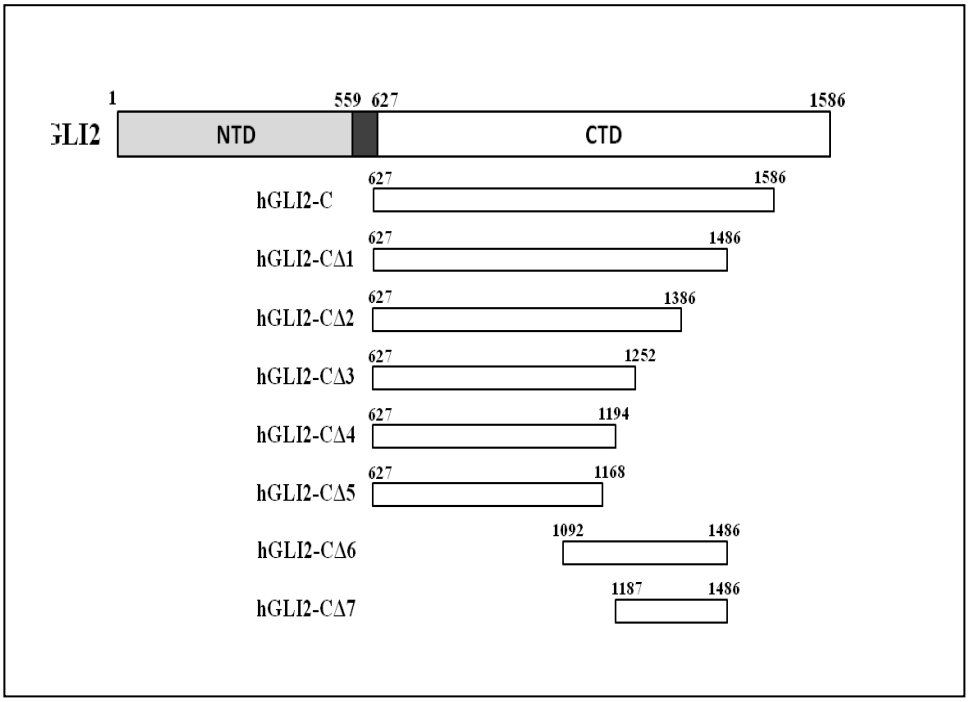


Figure 1. Gli proteins co-immuno-precipitate (IP) with AR protein. Western blots show that AR co-precipitates with myc-tagged Gli1, Gli2 and Gli3. (Upper Set) AR was immunoprecipitated from 293T cell extracts co-transduced with myc-tagged Gli1, Gli2 or Gli3. Anti-myc antibody (Top Panel) recognize Glis present in IP. (Bottom Set) Glis were immune-precipitated from 293T cell extracts co-transfected with AR. Anti-AR antibody (Bottom Panel) shows co-IP of AR with each of the tagged Gli proteins. Interaction was enhanced by the presence of MG132, a proteasome inhibitory drug (@ 5 micromolar).



Co-Precipitates With AR	Co-Activates PSA & PGC Promoters
+++	+++
+++	+++
+++	+++
+++	+
+++	+
+	-
-	-
+	++
-	-

Figure 2. Partial deletion variants of full-length Gli2 cDNA with N-terminal domain (NTD), central DNA-binding domain (black) and C-terminal domain (CTD) (on Left) were tagged with myc and were used to test co-immunoprecipitation with AR after co-transduction into 293T cells. The same variants were tested for their ability to co-activate reporter expression from PSA or PGC promoters (intensity identified by number of +) after co-transduction into 293T cells with full-length AR expression vector. Co-activation activity was lost earlier from these progressive deletion variants whereas Co-Precipitation was lost only with the most severe C-terminal deletion fragments. Co-activation and Co-precipitation activity was shown by a partial fragment from the C-terminal end that spends the deletion region associated with loss of Co-Activation and Co-Precipitation activities. Outcomes predict that the activation function of Gli2 for AR lies within the sequence between nucleotides (nts) 1486 and 1194 on Gli2 cDNA whereas the AR interaction domain lies between nts 1252 and 1168 on Gli2 cDNA.

2. *Hedgehog as an effector of testosterone biosynthesis in the prostate tumor microenvironment* ***(Specific Aim2 , Task 1 and Specific Aim 3, Task 2 and 3)***.

There is much interest in the potential for endogenous tumor androgen biosynthesis as an effector of advanced (CR) prostate cancer (3). Work in Aim 2 involved a comparative gene expression profiling of cyclopamine-treated vs vehicle treated prostate cancer cells (LNCaP and C42) using a microarray gene Chip procedure to identify other genes/gene pathways affected by cyclopamine. In the outcome of these preliminary studies (not shown here), we did note small but significant reductions in the expression of some steroid biosynthetic genes associated with cyclopamine treatment that might support the idea that inhibition of Hh signaling affected intracrine production of androgens by prostate cancer cells. This outcome further linked the relationship between the work in Aim 2 and Aim 3 (*test the role of Hh-based signaling in tumor autonomous androgen production*). To this end, we worked with the HPLC/Mass Spec Core in our institution to develop an HPLC-based assay to measure effects of cyclopamine on the production of testosterone (T) or dihydrotestosterone (DHT) from LNCaP AI prostate cancer cells cells (Aim 3, task 2). While the Core was able to get an identifiable signature for T or DHT spiked into cell culture medium, the test was insufficiently sensitive to identify the presence of endogenously synthesized T or DHT in conditioned mediums from untreated LNCaP AI cells (grown in the presence of charcoal stripped serum). Thus our Core was unable to develop a sufficiently sensitive assay to complete Task 2). We will discuss this situation later and discuss our plan to overcome the hurdle.

Because of our difficulties in measuring androgen output from cultured prostate cancer cells using the HPLC/MS method, we made a decision to switch our cell system from prostate cancer cells that require the sensitivity of this instrumentation to the use of human prostate stromal cells that were previously shown to produce sufficient T levels (from an adrenal precursory steroid) so that they can be measured using an

ELISA-based assay (4). Use of these alternative cells provides an additional benefit in that they are highly responsive to Hh agonists whereas prostate tumor cells are not (5). This allowed us to test whether stimulation of the cells with Hh agonist could induce T biosynthesis from prostate cells. First we conducted a comparative microarray- (CHIP) based gene profiling done using commercially-obtained primary human prostate stromal cells treated or untreated with the Hh chemical agonist, SAG. The outcome of this profiling effort showed a striking and specific upregulation by the Hh agonist of most of the genes involved in cholesterol biosynthetic pathway along with other select genes involved in androgen biosynthesis and metabolism from cholesterol in these cells (Figure 3). Following up on this observation, we have now shown that treatment with the Hh agonist essentially doubles T production from the adrenal precursor steroid, DHEA (Figure 4). Finally, we have found that transduction of the prostate stromal cells with active Gli2 expression vector also increased T output from DHEA and that siRNA against Gli2 blocks the ability of SAG to increase T output from DHEA (Figure 5). Thus, we have obtained evidence both at the gene level and at the steroid production level that active Hh signaling is involved in the production of endogenous androgens from prostate cells. We believe that this modified effort, while proving the point that we sought to establish in our original, unmodified Aim 3, also takes us in a very novel and interesting direction by establishing that paracrine Hh signaling, mediated by upregulation of Hh ligand production from prostate tumor cells following androgen deprivation, can stimulate tumor microenvironmental T production from tumor support cells. While this work was a transient deviation from our original Aim 3 due to the inability of our core facility to sensitively measure androgen production from prostate cancer cells, we will return to this effort and the tasks described in the original SOW with a move to a new Institution (The Prostate Centre at VGH, Vancouver, BC, Canada) with suitable and tested facilities and instrumentation for HPLC/MS measurement of T and DHT output from prostate cancer cells (6) that were described in the original SOW. Therefore, we do not intend to modify the SOW for Aims 2 and 3 but will complete these tasks in our new Institution.

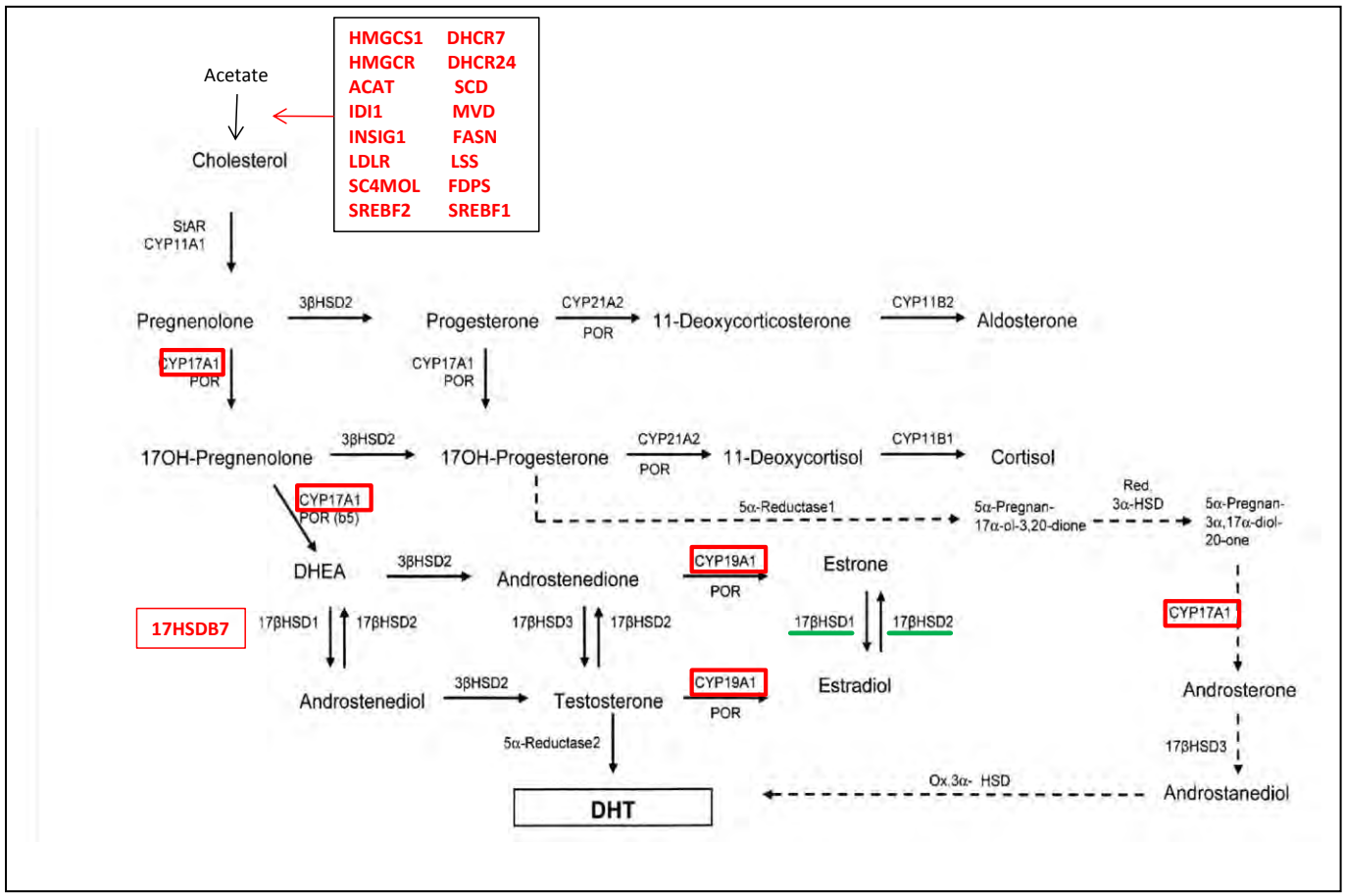


Figure 3, Above. Chart identifies genes involved in cholesterol (red in top box) or androgen (red boxes, below) that were significantly upregulated ($p < 0.05$) by treatment of primary human prostate stromal cells with 100 micromolar SAG, a Hedgehog agonist. Assessment was made in a comparative microarray gene expression profiling analysis that compared gene expression in vehicle-treated prostate stromal cells with SAG-treated prostate stromal cells. Profiling was done using the Affymetrix Human ST1.0 Gene Chip and data (results of 2 independent assessments of RNAs in each condition) and data was analyzed using the GeneSpring v.10 soft ware analysis program.

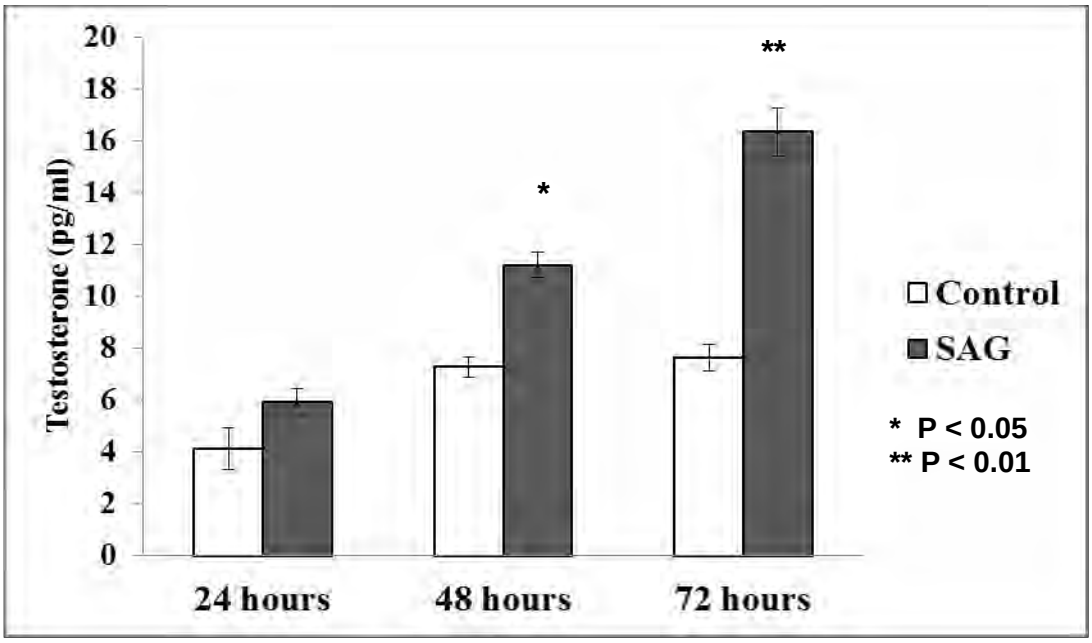


Figure 4 (Above). Testosterone levels in conditioned medium of cultured human prostate stromal cells treated with vehicle (ETOH, Control) or with 100 micromolar SAG in ETOH measured by a testosterone-ELISA Kit. Results show that treatment with the Hedgehog agonist, SAG, more than doubled testosterone output from the cells over a 72 hrs period.

3. *Further Analysis of the Role of β-catenin in Hh-mediated AR activation in androgen-deprived prostate cancer (***Aim 4, task 1 and 2)**.

In our application for this grant, we provided preliminary evidence (based on a single experiment) that cyclopamine, a Hh-inhibitor drug, appeared to suppress phosphorylation of β-catenin in treated prostate cancer cells and also reduced interaction of β-catenin with AR (assessed by co-immunoprecipitation studies). Since others published evidence that phosphorylated β-catenin might be a factor in hyperactive androgen signaling in androgen deprived prostate cancer cells (7), we proposed further experiments to confirm an action of cyclopamine on β-catenin phosphorylation and association with AR. Unfortunately, our experimental efforts in this regards were inconsistent. While we were able to repeat these findings in one additional experiment, in three other attempts, we failed to find reduced interaction of β-catenin with AR after cyclopamine treatment of LNCaP cells. Furthermore, an additional experiment failed to find any suppression of β-catenin phosphorylation upon treatment of LNCaP prostate cancer cells with cyclopamine. The inconsistencies in the outcomes of the multiply repeated experiments, even though they were done with great care and attention to reproducibility, fail to provide us with confidence that this is a useful pathway for further research and we cannot, at this time, support this mechanism for action of cyclopamine. Since we have found plausible and very reproducible evidence for other mechanisms of support of Hh for AR signaling, we will no longer pursue these experiments on β-catenin. We will, however, continue to pursue our efforts to assess the effects of cyclopamine on Fer kinase (Aim 4, tasks 3, 4 and 5) since there is a reasonable body of literature that suggests that Fer kinase is important to prostate cancer progression

through an unknown mechanism and because of the known interaction of Fer with the microtubule network that is affected by Hh activity or Smoothened inhibition (by cyclopamine or other drugs) in the continuation years.

4. *Complications Faced by Institutional (Ordway Research Institute) Financial Problems and Bankruptcy and Plans for the Future*

In December of 2010, the PI (R Buttyan) and the other faculty of Ordway Research Institute (ORI) was informed that the Institute was facing severe financial problems and that our ability to order scientific supplies was thereafter greatly restricted as the Institute was unable to pay any bills incurred without a financial rescue plan. The Institutional Director and the Chief Financial Officer described a feasible plan to accomplish this. However, for the next 3 months, we were forced to only order supplies from alternate companies that were not owed money by ORI so this allowed us to continue our work for that time. Unfortunately, the Institutional plans for solvency were not successful and, on April 28th of this year, ORI declared bankruptcy. The PI and all other staff associated with this project received a notice of employment termination on that day as were the PIs and staff from most other Divisions of the Institute. We notified the Project Manager of this event and further funding to the Institute was terminated. We feel this discussion is an important part of our progress report because our significant progress, despite the many difficulties presented by these actions, shows that we have the capability to carry out the project in a productive manner and that we have already addressed many of the tasks in our Aims despite these difficulties. The PI is happy to report that he accepted a position as a Senior Scientist at The Prostate Centre of the University of British Columbia and the Vancouver General Hospital in Vancouver, Canada and officially started in this position as of August 1 of 2011. The Prostate Centre is a remarkable UBC Center of Excellence for the study of prostate cancer that encompasses over 15 Principal Investigators, each studying different aspects of prostate cancer with the purpose of integrating research findings into new and more effective clinical treatments for prostate cancer. I am now seeking to continue the project at this site and I am afforded a much better and more well-equipped and prostate-centric working environment than was present at ORI. The enhanced environment includes a tested HPLC/MS Core system that has already been proven to be able to measure T and DHT production from cultured prostate cancer cells and this will allow us to complete all the tasks under Aim 3. Likewise, the advanced facilities and highly interactive environment will facilitate completion of the rest of the tasks as are now described in the modified SOW.

Key Research Accomplishments

- Created tagged Gli1 and Gli2 expression vectors and showed that they make appropriate proteins when transfected into prostate cancer or other cells (Task 1)
- Created stably transduced Smoothened, Gli1 and Gli2 LNCaP cell lines (and control vector transduced) that were used to show that these proteins (Gli1 and Gli2) affect androgen signaling in androgen deprived prostate cancer cells and allow androgen-independent growth (Task 1)
- Created a series of partially deleted Gli2 expression vectors that enable mapping of the Androgen Receptor (AR) binding site (New Task 1)
- Created a series of partially deleted AR cDNAs that have been used in mapping the Gli2 binding site.
- Used co-immunoprecipitation technique to Map specific interaction domains within the Gli2 protein and the AR proteins that may allow the design of small molecular weight inhibitors that might block this interaction (New Task 1)
- Successfully used a chromatin immunoprecipitation assay to show that Gli2 protein associates with the androgen response elements on genes that are regulated by androgens (Task 1)
- Identified suitable Smoothened, Gli1 and Gli2 siRNAs that knockdown expression of these genes in prostate cancer cells and showed that these siRNAs reduce reactivated androgen signaling in androgen deprived prostate cancer cells (Task 1)
- Showed that Gli-blocking drugs suppress reactivated androgen signaling in androgen deprived prostate cancer cells as well as the Smoothened-blocking agent, cyclopamine (Task 1)
- Conducted a comparative microarray profiling assay to identify genes affected by cyclopamine treatment of androgen deprived prostate cancer cells (Task 2)

- Successfully measured testosterone levels in conditioned medium of androgen-deprived prostate cancer cells using an ELISA assay and showed that the activation of Hedgehog signaling in prostate cells induces expression of genes associated with steroid and androgen biosynthesis and increases production of androgen from an adrenal precursor steroid (Task 3)
- Was unable to confirm the hypothesis that cyclopamine affects the phosphorylation of β-Catenin or its association with androgen receptor protein in cyclopamine-treated prostate cancer cells (Task 4)

Reportable Outcomes

1) Confirmed that the activity of cyclopamine on androgen signaling and androgen independent growth of prostate cancer cells involves Hedgehog Signaling Intermediate proteins (Published, Ref 1, Below)
2) Described the effectiveness of Gli-inhibitor drugs as a means of suppressing reactivated androgen signaling in androgen-deprived prostate cancer (Published, Ref 2, Below)
3) Established a plausible mechanism linking Hedgehog signaling to reactivated androgen signaling in androgen deprived prostate cancer cells. (Manuscript in Preparation)
4) Mapped the interaction sites between Gli2 protein and androgen receptor protein that may help identify new small molecules to block this interaction (Manuscript in Preparation)
5) Established that Hedgehog signaling can induce androgen biosynthesis from human prostate cells
6) Established the paradigm that paracrine Hedgehog signaling in the microenvironment of a prostate tumor can affect tumor-autonomous androgen production from surrounding benign support cells (Manuscript in Preparation).
7) Derived evidence that Hedgehog signaling mediates both cholesterol and androgen biosynthesis in prostate cells (Manuscript in Preparation).
8) Lack of evidence for reproducible effect of cyclopamine on β-catenin phosphorylation and/or association with the androgen receptor successfully refutes hypothesis (Manuscript in Preparation)

Conclusions

The work accomplished during the first year strongly supports the hypothesis that Hedgehog signaling induced by androgen deprivation can support reactivated androgen signaling in prostate cancer cells leading to increased propensity for androgen independent growth of these cells. Our data also supports the clinical testing of Hedgehog/Gli inhibitory drugs for treatments of prostate cancer patients with advanced disease, in conjunction with androgen deprivation therapy.

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Appendices (Follow)

Chen, M. Feurstein, M.A., Levina, E., Baghel, P.S., Carkner, R., Tanner, M.J., Shtutman, M., Vacherot, F., Terry, S., de la Taille, A. and Buttyan, R. (2010) Hedgehog / Gli supports androgen signaling in androgen deprived and androgen independent prostate cancer cells. *Mol. Cancer*, 9: 89-101. PMID: 20420697

Chen, M., Carkner, R. and Buttyan, R. (2011) The Hedgehog signaling paradigm in prostate cancer. *Exp. Rev. Metabol. Endocrin*., 6: 453-467. PMID: 21776292

MOLECULAR CANCER

RESEARCH Open Access

Hedgehog/Gli supports androgen signaling in androgen deprived and androgen independent prostate cancer cells

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Abstract

Background: Castration resistant prostate cancer (CRPC) develops as a consequence of hormone therapies used to deplete androgens in advanced prostate cancer patients. CRPC cells are able to grow in a low androgen environment and this is associated with anomalous activity of their endogenous androgen receptor (AR) despite the low systemic androgen levels in the patients. Therefore, the reactivated tumor cell androgen signaling pathway is thought to provide a target for control of CRPC. Previously, we reported that Hedgehog (Hh) signaling was conditionally activated by androgen deprivation in androgen sensitive prostate cancer cells and here we studied the potential for cross-talk between Hh and androgen signaling activities in androgen deprived and androgen independent (AI) prostate cancer cells.

Results: Treatment of a variety of androgen-deprived or AI prostate cancer cells with the Hh inhibitor, cyclopamine, resulted in dose-dependent modulation of the expression of genes that are regulated by androgen. The effect of cyclopamine on endogenous androgen-regulated gene expression in androgen deprived and AI prostate cancer cells was consistent with the suppressive effects of cyclopamine on the expression of a reporter gene (luciferase) from two different androgen-dependent promoters. Similarly, reduction of smoothened (Smo) expression with siRNA co-suppressed expression of androgen-inducible KLK2 and KLK3 in androgen deprived cells without affecting the expression of androgen receptor (AR) mRNA or protein. Cyclopamine also prevented the outgrowth of AI cells from androgen growth-dependent parental LNCaP cells and suppressed the growth of an overt AI-LNCaP variant whereas supplemental androgen (R1881) restored growth to the AI cells in the presence of cyclopamine. Conversely, overexpression of Gli1 or Gli2 in LNCaP cells enhanced AR-specific gene expression in the absence of androgen. Overexpressed Gli1/Gli2 also enabled parental LNCaP cells to grow in androgen depleted medium. AR protein co-immunoprecipitates with Gli2 protein from transfected 293T cell lysates.

Conclusions: Collectively, our results indicate that Hh/Gli signaling supports androgen signaling and AI growth in prostate cancer cells in a low androgen environment. The finding that Gli2 co-immunoprecipitates with AR protein suggests that an interaction between these proteins might be the basis for Hedgehog/Gli support of androgen signaling under this condition.

Background

When detected in the advanced stage, prostate cancer patients are treated with hormone therapies that reduce systemic androgen levels [1-3]. This action palliates the symptoms of metastases, induces regression of metastatic lesions and slows prostate tumor growth [4]. Over time, however, the cancer can recur in a castration resistant form (CRPC) that continues to grow despite the ability of hormone therapy to maintain systemic androgens at castrate levels and deaths from prostate cancer are inevitably associated with complications from this form of disease [5]. Progression of prostate cancer to CRPC appears to involve a reactivation of androgen signaling in the cancer

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cells [6-8] and a variety of mechanisms may account for residual androgen signaling in a low androgen environment. These include expression of variant forms of androgen receptor (AR) that are transcriptionally active without ligand [9,10], acquisition of an ability to endogenously synthesize androgens by the tumor cells themselves [11,12] or activation of aberrant AR transcriptional activity through cross-talk with alternate signaling pathways [6,13]. While all of these mechanisms are of interest from a scientific viewpoint, the ones that are readily targetable by drugs are the most clinically imperative as they offer an opportunity to test novel therapies to treat a disease that will kill almost 28,000 men in the United States this year. Recent reports that Abiraterone, an inhibitor of androgen biosynthesis, has clinical effects against castration resistant prostate cancer, reflects a potential treatment advance that might target tumor cell androgen biosynthesis [14]. Here we describe findings that suggest that inhibitors of the Hedgehog/Gli signaling pathway, currently in clinical testing for a variety of cancers, might also have a role for the treatment of castration resistant prostate cancer due to an ability to suppress reactivated androgen signaling in tumor cells.

Hedgehog (Hh) is best known for its role in tissue patterning and morphogenesis during embryonic development [15-18]. In the developmental situation, Hh is a ligand-driven process in which a ligand (referred to as a Hedgehog) engages the Patched 1 (Ptch) receptor on the cell surface and this relieves repression of Smoothened (Smo), a member of the extended G protein coupled receptor family [18]. Smo, when activated, then acts downstream to alter the processing and intracellular localization of Gli transcription factors and to increase Gli-mediated transcriptional activity. The plant-derived alkaloid, cyclopamine, is a prototype for a drug that antagonizes the Hh signaling process [19]. Cyclopamine antagonizes Smo activation and this action explains the teratogenic effects of this drug when it is ingested during pregnancy [20,21].

Aside from its role in development, Hh signaling also supports stem cells in adult tissues [22-24]. However, chronically hyperactive Hh/Gli signaling in adult tissues can be oncogenic, especially for the skin or brain [25,26]. Basal cell carcinoma of the skin and medulloblastoma are models for human Hh-mediated oncogenesis [27]. The aberrant Hh activity in these tumors can result from a loss of the Ptch gene or its function [28,29], mutations in Smo [30] or SuFu [31] that activate endogenous Hh signaling or cryptic overexpression of Gli proteins in tumor cells. For prostate cancer, the question as to whether Hh/Gli signaling plays any role is controversial. Although cyclopamine treatment or Gli knockdown suppresses the *in vitro* growth of prostate cancer cell lines or xenograft tumor growth in mice [32-34], the commonly used prostate cancer cell lines show little, if any, evidence for active canonical Hh signaling activity when they are grown in standard culture conditions [35,36]. For the androgen-growth dependent LNCaP prostate cancer cells and its variants, C4-2 and C4-2B, however, the situation was found to be changed by chronic exposure of these cells to androgen depleted medium. Androgen deprivation highly upregulated the expression and secretion of Hh ligands and increased endogenous expression of Hh/Gli target genes in these cells [37]. The clinical relevance of this observation is supported by the observation that Hh ligand production was found to be increased in prostate tumors by neoadjuvant hormone treatment [38]. Since cyclopamine suppresses the expression of Hh target genes in androgen-deprived LNCaP cells (37), this also suggests that active Hh/Gli signaling activity is awakened by growth under androgen deprived conditions. Others have observed that the high basal expression of Hh/Gli target genes in androgen independent (AI) variants of LNCaP was reduced by cyclopamine [39] and, collectively, the outcomes of these studies imply that Hh signaling in LNCaP cells is restricted to the androgen deprived or AI state. The question remains as to whether active Hh signaling has any biological consequences for the androgen deprived or AI prostate cancer cell. Here we show that, by manipulating the activity of canonical Hh signaling in androgen deprived or AI prostate cancer cells, we also affected the expression of androgen regulated genes and the ability of these cells to grow in the absence of androgen. Our results indicate that Hh/Gli signaling activity supports androgen signaling and AI growth in prostate cancer under low/no androgen conditions. Furthermore, we report that Gli2 protein can bind to AR and this interaction might define the point of cross-talk between the two signaling pathways.

Results and Discussion

Previously we reported evidence for conditional activation of canonical Hh signaling in androgen sensitive human prostate cancer cells by culture in an androgen depleted conditions [37]. Here, we used androgen sensitive parental LNCaP cells, other derivatives of LNCaP that are less dependent on androgens for growth (C4-2, LN3, LNCaP-AI) or androgen responsive VCaP cells that are unrelated to LNCaP, to study the effects of Hh signaling manipulation on the expression of androgen regulated genes in these cells. The LNCaP-AI variant cells that we used were independently isolated in our lab following long-term (> 1 year) culture of parental LNCaP cells in androgen depleted medium. These cells downregulate basal expression of Ptch1 when treated with cyclopamine (Additional file 1, Figure S1) so they appear to have basal-active Hh signaling activity similar to other AI derivatives of LNCaP that were previously described (39).

Initially, we tested the effects of the classic Hh inhibitor drug, cyclopamine on androgen regulated gene expression. All experiments were done using a medium that was depleted for androgens (phenol red-free RPMI with charcoal-stripped FBS) that could be re-supplemented with androgen (R1881) to mimic androgen-stimulated conditions. For parental LNCaP cells grown in androgen supplemented medium (+R1881), the presence of cyclopamine had no significant effects on the expression of four model androgen-regulated genes; KLK2, KLK3 [PSA] and PGC (androgen-inducible), or SHH that is repressed by androgen (Figure 1A). However, when these cells were switched to androgen depleted medium (-R1881) for 3 days, cyclopamine treatment had a distinct dose-dependent effect that further suppressed expression of KLK2, KLK3 and PGC and further increased expression of SHH (Figure 1A). Likewise, cyclopamine significantly downregulated expression of KLK2, KLK3 and PGC in the LNCaP-AI cells that are normally propagated in androgen-free medium, and it upregulated the expression of SHH in these cells (Figure 1A). Cyclopamine also suppressed the expression of KLK2 and KLK3 in VCaP, LN3 or C4-2B cells grown in androgen depleted medium for 3 days (Additional file 1, Figure S2), so the effects of cyclopamine on androgen regulated genes were not limited to LNCaP or its derivatives. We also tested whether a more water-soluble cyclopamine derivative, KAAD-cyclopamine, had a similar effect and found that this drug (at 0.5 or 1 μM) was as effective in reducing KLK2/3 and PGC expression in androgen-deprived LNCaP or LNCaP-AI cells as the 5 or 10 μM dose of cyclopamine (Additional file 1, Figure S3). Finally, we found that cyclopamine also significantly diminished the expression of a reporter gene (luciferase) from either of two androgen dependent promoters (Probasin [PRB] or PGC) in LNCaP or LNCaP AI cells in androgen depleted medium (Figure 1B) in a dose dependent manner. As for endogenous androgen-regulated genes, cyclopamine did not affect the expression of the reporter when cells were cultured in medium supplemented with 10 pM R1881 (data not shown).

Cyclopamine represses Hh signaling through its ability to antagonize Smo activation so we also tested whether Smo expression knockdown (using siRNA) could mimic the effects of cyclopamine with regards to suppression of androgen-inducible gene expression. LNCaP cells were transfected either with control (non-targeting) siRNA or with siRNA targeting AR or Smo and were thereafter maintained in androgen-depleted medium. AR siRNA selectively reduced expression of AR mRNA and protein (Figures 2A, C) but did not reduce the expression of Smo. Likewise, Smo siRNA reduced Smo mRNA levels but did not affect expression of AR mRNA or protein (Figure 2C). However, both AR and Smo siRNAs similarly reduced expression of KLK2 and KLK3 (Figure 2A). Further assessment of the effects of AR or Smo siRNA on expression of a luciferase reporter from either a Gli- or androgen-responsive promoter showed that AR knockdown selectively reduced expression of the androgen reporter but did not affect expression of the Gli reporter (Figure 2B). In contrast, Smo knockdown significantly reduced expression of both the Gli and androgen reporters (Figure 2B) in androgen deprived LNCaP cells. In summary, the above data shows that suppression of Hh signaling with a Smo antagonist, cyclopamine, or by reduction of Smo expression itself, suppresses expression of androgen inducible genes and induces expression of androgen repressed genes, but only when these human prostate cancer cells were cultured in a medium lacking androgen. The fact that Smo knockdown reduced expression of androgen regulated genes but did not affect expression of AR mRNA or protein suggests that some aspect of Hh signaling regulates the activity of the AR rather than its expression.

Since cyclopamine suppressed residual/reactivated androgen gene expression in androgen deprived and AI prostate cancer cells, we also sought evidence that this effect had biological consequences relevant to AI growth. First, we tested whether the presence of cyclopamine might prevent the development of AI cells from parental LNCaP cells chronically maintained in androgen depleted medium. LNCaP cells were seeded onto 10 plates at low density and then 5 plates each were switched to androgen depleted medium supplemented with vehicle (EtOH) or with 5 μM cyclopamine. The media were changed every 3 days. Within 2 months, cell numbers in the cyclopamine-treated cultures were significantly reduced compared to vehicle-treated cultures and most surviving cells in the cyclopamine-treated cultures were shrunken with optically dense nuclei that contrasted with the neuroendocrine cell-like appearance of cells in vehicle-treated cultures (Figure 3A). By the third month, cyclopamine-treated cultures had less than 1% of the cells of vehicle-treated cultures and all remaining cells showed the presence of the optically dense nuclei. No cells remained on cyclopamine-treated plates by 4 months of culture but the cells in the vehicle-treated cultures were increasing in numbers by this time and these cultures gave rise to growing lawns of cells by 6 months that typify AI growth. For overt LNCaP-AI cells, we found that treatment with 5 μM cyclopamine significantly inhibited their growth over a 10 day period (Figure 3B) but when cyclopamine treatment was accompanied by supplemental androgen (10 pM R1881), the growth rate of these cells was no different than vehicle treated cells. This indicates that the presence of androgen can overcome the growth-inhibiting effects of cyclopamine on overt AI cells.

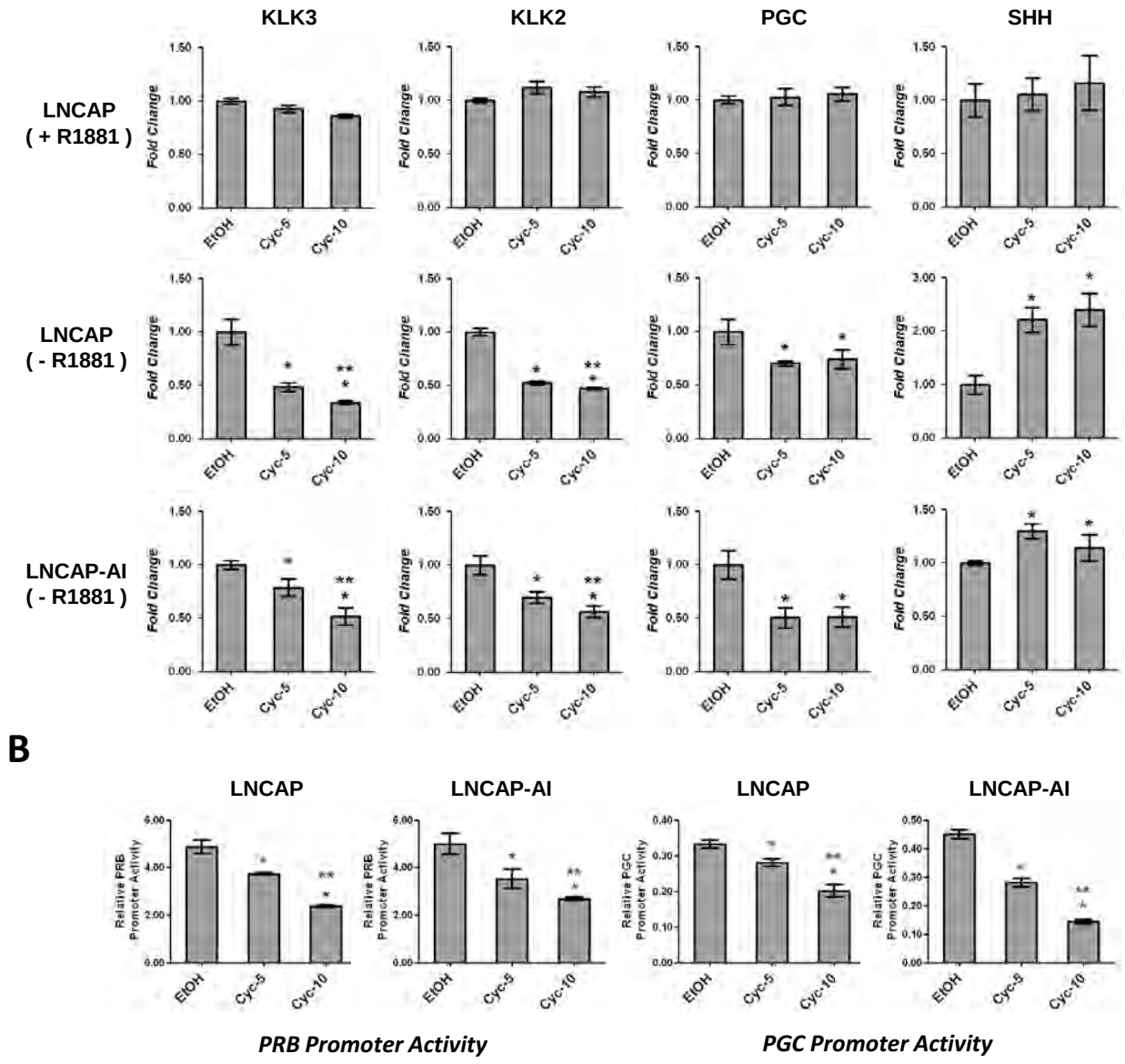

Figure 1 Effect of cyclopamine on androgen signaling in LNCaP cells. (A) Real time qPCR was used to measure relative expression of KLK3, KLK2, PGC or SHH mRNA in androgen-supplemented (+R1881) or androgen deprived (-R1881) LNCaP or in LNCaP-AI cells (-R1881) in the presence of vehicle (EtOH) or with 5 or 10 μM cyclopamine (Cyc-5, Cyc-10) (also see Additional file 2, Table S2). (B) LNCaP or LNCaP-AI cells were infected with probasin (PRB) or PGC promoter reporter vectors along with a CMV-GFP reference reporter and were cultured in androgen depleted medium with vehicle (EtOH) or with 5 or 10 μM cyclopamine (Cyc-5 or Cyc-10) for 72 hrs. Cell extracts were assayed for luciferase that was normalized by GFP intensity. Bars represent the means of triplicate experiments ± S.E. (* = $P < 0.05$ compared to vehicle control; ** = $P < 0.05$ between 5 and 10 μM cyclopamine treatment groups).

Finally, we sought to test whether overexpression of Gli1 or Gli2, transcription factors that lie at the endpoint of the Hh signaling process, might act oppositely to Smo antagonism/inhibition to increase androgen signaling or AI growth when LNCaP cells were grown in androgen free medium. Parental LNCaP cells were transduced with a Gli1- or Gli2- (Gli2ΔN) expressing lentivirus and these cells were compared to control cells transduced with empty virus to determine the effects of Gli overexpression on androgen regulated gene expression and cell growth in androgen depleted medium. The Gli overexpressing variants of LNCaP were also found to express significantly higher levels of KLK2 or KLK3 when compared to control (vector transduced) cells in androgen

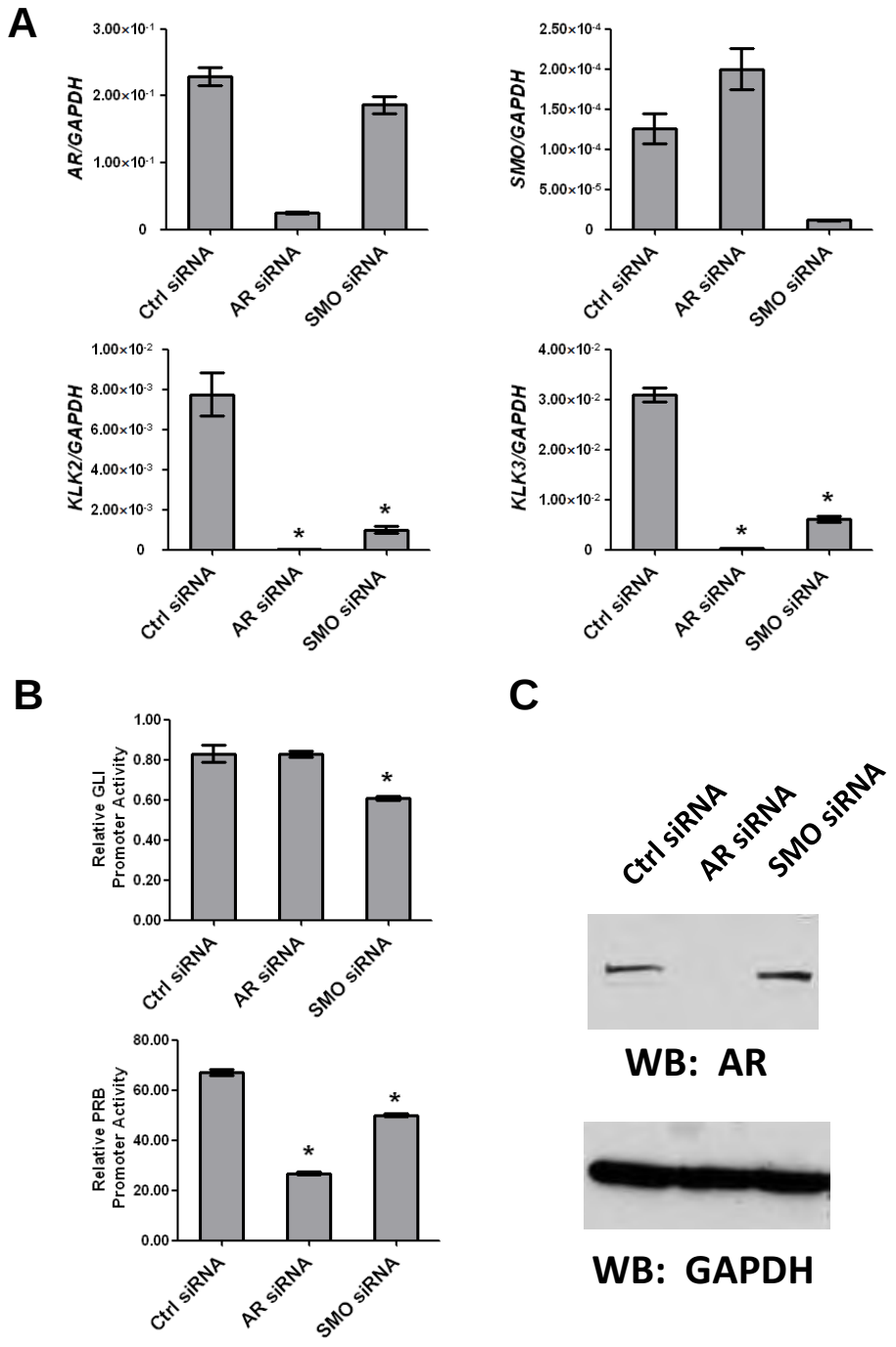

Figure 2 Smo knockdown affects androgen responsive gene expression in androgen-deprived LNCaP cells. (A) LNCaP cells were transfected with control (Ctrl) siRNA, AR or Smo siRNA and grown in androgen-depleted medium for 72 hrs. RNAs were extracted and assayed by real-time qPCR for expression of AR, Smo, KLK2 or KLK3. Bars represent the means of three experiments ± S.E. (* = $P < 0.05$ compared to control siRNA). (B) Cells transfected with siRNA were infected with a Gli or Probasin (PRB) FF luciferase reporter lentivirus along with a CMV-GFP lentivirus control reporter and were switched to androgen-depleted medium for 72 hrs. Cell extracts were quantified for luciferase activity that was normalized by GFP intensity. Bars represent the means of triplicate experiments ± S.E. (* = $P < 0.05$ compared to control siRNA). (C) Western blot shows effects of siRNA on expression of AR protein in cell lysates.

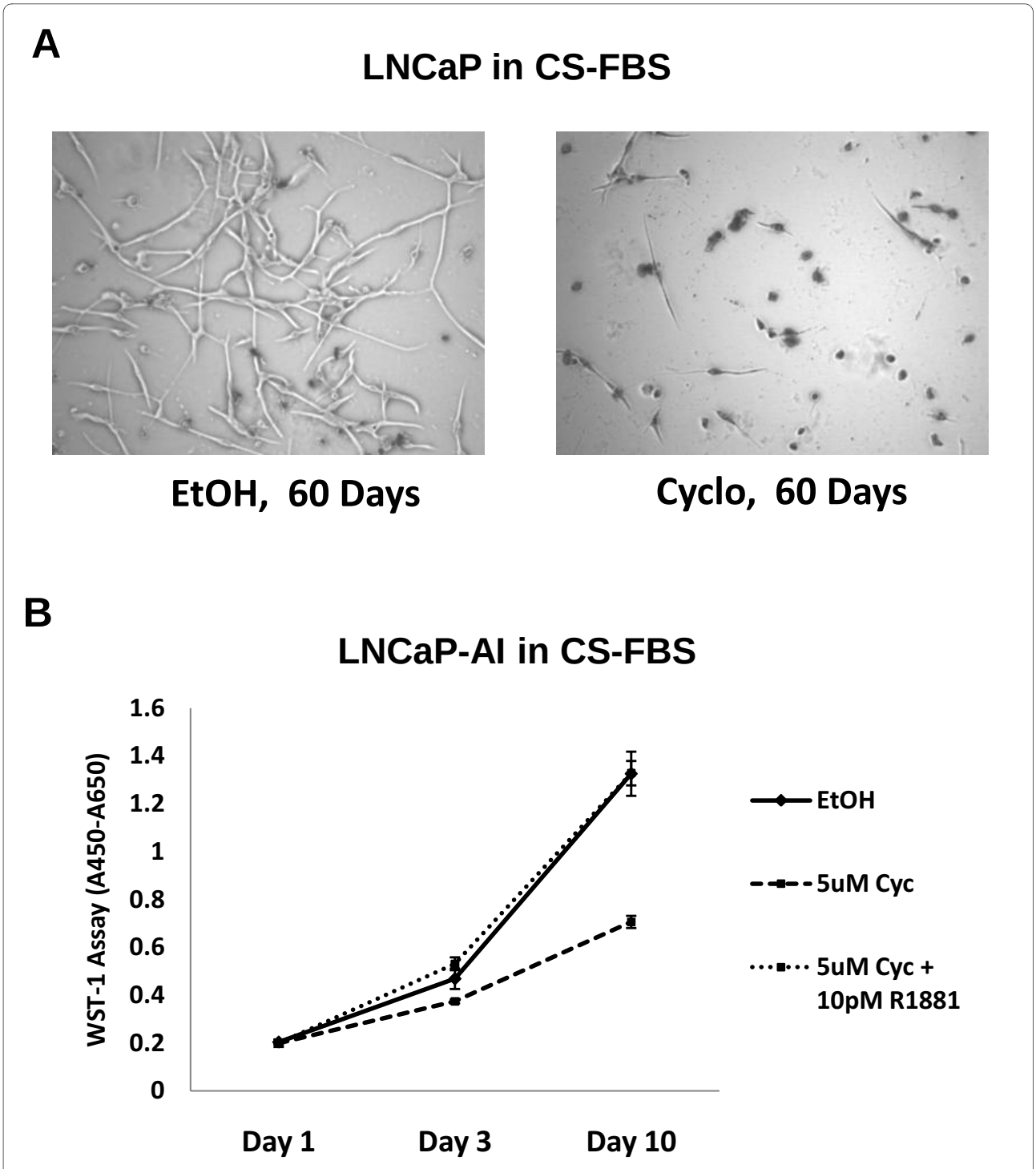

Figure 3 Cyclopamine prevents the development of AI prostate cancer cell growth and suppresses the growth of LNCaP-AI cells. (A) Phase contrast photomicrographs (40×) of LNCaP cells cultured for 60 days in androgen depleted medium (CS-FBS) supplemented with vehicle (EtOH) or 5 μM cyclopamine (Cyclo). Cell numbers in cyclopamine are greatly reduced and cells have optically dense, fragmented nuclei. (B) LNCaP-AI cells grown in androgen-depleted medium (CS-FBS) supplemented with vehicle (EtOH) or 5 μM cyclopamine. Cell numbers were counted at various days as indicated. Points represent the means of triplicate cultures ± S.E.

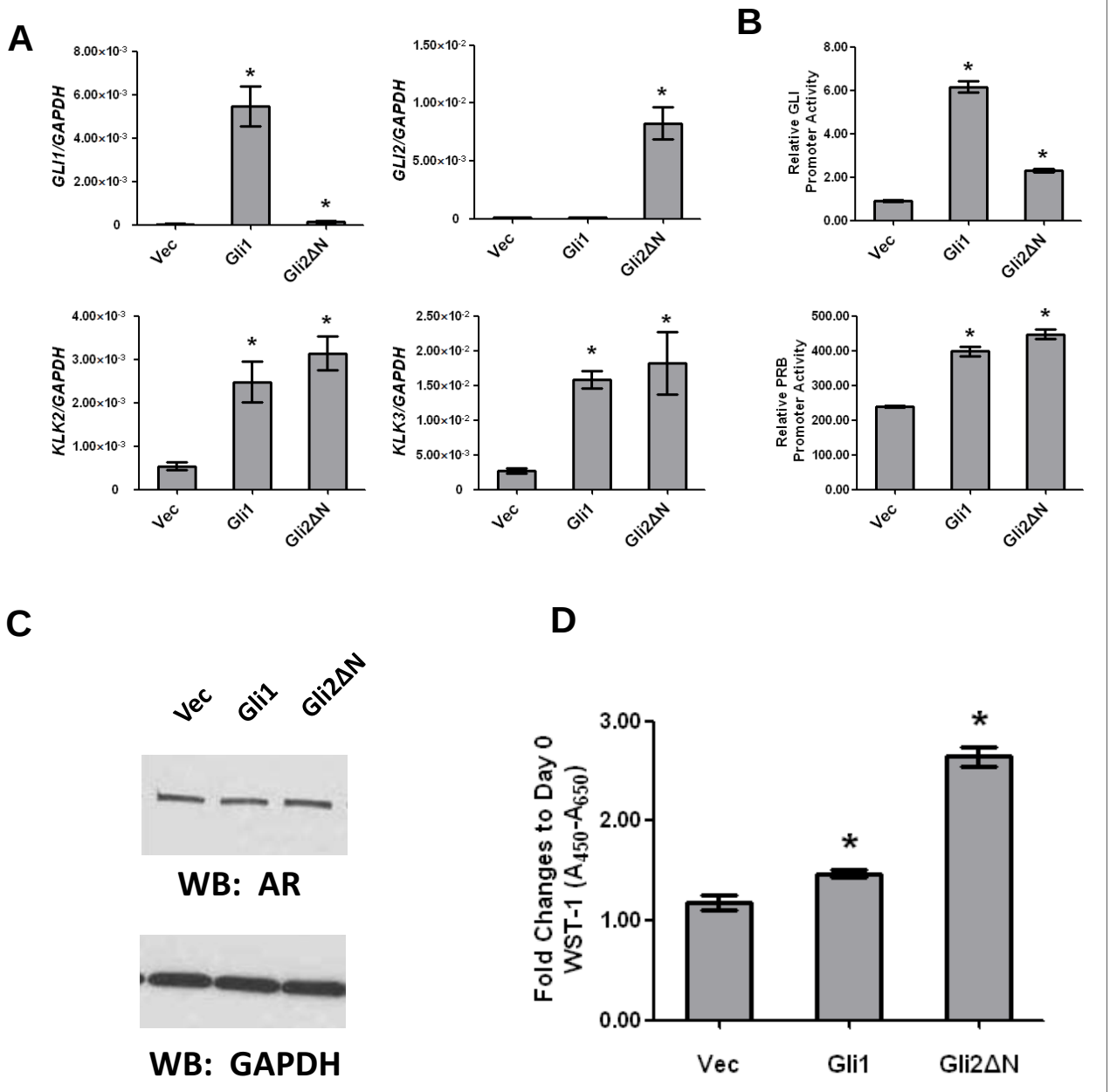

Figure 4 Gli overexpression affects androgen regulated gene expression in androgen-deprived LNCaP cells. (A) RNAs from control (Vec) or Gli1 or Gli2 (Gli2ΔN) overexpressing LNCaP cells cultured in androgen-depleted medium for 72 hrs were assayed by real-time qPCR for expression of Gli1, Gli2, KLK2 and KLK3. Bars represent the means of three experiments ± S.E. (* = P < 0.05 compared to vector control). (B) Cells were infected with a Gli or Probasin (PRB) reporter with CMV-GFP and switched to androgen-depleted medium for 72 hrs. Cell extracts were quantified for luciferase that was normalized by GFP intensity. Bars represent the means of triplicate experiments ± S.E. (* = P < 0.05 compared to vector control). (C) Western blot shows that Gli1 or Gli2 overexpression does not affect expression of AR protein. (D) Gli overexpression enables androgen independent cell growth. Control (Vec) or Gli1 or Gli2 (Gli2ΔN) overexpressing LNCaP cells were cultured in androgen depleted medium for 12 days and growth was measured by WST-1 assay and compared to Day 0. Bars represent the means of three experiments ± S.E. (* = P < 0.05 compared to vector control).

depleted medium (Figure 4A). Gli1 or Gli2 overexpressing LNCaP cells also expressed significantly higher levels of luciferase reporter from both AR and Gli dependent promoters compared to control cells (Figure 4B). Despite higher basal expression of androgen regulated genes, the Gli transduced cells expressed AR protein at equivalent levels to the control cells (Figure 4C) so here again, these effects appear to be independent of changes in AR expression. The Gli transduced LNCaP cells also showed significant increased growth in androgen depleted medium compared to the control cells (Figure 4D), though Gli2 cells appeared to be more robust than Gli1 in

this regard. Regardless of this differential hierarchy, this data shows that Gli function supports androgen regulated gene expression in a low androgen environment as well as AI growth.

The evidence that Gli1 or Gli2 overexpression upregulates androgen inducible gene expression and AI growth of androgen deprived LNCaP cells without affecting AR expression suggests that some function of the Gli proteins may support AR transcriptional activity in a low androgen environment. We tested for some potential direct interaction between these Gli and AR proteins in co-immunoprecipitation experiments. Human 293FT cells were transfected with an expression plasmid for full-length human AR, myc-tagged Gli2 or a combination of these plasmids. Forty-eight hrs later, extracts from the cells were immunoprecipitated with anti-AR or anti-myc antibody and the immunoprecipitates (IPs) were analyzed by Western blot for the presence of AR or myc-tagged Gli2. When the Western blot was probed with anti-AR, we found that AR co-immunoprecipitated with myc-tagged Gli2 only in extracts from cells co-transfected with both plasmids (Figure 5). Similarly, myc-tagged Gli2 was co-immunoprecipitated in the AR IPs from extracts of cells co-transfected with both plasmids (Figure 5). This apparent interaction between Gli2 and AR in the 293FT cells was not diminished by supplementation with 1 nM R1881.

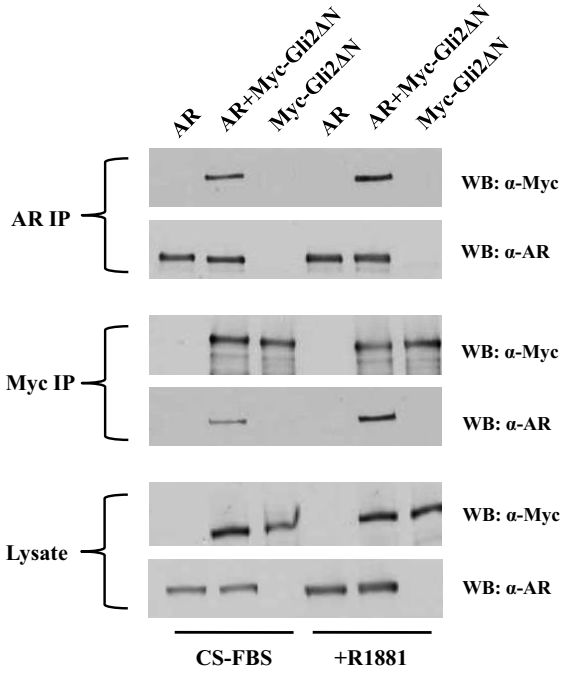

Figure 5 Co-immunoprecipitation of AR with Gli2 protein. Lysates of cells (293FT) transfected with AR, myc-tagged Gli2 (Myc-Gli2ΔN) or both for 48 hrs (under androgen-supplemented [R1881] or depleted [CS-FBS] conditions) were immunoprecipitated with α-AR or α-myc antibody. IPs or lysates were electrophoresed and blotted. The Western blot (WB) was probed with α-AR or α-myc antibody as indicated.

Here we provided evidence that aspects of the canonical Hh signaling pathway can play a role in supporting residual/reactivated androgen signaling in androgen deprived and AI prostate cancer cells and this finding has important implications with regards to both the mechanistic basis for AI growth in the castration resistant prostate cell and for treatment strategies for CRPC in patients. Smo inactivation by cyclopamine, a cyclopamine variant drug (KAAD-cyclopamine) or reduction in Smo expression by siRNA downregulated androgen inducible genes in androgen deprived and AI prostate cancer cells and these findings suggest that some action of Smo might be important for reactivation of androgen signaling under low androgen conditions. The effects of cyclopamine on androgen regulated genes was common to several types of human prostate cancer cell lines grown under androgen deprived conditions so the effect was not limited to LNCaP cells and derivatives. Cyclopamine also suppressed expression of reporter genes from two different androgen responsive promoters in LNCaP cells in androgen depleted medium and these findings support the idea that Smo activity supports AR-mediated transcriptional activity in the androgen deprived state. Finally, the modulatory effects of cyclopamine on AR regulated gene expression were consistent with the effect of this drug on AI growth. Chronic cyclopamine treatment prevented the development of androgen growth independent cells from parental androgen growth-dependent LNCaP cells and significantly inhibited the growth of an overt AI variant of LNCaP. The cyclopamine-mediated growth suppression was reversed by returning a low level of androgen to the cells, providing further evidence that effects of cyclopamine on development and growth of AI cells are based upon cyclopamines' actions on residual androgen signaling.

Smo action ultimately drives transcription by Gli family proteins so we also tested whether exogenous expression of active Gli had opposite effects of cyclopamine or Smo reduction. Here, our findings that Gli1 or Gli2 overexpression enhanced androgen regulated gene expression in androgen depleted medium and enabled AI growth for androgen growth-dependent cells strongly argues that the active Gli proteins resulting from Hh signaling play the most critical role in Hh-support of residual/reactivated androgen signaling regulation. Although the Gli2 overexpressing LNCaP cells exhibited more robust androgen independent growth than the Gli1 overexpressing cells, it is not possible to rank the effectiveness of the Gli proteins on growth control from this study since the cells may be expressing different amounts of transcriptionally active Gli protein. However, the recent report that Gli2 protein was abundantly expressed in tumor cells

from patients with AI (CRPC) prostate cancer [40] does provide further support for the idea that Gli2 protein expression might have a specific role in AI cancer cell growth in CRPC patients and Gli2 may be the preferred target for CRPC treatments.

With regards to the potential mechanism(s) through which Hh/Gli cross-talks to the androgen signaling pathway, it does not appear to involve changes in the expression of AR mRNA or protein as this was not affected by cyclopamine, Smo knockdown or Gli overexpression. However, the finding that Gli2 and AR proteins co-immunoprecipitate when they were co-expressed in 293T cells does suggest that Gli2 might directly interact with AR to influence the expression of AR target genes in the same manner that other co-activator proteins support AR function [41]. Previously Gli2 was shown capable of binding to CREB or to Zic family transcription factor proteins [42,43] so this finding extends the potential repertoire of transcription factors capable of interacting with Gli2. It is of further interest that the interaction between AR and Gli2 proteins was not diminished by androgen supplementation. Therefore, the lack of effects of cyclopamine on androgen regulated gene expression in androgen supplemented LNCaP cells might be due to some additional role of other upstream elements of the Hh signaling pathway that are only manifest in androgen depleted cells. Additionally, we must consider the possibility that Hh/Gli signaling is involved in the endogenous production of androgen (intracrine androgen biosynthesis) that is reportedly associated with AI prostate cancer cells [11], especially since Hh signaling is required for steroidogenesis in the testis and for androgen production by other types of cells [44,45]. This is an aspect that we will test for in future experiments.

Regardless of the mechanism(s) involved, the outcome of this research suggests that Hh/Gli inhibitors offer a specific means to target reactivated androgen signaling in CRPC and to test the idea that inhibition of anomalous androgen signaling in CRPC cells has therapeutic benefit for patients. Although cyclopamine is difficult to use as a therapeutic agent, several pharmaceutical companies are in the process of developing similar drugs that are easier to use in the clinical setting and some of these drugs are through Phase I testing [46]. Therefore, translation of these experimental studies to patients should be able to proceed fairly rapidly. Alternatively, there are non-canonical signaling pathways that increase Gli activity in cancer cells [47] so a clinical focus on Smo antagonists may not be sufficient to deal with all forms of CRPC. Reports of small molecular inhibitors of Hh/Gli signaling that act independently of Smo antagonism [48], suggests that Hh/Gli signaling provides a rich array of targets for the development of more effective treatments for CRPC.

Conclusions

Modulation of Hh signaling in prostate cancer cells by reduction of Smo expression or activity or by overexpression of active Gli proteins affected androgen signaling and the expression of androgen regulated genes in these cells but only when they were cultured in a low androgen medium. The effects of Hh modulation on androgen regulated gene expression in prostate cancer cells were consistent with the coordinate effects on AI cancer cell development and growth in low androgen medium but these effects were reversed by the presence of androgens. Since we have found that Gli2 protein, at least, interacts with the AR protein, the mechanism through which Hh signaling affects AR-dependent gene expression and AI cell growth may involve a direct interaction of AR with Gli proteins.

Methods

Cells and Culture

Human prostate carcinoma cell lines LNCaP and VCaP were obtained from the ATCC (Manassas, VA). LNCaP variants, LN3 or C4-2B were obtained from Curtis Pettaway, M.D. Anderson Cancer Center (Houston, TX) or ViroMed Laboratories (Minnetonka, MN), respectively. The LNCaP-AI variant was derived from parental LNCaP cells after more than one year growth in androgen-depleted medium. Cells were maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS) or switched to phenol red-free RPMI-1640 with 10% charcoal-stripped FBS (CS-FBS) for androgen-depleted conditions as previously described (37). The 293FT cells were obtained from Invitrogen, Inc. (Carlsbad, CA) and were maintained in DMEM with 10% FBS. Synthetic androgen, R1881 (methyltrienolone), was obtained from PerkinElmer Life Sciences (Boston, MA) and was supplemented to androgen-depleted medium at 10 pM where indicated. Cyclopamine was obtained from Enzo Life Sciences, Intl. (Plymouth Meeting, PA) and KAAD-cyclopamine from Toronto Research Chemicals Inc. (North York, ON, Canada). Cultured cells were imaged by a Leica DMIRE2 inverted microscope (Leica Microsystems Inc., Bannockburn, IL).

Generation of LNCaP Lines Stably Expressing Gli Transcription Factors

The ViraPower™ Lentiviral Expression System (Invitrogen) was used for generating replication-incompetent lentiviruses expressing recombinant human Gli1 or Gli2ΔN. All procedures were performed according to the manufacturers' protocols with modifications: 1) cDNAs encoding the full-length human Gli1 and the N-terminal-truncated human Gli2 were cloned from the plasmid GLI K12 [49] and pCS2-MT GLI2(ΔN) [50] (Addgene, Cam-

bridge, MA) into pLenti6 (Invitrogen); 2) For production of lentivirus in 293FT cells, 3 μg of pLenti6-Gli1, pLenti6-Gli2ΔN or pLenti6-Vec (empty vector control) were mixed with 9 μg of ViraPower Packaging Mix, and 36 μl of Lipofectamine-2000 (Invitrogen). The mixture was applied to 2 × 10^6 293FT cells in medium overnight. Transfection medium was removed and fresh medium was added for another 72 hours. Lentivirus containing medium was collected and filtered and used for infections; 3) LNCaP cells were seeded at 50% confluence overnight in preparation for viral transduction. Virus supernatants were added (diluted 1:5 with medium) and 48 hrs later, blasticidin was added at a concentration of 10 μg/ml for selection. Selection was carried out for 2-3 weeks and ~200 colonies were obtained and pooled as stably-expressing sublines, LNCaP-Vec, LNCaP-Gli1, or LNCaP-Gli2ΔN.

RNA Isolation and Reverse Transcription - Real-Time PCR Assays (RT-qPCR)

RNA was isolated from cells using the RNeasy Mini Kit with RNase-Free DNase digestion (QIAGEN, Valencia, CA). Reverse transcription was carried out using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) per the supplier's protocol. Real-time PCR was performed on an ABI 7900HT detection system (Applied Biosystems, Foster City, CA) using RT² SYBR Green/ROX qPCR Master Mix (SABiosciences, Frederick, MD) according to the manufacturer's protocol. The thermal cycling conditions were as previously described (37). The message number of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference for calculating specific gene messages. The sequences of qPCR primers used are listed in Additional file 2, Table S1.

Promoter activity assays

Firefly luciferase reporter vectors under the control of a promoter containing eight repeats of the Gli consensus sequence (pLLRM-GLI-Luc) was generated by sub-cloning the GLI-responsive promoter fragment from pGL3B/8XGliBS-lc-luc (JHU-73, ATCC) into a lentiviral luciferase reporter vector, pLLRM. Reporter vectors with rat probasin (PRB) or human Pepsinogen C (PGC) gene promoters and a reference construct expressing GFP under the CMV promoter (pLLCM-GFP) were prepared (Ohouo *et al*., in preparation) and were used to produce lentiviruses in 293FT cells as described above. Cells were lysed 72 hrs after infection with Passive Lysis Buffer (Promega, Madison, WI) and lysates were analyzed for luciferase activity with the 20/20 n Single Tube Luminometer (Turner Biosystems Inc., Sunnyvale CA) using a Luciferase Assay Kit (Promega). GFP intensity was measured by the BMG FLUOstar Optima plate reader (Imgen Technologies, Alexandria, VA) and used to normalize viral-infection efficiency.

Silencing AR and Smo expression in LNCaP cells by siRNA transfection

The siRNAs specifically targeting human Smo, human AR and control siRNA were purchased from QIAGEN. LNCaP cells were seeded at 70% confluence. siRNAs (40 pM) were mixed with 3 μl of SiLentFect Lipid Reagent for RNAi (Bio-Rad, Hercules, CA) in Opti-MEM I (Invitrogen) for 20 min and this was added to each well in 1.5 ml of medium. Medium was changed 24 hrs after transfection and 72 hrs later, cells were collected for total RNA isolation or lysed in RIPA buffer for Western blot analysis.

Western blot analysis

Cells lysates were assayed for protein and equal amounts of protein were analyzed by Western blot with appropriate antibodies. Each membrane was re-blotted with GAPDH antibody as a control for protein loading. Antibodies were used at the following dilutions: GAPDH at 1:5,000, AR at 1:10,000, and Myc at 1:5,000. Appropriate secondary antibodies conjugated to horseradish peroxidase were used at 1:10,000, and blots were developed by enhanced chemilluminescence reagent (Thermo Fisher Scientific Inc., Rockford, IL). Antibodies to GAPDH or AR receptor (H-280) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The monoclonal antibody to Myc-tag (4A6) was purchased from Millipore (Billerica, MA).

Cell Proliferation WST-1 Assay

Cells were seeded onto a 96-well plate at a density of 5,000 cells/well in CS-FBS media and were maintained for indicated days (media refreshed every 3 days). At appropriate times, 10 μl WST-1 (Roche, Indianapolis, IN) was added to each well and plates were kept at 37°C for two hrs. Color intensity was read at 450 nm (reference wavelength 650 nm) on the SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA)

Co-immunoprecipitation of AR and Gli2 in 293FT cells

Transfection of 293FT cells (2 × 10^6 cells) with AR or Gli2ΔN plasmids was carried out with Lipofectamine-2000. Cells were lysed in a 1% Triton X-100 lysis buffer with protease inhibitor cocktail (Roche) 48 hrs later. Aliquots of extract containing equal amounts of protein were precipitated at 4°C overnight with 50 μl Dynabeads Protein G (Invitrogen) pre-bound with 5 μg appropriate antibodies. Beads were washed by lysis buffer four times and immunoprecipitated proteins were eluted in 2× SDS sample buffer. The elutant was split into equivalent portions and blotted onto 2 membranes for Western blot analysis.

Statistical Analysis

Expression levels determined using RT-qPCR and promoter activity assay data were compared by comparison of the "means", wherein the data graphed or listed in the table represent the Means ± Standard Error (S.E.). The *Student t-Test* (one-tailed, equal variance) was employed for assessing statistical difference (defined as when $p < 0.05$) between data groups.

List of Abbreviations Used

AI: Androgen Independent (Growth); AR: Androgen Receptor; CRPC: Castration Resistant Prostate Cancer; Cyc: Cyclopamine; EtOH: Ethanol; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; Hh: Hedgehog; KLK2: Kallikreinin 2; KLK3: Kallikreinin 3 (Prostate Specific Antigen); IP: Immunoprecipitate; PRB: Probasin; PGC: Pepsinogen C; PSA: Prostate Specific Antigen; Ptch: Patched 1; SHH: Sonic Hedgehog; Smo: Smoothened; Vec: Vector;

Additional material

Additional file 1 Supplemental Figures S1-S3 and the Legends for the Figures.

Additional file 2 Supplemental Tables S1-S2; List of PCR primer sets used in experiments and real-time data for Figure1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MC conducted the majority of bench experimentation involved in this manuscript and assisted in experimental design and manuscript drafting and editing. MAF conducted some qPCR bench experimentation and reviewed the manuscript for accuracy. EL conducted promoter activity assays and reviewed the manuscript for accuracy. RDC conducted some qPCR bench experimentation and reviewed the manuscript for accuracy. MJT prepared some vectors used in the experimentation and reviewed the manuscript for accuracy. MS prepared some vectors used in the experimentation and reviewed the manuscript for accuracy. FV conducted confirmatory experimentation using the same cells in his laboratory and reviewed the manuscript for accuracy. ST conducted confirmatory experimentation using the same cells in the Vacherot laboratory and reviewed the manuscript for accuracy. AdlT provided funding for the confirmatory experimentation in France and reviewed the manuscript for accuracy. RB provided funding for the experimentation in the US, was responsible for experimental design and data oversight and review and drafted and edited the final manuscript. All authors read and approved the final manuscript.

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EXPERT REVIEWS

The hedgehog/Gli signaling paradigm in prostate cancer

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Hedgehog is a ligand-activated signaling pathway that regulates Gli-mediated transcription. Although most noted for its role as an embryonic morphogen, hyperactive hedgehog also causes human skin and brain malignancies. The Hedgehog-related gene anomalies found in these tumors are rarely found in prostate cancer. Yet surveys of human prostate tumors show concordance of high expression of hedgehog ligands and Gli2 that correlate with the potential for metastasis and therapy-resistant behavior. Likewise, prostate cancer cell lines express hedgehog target genes, and their growth and survival is affected by hedgehog/Gli inhibitors. To date, the preponderance of data supports the idea that prostate tumors benefit from a paracrine hedgehog microenvironment similar to the developing prostate. Uncertainty remains as to whether hedgehog's influence in prostate cancer also includes aspects of tumor cell autocrine-like signaling. The recent findings that Gli proteins interact with the androgen receptor and affect its transcriptional output have helped to identify a novel pathway through which hedgehog/Gli might affect prostate tumor behavior and raises questions as to whether hedgehog signaling in prostate cancer cells is suitably measured by the expression of Gli target genes alone.

Keywords: androgen signaling • cyclopamine • Gli • hedgehog signaling • prostate cancer • Smoothened

Hedgehog is a cell signaling pathway that is most noted for its involvement in embryogenesis. Increasingly, however, inappropriate hedgehog signaling activity is viewed as a factor in the development of human malignancy or as a factor involved in the acquisition of aggressive behaviors of already established tumors. Here, we review the putative role(s) of hedgehog signaling in prostate cancer. Prostate cancer is a challenging disease. Aside from the fact that it is the most common malignancy in males [201], it poses a considerable dilemma for public health policy with regards to screening and treatment issues. For example, even though prostate tumors are highly invasive, the majority of afflicted men experience prostate cancer as an indolent disease with a relatively slow growth rate [1]. Since it is usually diagnosed in men older than 60 years of age, the predominance of indolent prostate cancers raises questions regarding the effectiveness of prostate cancer screening efforts that are thought to identify large numbers of patients for whom the treatment may be more problematic than the tumor itself [2–4]. These facts highlight the need to understand the etiology that underlies the widespread occurrence of this disease and to develop a means of selectively diagnosing those individuals with aggressive form(s). Second, despite the abundance of indolent disease, owing to its overall high incidence, prostate cancer remains a leading cause of deaths from cancer in males [201]. This fact underscores the urgent need for better treatments for aggressive disease to reduce mortality. Finally, prostate cancer, in contrast to other human tumors, is distinguished by a remarkable dependency on androgenic steroids. Prostate cancer only arises in androgenically intact males, and, when it has spread beyond the confines of the prostate, is commonly treated by hormone therapies that deplete the patient's circulating androgenic steroid levels [5,6]. Acutely, androgen-deprivation therapies can be very effective and can shrink both primary and metastatic tumors while slowing the growth of residual tumor cells. With chronic use, however, hormone therapies usually prove to be only palliative; patients often recur with more aggressive, therapy-resistant disease referred to as castration recurrent prostate cancer (CRPC). Here the tumor cells are able to grow in a seemingly androgen-independent (AI) fashion, and this is the form of disease that is overwhelmingly associated with mortality from prostate cancer. Despite the behavior of CRPC tumor cells, whose ability to grow in castrated patients mimics that of tumor cells

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that are completely independent of androgens, there is extensive evidence that CRPC cells continue to utilize their endogenous androgen signaling system to drive their growth. Enigmatically, CRPC cells are believed to have acquired the means to maintain androgen signaling even though the systemic milieu of androgens in hormone-treated patients remains at castrate levels [7–10]. Since CRPC cells remain dependent on androgen signaling to grow, this dilemma creates the need to understand the molecular process(es) that enables androgen receptors (ARs) in the CRPC cell to continue to function in the castrate state. With this understanding, one might be able to conceive novel therapies to block the aberrant androgen signaling in CRPC cells and extend the effectiveness of hormone therapies in prostate cancer patients.

The focus here on hedgehog signaling in prostate cancer is driven by a growing body of literature that addresses various aspects of the signaling pathway in prostate tumors or in prostate cancer cells. This literature is plagued by contradictions and controversies, yet, despite these problems, many investigators continue to view the outcomes of their studies as evidence for involvement of hedgehog signaling in prostate cancer development or in progression of prostate tumors to aggressive or therapy-resistant states. In addition, the outcomes of some preclinical studies that showed some striking effects of hedgehog-blocking drugs in animal-based prostate cancer models treatments give strong reason to consider whether these types of therapies might have value for prostate cancer patients, especially those with advanced or therapy-resistant disease.

Abnormal (hyperactive) hedgehog signaling is already established as being a causative factor for the development of certain types of human skin, brain or cartilage-derived tumors (discussed later). Likewise, published literature supports the potential for the involvement of particular aspects of the hedgehog/Gli signaling pathway in other types of solid human tumors [11–16]. Here we will first address the nature of hedgehog signaling in normal and malignant cells and then describe the literature that suggests that hedgehog contributes to human prostate cancer. We will address the controversy as to whether hedgehog acts in prostate cancer exclusively through a paracrine response pathway that mimics hedgehog's involvement in normal prostate development or whether there is any evidence to support a role for a tumor cell-autonomous hedgehog signaling process similar to that found in basal cell carcinoma and medulloblastoma. We will also propose that hedgehog may have an especially important role in promoting progression of prostate cancer to CRPC, at least partly through Gli support of abnormal androgen signaling in tumors of patients subsequent to hormone therapy. While the validation of any potential relationship between prostate cancer and hedgehog signaling or between the aggressive behavior of the CRPC cell and hedgehog/Gli might provide insights leading to improved diagnosis or prognostication of disease behavior, the availability of several small-molecule inhibitors that target hedgehog/Gli at different parts of the signaling pathway suggests that the most useful benefit in exploring this relationship lies in the possibility of using hedgehog-/Gli-blocking drugs to treat patients with advanced or hormone therapy-resistant disease who currently have a very poor prognosis.

Overview of the hedgehog signaling pathway

Hedgehog is considered to be one of the primal cell signaling pathways that regulates cell fate during embryonic development (along with Wnt and Notch) [17–19]. Originally discovered in *Drosophila*, this signaling pathway acquired its name from the distinctive morphology of certain mutant larvae that were characteristically short and stubby with clustered, spine-like denticles that occurred as a consequence of disruption of the normal anterior–posterior segmental pattern formation during embryogenesis [20]. This developmental anomaly was then attributed to a mutation in a drosophila gene termed 'hedgehog' that encodes a secreted polypeptide (ligand) that can initiate hedgehog signaling in receptive drosophila cells [21]. We now know that some form of hedgehog signaling is evolutionarily conserved throughout metazoans and that hedgehog is an important tissue morphogen that participates in the establishment of embryonic polarity and the early patterning of tissues that sets the stage for acquisition of adult tissue structure and function.

Canonical hedgehog signaling is initiated by peptide ligands that are still referred to as hedgehogs, and it serves, at the end point, to activate transcription from the Gli family of transcription factors in responsive cells. Humans have three gene homologs that encode hedgehog ligands (Sonic [Shh], Indian [Ihh] or Desert [Dhh] hedgehog) [22,23]. Shh is the most well studied and is predominant with regards to its more widespread expression throughout different tissues of the body, although all can similarly engage with receptor to initiate the signaling process. Shh is synthesized as a propolypeptide that is processed by a unique autocatalytic reaction in which the C-terminal domain catalyzes a cholesterol-dependent internal cleavage of the pro-form that simultaneously attaches a cholesterol moiety to the cleaved N-terminal domain [24]. The autocatalysis is not sufficient for secretion of the mature ligand; this requires the action of an independent membrane protein referred to as Dispatched [25]. Cholesterol-modified mature Shh is inherently highly hydrophobic and this can limit its diffusion away from the cells that secretes it. The short-acting nature of the hedgehog signaling process in early development helps to promote the formation of patterns in tissues that are based upon ligand diffusion gradients that restrict ligand access to target cells more distal from the hedgehog-secreting cells.

The signaling process proceeds when the mature ligand engages a receptor on a target cell and, for hedgehog, proteins of the Patched (Ptch) family serve this purpose. Ptch proteins are large, 12-pass membrane proteins, and humans encode two homologs [26], Ptch1 and Ptch2, with differing affinities for hedgehog ligands and differential expression in various tissues of the body. A diagram of the general intracellular process that accompanies hedgehog signaling is shown in Figure 1. It should be noted that the brief schema described here is specific for vertebrate-derived cells as evolution from invertebrates was accompanied by modifications that tether the proximal stage of hedgehog signal processing to the subcellular organelle referred to as the primary cilia [27,28]. The integration of hedgehog signaling into the primary cilia provides vertebrate

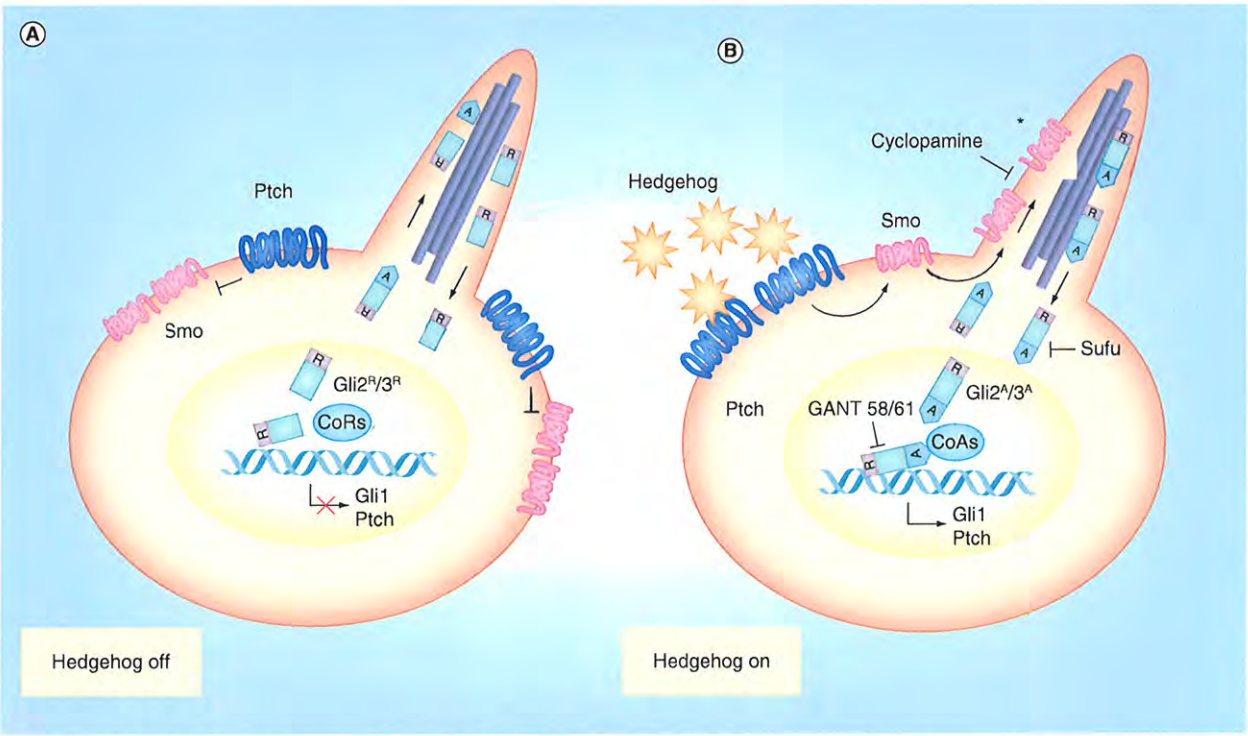

Figure 1. Schematic of the hedgehog signaling process in a target cell. (A) Hedgehog-off. In the absence of hedgehog ligand, Patched gates the movement of Smoothened into the primary cilia and prevents its activation. Without activated Smoothened, Gli2/3 proteins traffic into the cilia where they are processed to remove the C-terminal activation domain. Lacking this domain, the truncated Gli proteins exit the cilia and migrate into the nucleus where they bind to Gli-response elements on DNA and attract a transcription corepressor protein complex that blocks transcription of Gli-target genes. **(B)** Hedgehog-on. Hedgehog ligand binds to Patched and enable Smoothened to traffic into the primary cilia where it becomes activated (*). With activated Smoothened in the primary cilia, Gli protein processing to the repressor form is inhibited, Gli2/3 proteins exit the primary cilia with an activation domain intact and they can enter the nucleus, bind to Gli-response elements on DNA and attract a transcription coactivator protein complex that enables transcription of Gli target genes.
GANT: Gli antagonist; Ptch: Patched; Smo: Smoothened.

cells with unique opportunities to regulate the signaling process, but the linkage also has some important implications for our understanding of hedgehog action in human tumors, as will be discussed later. Likewise, vertebrates have a more complex end-response to hedgehog signaling through evolutionary divergence of the function of the invertebrate Ci transcription factor that is activated by hedgehog onto three different Gli proteins (Gli1, 2 and 3) in vertebrates [29,30]. Since the topic of this treatise is human prostate cancer, hereafter our discussion will focus on the signaling pathway as it is known to function in higher vertebrates (mouse through humans).

Ligand engagement of Ptch relieves repression of the Smoothened (Smo) protein that is required for further signaling. Smo, a seven-pass transmembrane protein of the extended G-protein-coupled receptor family, has an active and an inactivate state that appears to be defined both by its location within the cell (inside or outside of the primary cilia) [31] and by other modifications that may include its ability to capture oxysterols at an active site [32,33]. Smo activation requires two steps that were operationally defined by certain low-molecular-weight compounds that disrupt the activation process [34]. The first step involves the movement of Smo proteins from the plasma membrane and endoplasmic vesicles into primary cilium and here unliganded Ptch acts as a gatekeeper that restricts access of Smo to the primary cilium. Ptch action in this regard is mimicked by the drug, SANT-1, which similarly suppresses ciliary accumulation of Smo, even in the presence of ligand [35,36]. Once in the primary cilia, however, Smo activation requires a secondary step that is also regulated by Ptch, and this activation step is operationally defined by inhibition with cyclopamine or derivatives that allow Smo ciliary accumulation but prevent any further downstream signaling activities. The nature of the secondary Smo activation event remains enigmatic, although it probably involves a conformational shift and/or a change in Smo interaction with other ciliary proteins that are involved in hedgehog signal processing. Regardless of our understanding of this particular event, the presence of active Smo within primary cilia induces a functional change in the organelle that fundamentally alters the manner in which the two dominant Gli proteins, Gli2 and Gli3, are post-translationally processed.

Table 1. Genes that are known to be regulated by Gli binding.

GLI1	[113]
PTCH1	[114]
HHIP	[115]
CDKN2A/p16	[116]
CCND2/cyclinD2	[117]
MYCN/N-myc	[118]
CDK2	[119]
FOXA2	[120]
FOXM1	[121]
FOXE1	[122]
JUN	[123]
NKX2–1/Nkx2.1	[124]
NKX2–2/Nkx2.2	[124]
EGR2/Krox20	[125]
PRDM1/Blimp1	[126]
IGFBP3	[127]
IGFBP6	[117]
SFRP1	[128]
FST	[129]
SPP1/OPN	[117]
RAB34	[124]
RGS4	[127]
BCL2	[130]
EDN2/ET-2	[131]
JUP/PKGB	[117]
FBN2	[127]

As transcription factors with shared function, all Gli proteins have a homologous internal DNA-binding domain that recognizes and binds a cis-regulatory consensus motif on DNA: G–A–C–C–A–C–C–A [37]. The lack of this consensus sequence within or near any given gene does not preclude regulation by Gli since functional nonconsensus binding sites are also described [38]. Given their nature as transcription factors, all Gli proteins also possess activation domains within their C-terminal region that interact with other transcriptional accessory proteins needed for the chromatin remodeling involved in active transcription. Outside of this organizational similarity, however, there are distinct differences between the three homologs that provide the basis for separation of functions in the Gli-mediated transcription process. For one, the proteins encoded by Gli2 and 3 also possess repressor domains within their N-terminus that can preferentially attract corepressor protein complexes to the DNA-binding sites when the activation domain is proteolytically removed [39,40]. It is the relative efficiency with which these two Gli forms are specifically proteolyzed that distinguishes the inactive versus the active hedgehog signaling state. In the absence of activated Smo, Gli2 and 3 proteins traffic into primary cilium where they are modified into repressor forms [41]. This process is initiated by a series of sequential phosphorylations, initiated by protein kinase A and then followed by glycogen synthase kinase-3-β and casein kinase 1. Following phosphorylation, the Gli2/3 repressor forms are generated by proteolysis that may be guided by site-directed ubiquitylation under the control of SCF-β^{TRCP} [42]. The Gli2/3 modification and proteolytic process also requires the presence of certain ciliary kinesin motor proteins to shepherd Glis through the primary cilium and to scaffold the modification complex during the process [27]. The Gli2/3 proteins are also distinguished by their differing contributions to the repressive or activated Gli state of a cell. Whereas native Gli2 is a more avid transcriptional activator than native Gli3, cleaved Gli3 is a stronger transcriptional repressor when compared with cleaved Gli2, so the intensity of the response to hedgehog signaling in a target cell also depends upon the relative expression levels of the two different proteins in that cell. Gli proteins are also targeted for ubiquitylation by the SPOP ubiquitin ligase [43] but it is unclear whether proteasomal degradation under this element is involved in the specific generation of repressor forms rather than their generalized degradation along with Gli1 [44]. In summary, the presence of activated Smo within the primary cilium suppresses the generation of the Gli2/3 repressor forms so they accumulate within the primary cilium in this state. They are also much more likely to exit the cilium with an intact C-terminal domain that is able to enter the nucleus, bind to Gli response elements and capture the chromatin accessory proteins required for an active transcription complex.

Given the importance of hedgehog/Gli signaling for vertebrate development and cancers, there is considerable interest in the targets of active Gli-mediated transcription. Here, it is somewhat ironic that the most well-recognized targets of active Gli transcription include Gli1 and the *Ptch* genes that are mechanistically involved in the signaling process [45]. The nature of the Gli1 protein, which lacks a repressor form, and its short-lived character suggests that it functions mainly as a means for amplifying the output of the hedgehog signaling process once it is initiated. Indeed, this function is consistent with lack of an overt phenotype in Gli1-knockout mice whereas Gli2- or Gli3-knockouts are more severely affected [46,47]. By contrast, Ptch upregulation by active hedgehog provides a means to eventually diminish the activity of the signaling process once initiated, so this action appears to be part of a negative feedback loop controlling hedgehog activity in any given target cell. Other genes reported to be hedgehog targets include hedgehog-interacting protein (*HIP*), whose gene product also feeds back to diminish local signaling activity; cell cycle regulators, including N-myc, cyclin D1 and D2, which may partially explain hedgehog effects on

cell growth; effectors of other developmental signaling pathways including Wnt and Notch ligands and other gene products (bcl-2, FOX transcription factors, bone morphogenetic proteins and follistatin) (Table 1) that are probably associated with differentiated states. In summary, the spectrum of known hedgehog target genes reveals the autoregulating nature of the signaling pathway and explains its obvious involvement in developmental organization of tissues, cell growth and differentiation.

The complex and unique characteristics of the basic hedgehog signaling process, described in the previous section, allows for its regulation at many alternative steps. These include interference with hedgehog ligand processing, release or receptor binding by effectors of sterol biosynthesis [32] or direct interference with mature ligand function by the presence of the HIP protein that binds to ligands and prevents their interaction with receptors [48]. For the target cell, hedgehog signaling can be facilitated by the presence of heparin proteoglycans and lower affinity hedgehog coreceptor proteins that include CDON and BOC [49]. Further downstream, integration of vertebrate hedgehog signaling into the primary cilium means that signal processing requires the activities of numerous ciliar transport proteins to shuttle Gli proteins into and out of the cilium [41,50,51]. Genetic ablation of individual ciliar transport proteins in mice confers phenotypes that are reiterative of mutations in the primary hedgehog regulatory genes. End-stage Gli transcriptional activity is also affected by acetylation or sumoylation of the Gli proteins [52,53]. Finally, Gli transcriptional function is tempered by the presence of the multifunctional SuFu protein that can bind and sequester Gli active forms in the cytoplasm or attract transcriptional corepressors to activator Gli complexes already bound to chromatin [54,55]. The multiplicity of alternative regulatory sites along the hedgehog signaling cascade provides copious opportunities for signal facilitation or interference and it complicates attempts to understand the reason that hedgehog signaling abnormalities strongly underlie certain types of developmental defects or malignancies but not others.

Another notable aspect of hedgehog signaling is its remarkably sensitivity to small-molecule manipulation. This is mainly attributable to the unique nature of the Smo molecule, whose activity is strongly influenced by its association with sterols or other low-molecular-weight compounds. Sterol-like compounds, such as SAG [35] or purmorphomine [56], promote the activated Smo state and these molecules provide an alternative means of antagonizing hedgehog for experimental purposes. By contrast, sterols modeled after the phyto-derived jerveratrum alkaloid, cyclopamine, strongly inhibit Smo activation and these drugs are frequently used experimentally to antagonize hedgehog signaling [57]. The evidence that hyperactive hedgehog signaling plays a role in human cancers has been a tremendous impetus for the discovery of novel compounds that might be used for the purpose of therapeutics and these efforts have resulted in the identification of numerous other low-molecular-weight compounds that can antagonize hedgehog or block Gli action. Since many of these newer compounds are being considered for clinical utilization in oncology, we will assess the spectrum of potential hedgehog/Gli targeting agents in a later section of this article.

Hedgehog in prostate development

Hedgehog's importance as a developmental morphogen for vertebrates is established by the striking developmental anomalies that are associated with abrogation of pathway activity. Loss of Shh, Gli2 or Gli3 function in mutant or knockout mice can be embryonically lethal or result in the death of the neonate shortly after birth associated with developmental defects that include holoproencephaly/cyclopism [58], spinal cord anomalies and other neuronal deficits [59], defects in the formation of the axial skeleton and limbs [60], underdeveloped lungs, and anorectal malformations that include persistent cloaca [61], depending on the severity of the pathway ablation. For males, sexual accessory tissue development is also affected by hedgehog deficiencies and this effect includes hypodevelopment of the prostate gland.

The prostate gland is derived from the embryonic urogenital sinus (UGS) and Shh is expressed in rodent and human UGS and in the buds and ducts that outgrow from it during the process of prostate organogenesis and maturation [62]. Embryonic male mice that lack functional Shh as a consequence of homozygous mutation fail to show the early inductive budding from the UGS that initiates prostate formation [63,64]. However, it is remarkable that inductive budding can be restored simply by supplementing testosterone to the female mouse (*in vivo*) or to isolated mutant male UGS tissues (*in vitro*) [63]. These observations are highly consistent with a requirement of hedgehog for embryonic testicular steroidogenesis and fetal androgenization that guides the inductive phase of male sexual accessory tissue development [65] and they are inconsistent with the idea that any prostate-autonomous hedgehog activity is required for initial organogenesis. Despite the evidence that prostate-autonomous Shh is unnecessary for UGS inductive budding, later embryonic ductal branching and neonatal maturation of the rodent prostate gland is markedly hampered by the lack of Shh, even when supplemental testosterone is provided. Thus, the secondary budding and ductal extension associated with late embryonic and neonatal prostate development is dependent upon prostate-autonomous hedgehog signaling. This developmental situation may be analogous to the regrowth of the regressed prostate in chronically castrated adult rodents that occurs subsequent to testosterone replenishment. Here, cyclopamine treatment was shown to block the androgen-stimulated regrowth of the regressed adult mouse prostate associated with testosterone replacement and this outcome suggests that testosterone replacement induces hedgehog expression needed for prostate ductal expansion in adults [66].

With regards to the nature of the hedgehog signaling process in the developing prostate, *in situ* hybridization and immunohistochemical analyses of embryonic or neonatal mouse and rat tissues tends to localize expression of Shh to the epithelium of the rodent UGS and to the growing tips of the prostate epithelial buds as they invade into the surrounding mesodermally derived mesenchyme [67–70]. By contrast, Ptch and Gli1 (the surrogate Gli target gene) were found to be mainly expressed by UGS mesenchyme or stromal cells adjacent to buds of the developing prostate gland that also stain positive for smooth muscle actin. The striking juxtaposition of ligand expression restricted to the developing

prostate epithelium with receptor and target gene expression that is mainly found in the adjacent mesenchyme shows that hedgehog encompasses a typical paracrine signaling process in the developing prostate that is characteristic of the hedgehog signaling paradigm in other types of developing tissues. There are, however, some reports that also find reduced expression of Ptch1 and Gli1 in the epithelium at bud tips [67] and these findings raise questions that extend to human prostate cancer tissue studies as to whether there may be some autocrine-like hedgehog activity in prostate epithelial cells that manifests exclusively under conditions of rapid growth.

Hedgehog & human cancers

Genetically manipulated mouse models have established an oncogenic role for hedgehog signaling in certain tissues that is remarkably predictive of the occurrence of proven hedgehog-driven tumors in humans. Mice with haploinsufficieny of Ptch1 [71,72], or those with haploinsufficiency of SuFu when combined with p53 haploinsufficiency [73], develop a common spectrum of cutaneous, brain and cartilaginous tumors that corresponds to the specific types of gene anomalies found in basal cell (skin) carcinoma (BCC), medulloblastomas or rhabdomyosarcomas in humans [74]. These types of tumors often have reduced Ptch1 expression associated with loss of heterozygosity at 9q22 (the *Ptch1* locus), which may or may not be associated with a mutation in the remaining *Ptch* allele [75]. Likewise, inactivating mutations in Ptch or SuFu underlie the Gorlin syndrome that predisposes to the development of BCC and/or medulloblastoma [76,77]. Conversely, mutations in the *Smo* gene that confer gain-of-function to the encoded protein are also found in human BCCs and, rarely, in medulloblastomas [78], but exogenous targeted expression of a mutant human *Smo* gene from BCC in transgenic mice similarly induces cutaneous carcinomas, medulloblastomas and rhabdomyosarcomas. Collectively, the reiteration of tumor development in mice by the same genetic aberrations that are found in human tumors of the same class validates the oncogenic nature of unrestricted hedgehog/Gli signaling in this limited subset of tissues. Although these types of genetic lesions confer the appearance of 'autocrine-like' auronomous hedgehog signaling activity in the tumor cell, the abnormal activity is independent of the presence of hedgehog ligands in the tumor microenvironment.

Despite the lack of prevailing evidence for the occurrence of genetic lesions of the type previously described in most other types of solid human tumors, considerable interest remains in the potential roles of hedgehog or Gli, especially for lung, breast, pancreas, colon and prostate carcinoma [12,13,66,79,80]. As will be discussed for prostate cancer, the evidence for association usually encompasses findings of high expression of ligand and/or hedgehog target genes in tumor cells or findings that hedgehog/Gli inhibition, usually by cyclopamine or by Gli expression knockdown, suppresses cell growth *in vitro* or *in vivo* as tumor xenografts in mice. The outcomes of these experiments are often used to support the idea that some form autocrine-like hedgehog signaling is constitutively active in these other types of solid tumor cells. Unfortunately, much less effort is made to establish whether, indeed, any or all of these tumors demonstrate any actual autonomous hedgehog signaling activity, and experimental evidence more strongly implicates that these tumor systems are more influenced through paracrine hedgehog [81], much like in the tissues from which these tumors develop. The situation for tumors other than BCC, medulloblastoma or rhabdomyosarcoma is especially complicated by observations that Gli expression can be regulated independently of hedgehog signaling. TGF-β-, β-catenin- and hyperactive RAS/RAF/MEK/ERK-mediated signaling upregulates Gli expression/activity in tumor cells independent of the presence of hedgehog ligand [70,82,83] and hyperactivity of these alternate cell signaling pathways is known to occur in many different types of cancer. Given the existence of alternative pathways to Gli expression, one should certainly consider whether simple overexpression of Gli, when combined with post-translational processing deficits that fail to generate Gli repressor forms, would be sufficient to explain Gli involvement in them without invoking further upstream hedgehog activities. This is a paradox that we will explore in our focus on prostate cancer.

Finally, the requirement for the primary cilium to process canonical hedgehog signaling in normal cells raises other questions regarding the existence of active hedgehog signaling in cancers that may lack hedgehog-activating mutations since primary cilium are mainly formed on growth-arrested cells whereas cancer cells, especially in culture, usually lack these organelles [84]. The apparent absence of primary cilium in dividing cancer cells then raises critical questions as to how Smo might transition to the active form in cancer cells without activating mutations or evidence of other hedgehog signaling anomalies, and this is an area of research in which we hope to have advances in the coming years.

For those tumor systems that are commonly associated with hyperactive Smo function (due to loss of Ptch function or Smo mutations), there is good reason to consider the testing and use of Smo-targeting agents as anticancer therapeutics. Whereas there was some initial interest in the use of cyclopamine in clinical practice, this agent has critical attributes that make it unfavorable for this purpose and these include its poor availability through nonvenous routes, as well as concerns that it has off-target effects, especially at higher doses [85]. Nonetheless, the remarkable sensitivity of Smo to small-molecule inhibition has encouraged discovery efforts to identify agents that act in a similar way to cyclopamine (by inhibiting Smo activation) with a more favorable clinical profiles. Two contemporary Smo-targeting agents, GDC-0449 and IPI-926, are already subject to clinical testing in human patients [86–88]. Use of GDC-0449 alone in Phase I testing has already demonstrated evidence of objective responses for some cancers [88] and investigators are already considering the possible benefit of combining Smo-targeting drugs with other targered therapeutics for cancers [89] to improve the response. Considering the evidence that many solid tumors benefit from a paracrine hedgehog signaling environment, Smo-targeting drugs could provide an adjuvant therapy to suppress the hedgehog signaling microenvironment of the tumor and open clinical trials for GDC-0049 are actively accruing patients with these alternate solid tumors. Similar effects might be afforded by agents that

target hedgehog ligand processing and interaction with receptors. Robotnikinin, a drug that blocks the interaction of Shh with receptors [90], is of this class. Further down the pathway, the knowledge that Gli activity may be an important factor in tumor biology, independent of hedgehog signaling, has also driven discovery efforts to identify drugs that can block this activity, and the Gli antagonists (GANTs; -58 and -61) [91], and, more recently, the HPI class of drugs [92] that interfere with Gli trafficking and transcription, may have clinical applicability. Finally, the actions of arsenic trioxide, which is being tested as a solid tumor therapeutic [93], may also include the inactivation of Gli function in cancer cells [94,95] so this drug may provide an alternative option for hedgehog targeting in cancers.

Overview of hedgehog/Gli in prostate cancer

The involvement of hedgehog signaling in prostate development forms a foundation for considering whether hedgehog/Gli might have some role in prostate malignancy. This concept received substantial impetus from two early reports of cyclopamine- or Shh antibody-mediated suppression of prostate cancer cell growth *in vitro* and *in vivo* [66,96], and the outcomes of these experimental studies were viewed as evidence for an active autocrine-like hedgehog signaling process in these cell lines. This conclusion should now be reconsidered, especially in light of the concerns discussed previously. A review of relevant literature on this topic with these new perspectives shows remarkable weaknesses in the argument that autocrine hedgehog has an important role in the development of prostate cancer. For one, the genetically altered mouse models that were so useful for establishing a relationship between abnormally hyperactive hedgehog signaling and the development of skin and brain malignancies have not shown any evidence that such aberrations lead to the development of prostate neoplasia or malignancy. It is especially notable that even mice with a prostate (epithelial cell)-specific knock-in of gain-of-function mutated *Smo* gene that is oncogenic when expressed in skin, brain or cartilage, demonstrated no evidence for any type of prostatic pathology [97]. In fact, at this time, the only report of an animal (mouse) model that develops prostate cancer from a hedgehog manipulation involves the direct introduction of a constitutive Shh expression vector into mouse prostate by tissue electroporation [98]. These adult mice uniformly developed prostate intraepithelial neoplasia that rapidly progressed to metastatic prostate adenocarcinoma over time. While this outcome is remarkable and does support the potential for unrestricted hedgehog in prostate cancer development, the electroporation technique lacks the cell-targeting specificity to show that overexpression of Shh in the tissue was acting through any autonomous effect on the prostate epithelium and the outcome could easily be a consequence of an unrestricted hedgehog stimulation of the prostate stroma that destabilizes the tissue, leading to cancer.

With regards to actual human prostate tumors or prostate cancer cell lines, there are no studies identifying abnormalities in *Ptch* or *Smo* genes similar to those found in BCC or medulloblastoma. Allelic loss of 9q22 and/or *Ptch* mutations are not described for this disease, and reports of *Smo* mutations are similarly lacking, although there is no reason to believe that a screening effort to identify the presence of *Smo* gene lesions was ever suitably undertaken for prostate cancers. Perhaps the only description of hedgehog-related gene aberrations in prostate cancer involves the finding of two prostate tumors with loss-of-function mutations in the *SuFu* gene [99]. These mutations were found in a small cohort of tumors in which SuFu immunostaining was also notably reduced. Of further note, the human *SuFu* gene lies in a chromosomal region (10q24.32) that encompasses an area of frequent allelic loss in prostate cancer. While these coincidences are insufficient to establish a more widespread pattern involving loss of SuFu in prostate cancer development or progression, they do at least establish precedence to seek further evidence that changes in the *SuFu* gene or in reduced expression of the encoded protein may be a factor in the disease.

Given the paucity of evidence for disruption of genes encoding intermediate hedgehog signaling elements in prostate tumors, what can be learned regarding hedgehog involvement in prostate cancer from gene-expression studies of human prostate tumor specimens? Unfortunately, varied outcomes from the numerous published efforts that describe and quantify expression of hedgehog-related genes in prostate tumors challenge efforts to provide consensus on this issue. There are general concerns that the so-called 'normal' regions of human prostate specimens that are available for study might be affected by the common prostate benign disease states that might also invoke abnormal hedgehog responses [100] and this raises questions regarding the establishment of normal prostate basal expression levels for any of these genes. Approaches that assess RNA levels by *in situ* hybridization are complicated by the uneven cellular architecture of a prostate tumor (in which the cellularity of the stroma can appear sparse compared with the adjacent epithelium) and this might account for the conflicting findings of Gli1 RNAs localized to benign and malignant prostate epithelium in one study [96] versus selective expression in the stroma around tumors in another [100]. Likewise, quantitative reverse-transcriptase PCR approaches that involve bulk extraction from tumor tissues are complicated by the comixtures of tumor and benign stromal cells in the specimens that complicate analysis, so it is difficult to comment on observations based on this approach. *In situ* immunohistochemical approaches using antibodies against hedgehog-related proteins offer the potential for higher detection specificity, with appropriately validated antibodies, but this approach suffers from a diminished ability to quantify outcomes.

With these considerations, the observations of Azoulay *et al.*, who evaluated hedgehog ligand expressions in a cohort of 231 different prostate tumors, some of which were obtained from patients treated with hormone therapies, were remarkable [101]. They described a significant correlation between high(er) expression of Shh in malignant epithelium with tumor grade or metastasis to lymph nodes. Sheng *et al.* evaluated 55 different tumors for multiple parameters, including Shh, Ptch1 and HIP expression (the latter being surrogate Gli targets) [99]. Here, the investigators described elevated immunostaining for Shh in malignant epithelium compared with benign epithelium, with increased Ptch1

and HIP expression in tumor cells that correlated with tumor grade. Narita *et al.* characterized Gli2 expression in 21 localized prostate tumors from androgenically intact patients compared with 14 BPH specimens and described a significant increase in Gli2 immunostaining in the malignant compared with the benign epithelium [102]. Overall, the most validated studies appear to support that expression of Shh in prostate tumor cells tends to increase as a function of tumor grade (and potential for metastasis), that prostate tumor cells tend to show higher Gli2 expression and productive Gli transcriptional activity compared with their benign counterparts, and that Gli2 expression rises further in therapy-resistant tumor cells. These outcomes then suggest that a more active hedgehog signaling microenvironment around a prostate tumor in conjunction with increased tumor cell Gli activity is associated with aggressive cancer cell behaviors that include potential for metastasis and therapy resistance. The outcomes do not, however, sufficiently establish that there is any direct association between the overexpression of hedgehogs in more aggressive prostate tumor cells and the enhanced Gli expression/activity that is also reported to be found in prostate tumor cells.

What can be learned from study of human prostate cancer cell lines? Use of some of the lines as xenografts in mice has revealed additional features of hedgehog effects that provide insight to the *in vivo* situation. For one, overexpression of the ligand (Shh) in LNCaP cells significantly increased the *in vivo* tumor growth rate of tumor xenografts compared with control xenografted LNCaP cells [100]. This indicates that the higher expression of Shh found in prostate tumors of higher grade has the potential to impact on prostate tumor growth rates. The fact that similar tumor growth acceleration can also be achieved by comixing unmodified LNCaP cells with UGS mesenchymal cells lacking Gli3 repressor ($Gli3^{-/-}$) [103] certainly shows that signaling action through the paracrine pathway, at least has the potential to significantly contribute to the hedgehog-mediated tumor growth acceleration effect. Finally, observations that the treatment of mice with Sbh-targeting antibodies, cyclopamine, Gli2-targeting antisense oligotides [102] or Gli-blocking drugs of the GANT class significantly inhibit the growth of prostate tumor cell xenografts (CWR22rv1 or PC3 cells) identifies the potential for use of hedgehog-/Gli- suppressive therapeutics for prostate cancer treatment, although, to date, no actual clinical trials using hedgehog-blocking approaches for prostate cancer patients have been reported.

Evaluation of prostate cancer cell lines in a culture setting provides a means of testing for the presence of any autocrine-like hedgehog signaling activities in the cells and whether activation or interference at various sites of the signaling pathway affects hedgehog target genes or cell growth outside the influence of a paracrine signaling environment. For the most commonly utilized human prostate cancer cell lines (LNCaP and derivatives, DU145, PC3 or CWR22rv1) grown in culture, Shh, Gli1/2 and other key hedgehog target genes (*Ptch1*, *Gli1* and *HIP*) are, in general, reported to be expressed in most, although there is wide variability in individual levels among the different lines. The most comprehensive survey for basal expression of hedgehog effector genes (mRNAs) in the common prostate cancer cell lines was published by Zhang *et al.* [104] and this survey showed no overt concordance between the expression of hedgehog ligands (Shh or Ihh) and the basal expression of hedgehog surrogate targets (Gli1 and Ptch1), except for HIP; no concordance in the expression of the different hedgehog target genes in any of the lines; and, finally, no concordance between the expression of any of the Gli RNAs with Ptch or HIP expression. Likewise, the common prostate cancer cell lines were shown to be refractory to treatment with recombinant Shh protein or to adenoviral transduction of a mutated *Smo* gene [104]. Collectively, these findings do not lend support to the presence of a basally active or even an accessible endogenous hedgehog signaling process in any of the cell lines evaluated based upon the idea that the activity of the pathway is solely indicated by expression levels of known Gli target genes. Conceptually, the lack of evidence for intermediate hedgehog signaling activity in prostate cancer cell lines based upon these considerations then challenges the idea that cyclopamine treatment, which invariably affects the growth of these cells *in vitro*, is functionally targeting an active hedgehog signaling process guided by Smo activation. Here again, the failure of cyclopamine to suppress expression of hedgehog target genes (*Ptch1*, *Gli1* or hedgehog reporter) in the cultured prostate cancer cell lines [104,105] provides additional support for the lack of intermediate signaling pathway activity in the cancer cell lines, as long as one can be reassured that pathway activity is exclusively reflected by the relative expression levels of Gli target genes. As we will discuss later, this may not always be the case, at least in prostate cancer cells that express the AR protein. Regardless of these concerns, there are prominent indications that Gli proteins, at least, play some role in the growth potential of prostate cancer cells. Suppression of Gli1 or Gli2 expression using gene-specific si-/shRNAs or antisense oligotides significantly reduced their *in vitro* growth rate and invasiveness [102,106,107] and increased the propensity for apoptosis. The mechanism supporting the presence of active Gli in these cells remains uncertain.

Hedgehog/Gli & androgen cross-talk in prostate cancer

The androgen signaling pathway that is so central to prostate cancer is remarkably interactive with other cell-signaling pathways. These interactions often occur at the level of the AR protein where AR activity can be increased under stimulation of signal-activated protein kinases [108] or by interaction with other pathway-regulated transcription factors, as is exemplified by β-catenin in the Wnt signaling pathway [109]. These signaling interactions are especially notable when they support promiscuous androgen signaling under low androgen conditions, as this allows for the possibility that the secondary signaling pathway is a druggable target for suppression of CRPC. Recently, we learned of a unique bi-directional interaction between androgen and hedgehog signaling in prostate cancer cells. The nature of this interaction is defined by the androgenic milieu of the prostate cancer cell and it appears to have the potential to produce a more active paracrine hedgehog microenvironment of a tumor in hormone-treated patients and, at the same time, promote promiscuous activity of the tumor cell AR that enables androgen-independent growth.

The nature of this interaction is first defined by evidence that hedgehog ligands are androgen-repressed genes in prostate cancer cells. Using the example of cultured prostate cancer cell lines that express AR and are growth-responsive to the presence of androgens in their medium, expression of mRNA encoding hedgehogs was found to be markedly increased by a switch to androgen-depleted medium [101,110]. For LNCaP cells, androgen depletion upregulated Shh by 30,000-fold, and the expression of Ihh and Dhh was also upregulated, although not to this extent. This response was not unique to LNCaP; other androgen-responsive prostate cancer cells demonstrated similar changes in hedgehog expression when treated in this manner. Moreover, the changes in Shh mRNA were accompanied by similar increases in the expression and release of the mature Shh polypeptide with intact paracrine function, shown by the finding that the conditioned growth medium from androgen-deprived, but not androgen-supplemented, LNCaP cells was able to elicit a hedgehog response from mouse fibroblasts [110]. The clinical relevance of these *in vitro* findings is supported by the previously mentioned survey of hedgehog expression in human prostate tumors [101], which included a group of tumors obtained from patients who had been adjuvantly treated with hormone therapy prior to surgery. Here, hormone treatment essentially doubled the percentage of tumors found to express Shh or Dhh in malignant epithelium compared with untreated tumors.

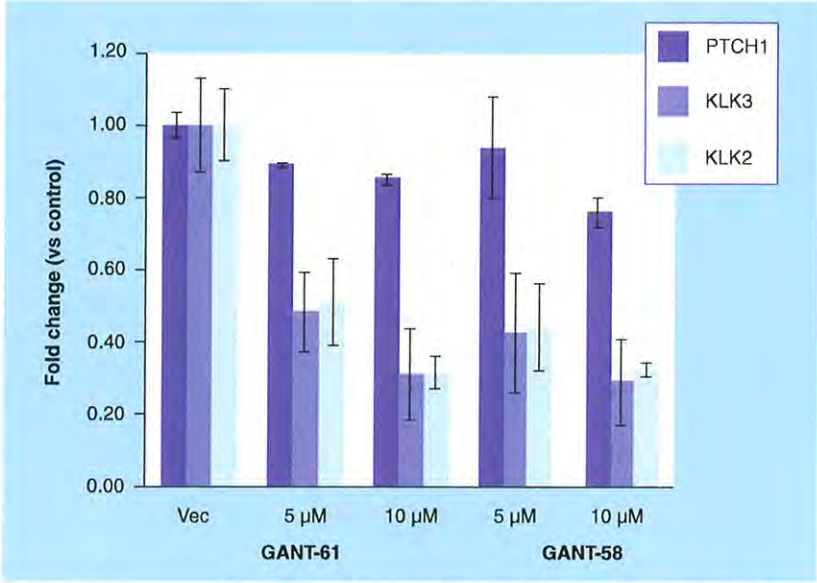

Figure 2. Suppression of androgen-dependent gene expression in androgen-deprived prostate cancer cells by the Gli-suppressing drugs GANT-58 and 61.
LNCaP cells were seeded onto plates overnight in RPMI-1640 medium containing 10% fetal bovine serum, then switched to an androgen-depleted medium as was previously described [110] containing dimethyl sulfoxide vehicle only (Vec) or GANT-58 or GANT-61 dissolved in dimethyl sulfoxide at the indicated concentrations and was incubated for an additional 72 h. RNAs were then extracted from these cells and were assessed by quantitative real-time PCR for the expression of KLK2 or KLK3 (prostate-specific antigen), as described, and the results are normalized to expression of GAPDH in the same samples. Each point indicates the results from triplicate cultures.
GANT: Gli antagonist.

In addition to its effect on hedgehog expression, androgen deprivation was also shown to significantly increase the expression of Gli2 mRNA in LNCaP and other prostate cancer cell lines [110]. Considering the fact that this action was also accompanied by upregulated Ptch1 expression, one might reasonably suppose that the coincidental increases in Shh, Gli2 and Ptch expression represent the activities of an autocrine hedgehog cascade initiated by androgen deprivation. Indeed, since cyclopamine treatment conferred a small but significant decrease in Ptch expression under this condition [110], the outcome further supports the idea that androgen deprivation is associated with a reawakening of some autocrine-like activity in prostate cancer cells. Arguing against this is the fact that Gli1 mRNA expression was significantly decreased by this same condition and it is difficult to explain the striking discordance in the response of these two foremost Gli target genes (*Gli1* and *Ptch1*), unless one invokes different regulatory mechanisms for each gene operating in the confines of the androgen-deprived cell. This remains an unresolved issue, which is further complicated by the evidence that active hedgehog/Gli affects androgen signaling in prostate cancer cells.

The notion that hedgehog/Gli also affects androgen signaling originated from observations of a dose-dependent effect of cyclopamine on the expression of androgen-regulated genes [111] in LNCaP and other prostate cancer cells. Here, cyclopamine treatment was shown to specifically suppress expression of kallikrein-related peptidase (KLK)2, KLK3 and PGC in androgen-deprived, but not androgen-supplemented, LNCaP cells, whereas it further induced expression of Shh, which represents an androgen-repressed gene. Cyclopamine had similar effects on expression of luciferase reporters from androgen-dependent promoter elements in these cells. These effects were most pronounced in androgen-deprived cells in which Gli2 levels were elevated. Whereas questions remain regarding cyclopamine's specificity and its mechanism of action in prostate cancer cell lines, a similar outcome was observed after knockdown of Smo expression using siRNA. The fact that this effect also involves elements of hedgehog (Gli activity) downstream of Smo is indicated by the ability to suppress androgen-dependent gene expression by specific reduction of Gli2 expression or by treatments with the Gli inhibitor drugs, GANT-58 and -61 (Figure 2). Here, it is notable that the GANT drugs did not significantly affect expression of Ptch1. Finally, in the reverse paradigm, exogenous expression of Gli1 or Gli2 in androgen-deprived prostate cancer cells not only increased the expression of androgen-dependent genes but also enabled these cells to grow in an androgen-deficient

medium [111]. Collectively, the outcomes of these studies support the presence of a Smo-dependent signaling process, at least in androgen-deprived prostate cancer cells, which cross-talks with the androgen signaling pathway through Gli to affect androgen-regulated gene expression. The involvement of Gli in the regulation of androgen-dependent genes suggests that the effect might be mediated by some form of Gli/AR interaction. Indeed, coimmunoprecipitation or two-hybrid analysis shows that Gli1 or Gli2 can directly bind to the AR protein [111,112]. Based on these reports, the Gli proteins may have AR coactivation functions that contribute to androgen signaling, especially in the androgen-deprived state.

Expert commentary

Since its discovery in 1980, we have learned a great deal regarding the mechanistic aspects of hedgehog signaling and its role in vertebrate development. In addition, we have come to accept its causative role in some forms of human cancer. The association of hedgehog signaling abnormalities with human tumors has spurred the development and testing of clinically useful drugs that target hedgehog/Gli, some of which are already demonstrating efficacy as cancer therapeutics. However, our current knowledge regarding the role of hedgehog/Gli signaling in prostate cancer remains relatively limited to the notion that the disease, once acquired, benefits from a paracrine hedgehog signaling influence that is driven by the production of hedgehog ligands by prostate tumor cells that act on adjacent benign (stromal) cells and feeds back to the tumor, stimulating tumor cell growth and metastasis. With regards to prostate tumor cells themselves, there is little evidence for the types of mutations or defects in hedgehog signaling genes that are found in human skin and brain tumors, but this does not rule out the possibility that genetic anomalies in other hedgehog-regulating genes might be a factor in the disease. Furthermore, the indications that tumor Gli activity has a role in advanced/aggressive disease are relatively convincing, but there are many reasons to be skeptical as to whether the hyperactive Gli is a consequence of tumor cell-autonomous hedgehog signaling through an active autocrine-like signaling process. Recent findings that the hormone therapies used to treat advanced prostate cancers have the potential to augment the paracrine hedgehog signaling microenvironment of a prostate tumor, in conjunction with the findings that Gli proteins can interact with AR and confer androgen-independent growth behavior on human prostate cancer cells, support the consideration of hedgehog-blocking drug therapy used in conjunction with hormone therapy for patients with advanced/therapy-resistant disease. While drugs that target Smo are now clinically available and should be effective for suppression of hedgehog paracrine effects, the questions regarding the source of Gli activity in prostate cancers suggest that drugs that specifically target Gli may be more useful than Smo blockers alone as they might act on the paracrine hedgehog tumor microenvironment, as well as on tumor-autonomous Gli, allowing effective disease control when used as an adjunct to hormone therapy.

Five-year view

The availability of clinically tested drugs that target hedgehog/Gli suggests that clinical trials of hedgehog therapeutics for prostate cancer are likely to advance faster than the resolution of critical research issues that might guide the most effective application of these therapies. With this perspective, the field requires research advances in three focus areas to help resolve the hedgehog/Gli contribution to prostate cancer. The first involves further exploration of the hedgehog paracrine effect in prostate cancer. Here, the knowledge that hedgehog expression is induced by inflammation, as is common in the prostate, suggests that hyperactive paracrine hedgehog could explain the link between prostate inflammation and prostate carcinogenesis and identify a role for hedgehog in prostate cancer etiology. Development of this concept should encompass surveys of human prostate tissues to correlate the presence of prostate inflammation with hedgehog expression in adjacent epithelium and involve attempts to create a mouse model of prostate cancer by conditional targeted overexpression of Shh in the adult prostate epithelium. Further work is needed to identify the paracrine hedgehog-induced substances that are produced by hedgehog-stimulated tumor support cells that induce prostate tumor growth. The second area of focus involves addressing the source of Gli hyperactivity in prostate cancer cells and defining the extent to which increased tumor-autonomous Gli activity is associated with progression to aggressive (metastatic) disease. We have described the considerations leading many to questions about whether intermediary hedgehog signaling is even possible in prostate cancer cells and the evidence that Gli expression is not solely dependent upon an active hedgehog signaling process in prostate or other solid tumors. Can we then attribute Gli overexpression in prostate cancer to some specific alternate signaling process that increases with disease progression? The third area of research involves expanding our understanding of the cross-talk between hedgehog/Gli and its consequences for androgen signaling in prostate cancer cells. Research in this area should attempt to dissect the interaction sites of Gli with AR and define the extent to which the alternate Gli forms can coactivate or corepress AR transcription. More work is needed to resolve the question of the extent to which Gli is hijacked by the AR in prostate cancer cells and whether Gli activity is best measured in these cells by expression of androgen-regulated, rather than Gli-regulated, genes. Finally, the evidence that reduction in Smo expression in prostate cancer cells affects the expression of androgen-regulated genes also suggests the need to better understand Smo function in the context of the prostate cancer cell.

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Key issues

- Hedgehog signaling regulates the activities of Gli transcription factors.
- Paracrine hedgehog signaling guides developmental growth of the prostate gland.
- Gene anomalies that dysregulate hedgehog signaling are causative of some forms of human cancers.
- These gene anomalies are rarely found in prostate tumor cells.
- Aggressive prostate tumor behaviors correlate with high expression of hedgehog ligands and Gli2.
- Overexpression of Sonic hedgehog increases the growth of human prostate cancer xenografts in mice, and treatment of mice with hedgehog/Gli inhibitors strongly inhibits tumor xenograft growth.
- Knockdown of Gli1 or Gli2 expression reduces prostate cancer cell growth *in vitro*.
- Gli proteins (1 and 2) bind to the androgen receptor and affect androgen signaling in prostate cancer cells.
- Overexpression of Gli2 allows androgen-independent growth of prostate cancer cells *in vitro* and may be a factor in the development of castration-resistant prostate cancers.

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• of interest
•• of considerable interest

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