The pro-cancer function of soluble guanylate cyclase alpha-1 (sGC1) in prostate cancer progression

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The Pro-cancer Function of Soluble Guanylate Cyclase Alpha-1 (sGCα1) in Prostate Cancer Progression

by

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A large body of evidence demonstrated that AR involves in the development of CRPC despite of the hormonal treatment. One common scenario is AR overexpression, which is observed in 22%-30% of castration-resistant prostate cancer (CRPC). Thus, we postulate that expression of a hyperactive AR would be sufficient to drive hormone-dependent prostate cancer cells to castration-resistant ones. To test this hypothesis, we generated LNCaP cell lines that stably overexpress a VP16-AR hybrid protein. Interestingly, VP16-AR cells yielded androgen-independent cell proliferation, while under the same growth conditions the parental LNCaP cells exhibited only androgen-dependent growth, suggesting that hyperactive AR is sufficient to transform prostate cancer cells to CRPC. Our lab has identified soluble guanylate cyclase alpha-1 (sGCα1) as a novel androgen-regulated gene involved in prostate cancer cell proliferation. The
classical biological function of guanylyl cyclase (GC) is to catalyze cGMP synthesis in nitric oxide (NO) signaling. Our data demonstrated that the sGCα1 effect on prostate cancer cell growth is independent of the classical mediators of NO signaling, suggesting that sGCα1 acts via a novel signaling pathway. Interestingly, a gene expression profile from primary prostate tumors demonstrated that the expression of sGCα1 and p53 is highly correlated. Importantly, immunohistochemical results showed that p53 is colocalized with sGCα1 in the cytoplasm of localized prostate tumor specimens. Overexpression of sGCα1 led to p53 cytoplasmic accumulation, whereas knockdown of endogenous sGCα1 resulted in redistribution of p53 in the nucleus, suggesting that sGCα1 modulates p53 subcellular localization. Our data show that sGCα1 represses p53 transcriptional activity distinct from its guanylate cyclase function. PCR array analysis indicated that sGCα1 regulation of p53 affects the expression of certain p53-regulated genes involved in apoptosis. Interestingly, LNCaP cells that stably overexpress sGCα1 exhibited a marked induction of Akt kinase, a mediator of PI3K signaling that is very important in the proliferation of prostate cancer cells. Thus, we propose two mechanisms by which sGCα1 mediates prostate cancer progression: first, sGCα1 compromises p53 transcriptional activity by retaining p53 in the cytoplasm and blocking apoptosis; the second is that sGCα1 induces activation of Akt and its downstream targets, leading to enhanced cell proliferation. In view of the pro-cancer roles of sGCα1 in mediating prostate cancer progression, we attempted to disrupt sGCα1 activity using interacting peptides. Four peptides, designated as Peptide A, B, C, and D, were synthesized, based on four known sGCα1-interaction domains found on sGCβ1, its heterodimerization partner mediating NO signaling. Interestingly, only Peptide A had a rapid and strongly
cytotoxic effect on both androgen-dependent and androgen–independent prostate cancer cells. Interestingly, Peptide A had little or no effect on cancer cells lacking sGCα1 expression, suggesting that this peptide acts via endogenous sGCα1. In support of this, immunocytochemistry analysis showed a cytoplasmic co-localization of Peptide A and sGCα1. Importantly, an LNCaP xenograft mouse model showed that the treatment with Peptide A-8R completely stopped the tumor growth. Therefore, Peptide A may be a novel therapeutic agent against prostate cancer.
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Introduction

Androgens and Androgen Receptor in Normal Prostate

Androgens play a pivotal role in the development and maintenance of the prostate gland and secondary male characteristics, as well as male sexual differentiation and spermatogenesis (1). Androgens, a type of steroid hormone, are made mostly in the testes (>95%), and a small portion of them is synthesized in the adrenal glands. Testosterones are the major type of androgen circulating in the blood and converted into dihydrotestosterones, a more active form, in the prostate by the 5α-reductase (1).

Androgens cannot work alone but exert their effect by binding to the androgen receptor (AR) and together sustain the normal prostate development and function (2).

Androgen receptor, a member of nuclear receptor, is comprised of distinct functional and conserved domains, designed as A-E (see Figure A below).

Figure A: Structural and function domains of the human androgen receptor indicated as A–E. Numbers above the bars refer to the amino acid residues starting from the N-terminus (N) to C-terminus (C). NTD, DBD and LBD represent N-terminal domain, DNA binding domain and ligand binding domain, respectively. AF-1 and AF-2 are two distinct domains that harbor transcriptional activation function.
The A/B region, commonly referring to the N-terminal domain (NTD) is composed of a transcriptional activation function-1 (AF-1), which is responsible for most of the AR transcriptional activity. The C region, a DNA binding domain (DBD), consists of 2 zinc finger motifs that bind to androgen response elements (AREs) of target genes. The D region, a hinge connecting the DBD and the LBD, contains the nuclear localization signal (NLS). The E region, also known as the ligand binding domain (LBD), is comprised of the transcriptional activation function-2 (AF-2), which has much weaker transcriptional activity than AF-1 (Reviewed in 3).

Without the androgen, AR, primarily localized in the cytoplasm is accompanied with heat shock proteins (HSPs) (Reviewed in 4). This cytosolic AR protein may be degraded in a proteasome-dependent manner (Reviewed in 4). Upon the ligand activation, the cytoplasmic AR is disassociated from HSPs, causing AR homodimerization, phosphorylation and subsequent translocation to the nucleus (Reviewed in 4). AR binds to AREs in promoter region of its targets, followed by the assembly of the transcription initiation complex, resulting in the initiation of transcription (5).

**The AR Signaling in the Castration-Resistant Prostate Cancers**

The prostate is one of the most important AR target organs. Alteration of AR activity can lead to prostatic diseases, including prostate cancer, which is the second leading cause of cancer deaths among males in United States. Based on the progression or development of the prostate cancer, this disease is classified into the androgen-dependent, also referring to the hormone-sensitive and the androgen-independent, also
known as castration-resistant prostate cancers (CRPC). Because, in the early stage, the proliferation and the survival of prostate cancer cells depends on androgens (Reviewed in 3), the androgen-sensitivity property makes the disease treatable by androgen-ablation therapy, a type of procedure whose purpose is to reduce androgen production. Importantly, when prostate cancers relapse from the initial hormone depletion treatment, tumors gain the ability to survive in the limiting androgen condition. The latter stage is known as CRPC. Interestingly, despite withstanding the androgen-ablation therapy, CRPC remains the dependency of the AR signaling (6). Various mechanisms by which AR sensitizes CRPC in low serum androgen will be reviewed in the following content.

**AR Gene Mutations**

The somatic mutations in AR gene have been documented in great detail on the Androgen Receptor Mutations Database ([http://androgendb.mcgill.ca/](http://androgendb.mcgill.ca/)). Some of the AR mutations lead to disrupt AR functions and others increase AR activity. For example, the Gain-of-Function mutations in the AR gene may result in prostatic diseases, including prostate cancer. These mutations may result from enhanced the ligand-activated AR binding ability or AR-coactivator interactions. One of the first reports studied AR mutations in the androgen-dependent LNCaP cells, which harbor a T877A mutation within the ligand-binding domain (LBD). As a result, this AR mutant broadens ligand selections (7). Instead of androgens, AR can be activated promiscuously by other steroid hormones, such as glucocorticoids, progesterones and few anti-androgens.

In addition to the LBD mutation, another AR mutation (E231G), which is found in the highly conserved N-terminal region, increases AR interactions with coactivators,
such as ARA70. Importantly, transgenic AR-E231G mice rapidly developed prostatic intraepithelial neoplasia (PIN) and underwent invasive and metastatic disease in 100% of mice, implying that the altered of AR interaction with co-regulators plays an important role in prostate carcinogenesis (8). In summary, these data suggest that Gain-of-Function AR mutations increase its transcriptional sensitivity in the low androgen microenvironments.

**AR Amplification/Overexpression**

Edwards et al (2003), described the AR gene and AR protein levels from matched paired hormone-sensitive and hormone-refractory tumors. Interestingly, the gene and protein expression from this patient cohort indicated that AR protein levels statistically were higher as diseases progress from hormone-sensitive to hormone-refractory stage (9). A mouse xenograft model was used to demonstrate that the AR overexpression can convert prostate cancer growth from hormone-sensitive to hormone-refractory (10). Furthermore, Sawyers and colleagues studied the global gene profiling of prostate cancer xenograft models, a panel of seven isogenic hormone-sensitive and hormone-refractory human prostate cancer xenograft pairs. Interestingly, only AR gene expression was overexpressed in hormone-refractory and the AR protein showed a modest increase (10). Importantly, the mouse xenografts that exogenously overexpressed a modest level of AR provided a resistance of the anti-androgen therapy and converted bicalutamide into a weak agonist (10). These data suggest that the AR overexpression confers prostate cancers a castration resistant capacity.
**Overexpression of AR Coactivators**

AR directly interacts with a number of coactivators to enhance AR-dependent transcriptional activity. Among those, the p160 family including glucocorticoid receptor-interacting protein 1 (GRIP1), the steroid receptor coactivator-1 (SRC-1) is the best-studied. The p160 coactivators interact with the AF-1 or the LBD of the AR, thereby enhancing ligand-dependent AR transcription of target genes (11). Furthermore, another type of coactivator alters the ligand binding specificity of AR. This class belongs to the AR associated proteins (ARA) family, such as ARA55 and ARA70. For example, ARA70 has been shown to enhance AR transcriptional activity in the presence of androst-5-ene-3beta,17beta-diol (Adiol), a precursor to testosterone, suggesting that ARA70 broadens the AR ligand binding specificity (12). Therefore, the overexpression of AR coactivators may promote prostate cancer against the castration.

**Adrenal Androgen Synthesis**

Since androgen depletion therapy can reduce serum androgens to castrate levels (<5%), Balk and his colleagues reported that adrenal androgens synthesized within tumors, also known as intratumoral androgen synthesis, compensate for loss of androgens (13). Few key enzymes, which play an important role in catalyzing adrenal androgen synthesis, were amplified in tumors. For example, the AKR1C3 gene, which is responsible for the intracellular conversion of adrenal androgens to testosterone, was overexpressed in the 33 androgen-independent metastatic tumors as compared to 22 primary tumors (13). Additionally, a recent review by Ang et al argued that CYP17 overexpressed in the advanced prostate cancers catalyzes the synthesis of androgens.
(Testosterones) from adrenal glands and prostatic tumors (14). The conversion of testosterones to dihydrotestosterones depends on 5α-reductase (5αR) isoenzymes, 5αR1 and 5αR2, which are encoded by two distinct genes SRD5A1 and SRD5A2, respectively. Interestingly, 5αR1 plays more important role than 5αR2 does in prostate tumors. For example, a microarray analysis indicated that the SRD5A1 gene is overexpressed in bone marrow metastases from castration-resistant prostate cancer (13). Taken together, the intratumoral androgen synthesis may offer castration-resistant prostate cells an alternative source to obtain androgens and adapt to an androgen deprivation condition.

**AR Cross-Talk with Cytokines and Growth Factors**

The AR protein in the CRPC tumors can be activated/regulated in an androgen-dependent manner or by cell surface receptors. Among these receptors, the epidermal growth factor receptor (EGFR) or HER2 is the most important and widely studied. Indeed, a clinical report from Mellado’s Laboratory demonstrated that 69 patients with high levels of the HER2 extracellular domain in serum are associated with a poor clinical outcome in the castration-resistant prostate cancer (15). Sawyers and colleagues (2004) described that the HER2 signaling is essential in the regulation of the AR protein stability and optimizing AR transactivation functions at limiting concentrations of androgens (16). In addition to the HER2, Src, a nonreceptor tyrosine kinase, is involved in the regulation of AR transactivation. Asim et al (2008), showed that Src was overexpressed in androgen-independent prostate cancer cells as well as in a transgenic mouse model. Blocking Src kinase signals using PP2, a specific inhibitor compromised AR transactivation, suggesting that Src signals subsidize AR function in CRPC (17).
Additionally, Src kinase cross-talks with AR and facilitates AR the tyrosine phosphorylation, thereby increasing AR genomic activity (18). Collectively, these data suggest that the crosstalk between AR and Src protein kinases increases AR transcriptional function in the minimal androgen conditions.

**Akt Signaling Pathway and Prostate Cancer**

Akt, also referred to as the protein kinase B (PKB) is an important protein kinase in the control a variety of cellular functions (19). Akt can be activated by insulins, and various growth factors in a phosphoinositide 3-kinase (PI3K)-dependent fashion. The activation of Akt relies on its at two specific regulatory sites, Thr308 and Ser473 (20). The kinase involved in the phosphorylation of the Thr308 and Ser473 sites is 3-phosphoinositide-dependent kinase (PDK1) (21). Activated Akt promotes cell survival or blocks apoptosis by phosphorylating or inactivating its downstream targets, including Bad (22), forkhead transcription factors (23), c-Raf (24) and caspase-9. Another Akt downstream target is that GSK-3β, a cytoplasmic serine/threonine protein kinase is also involved in various signaling pathways important for development, such as Wnt/β-catenin, Hedgehog, Notch and insulin (25,26). Moreover, GSK-3β has been shown to modulate the expression of cyclinD1, a positive regulator of cell cycle progression (Reviewed in 26).

The Phosphatase and tensin homolog (PTEN), a negative regulator in the Akt signaling pathway prevents the cell from undergoing uncontrolled growth. However, PTEN is frequently mutated or deleted in a number of human cancers, including prostate cancer. Importantly, the mutation of PTEN is found in 30% of primary prostate cancer.
(27) and 63% of metastatic prostate tumors (28). Recently, Sawyers and colleagues described that transgenic TMPRSS2-ERG mice were not sufficient to develop the prostatic intraepithelial neoplasia (PIN) but with PTEN loss, suggesting that the cooperativity between the Akt pathway activation and the ERG aberration in the prostate tumor initiation (29).

*Therapeutics Targeting the AR Signaling in CRPC*

Current androgen ablation therapy is to lower androgen production by blocking androgen synthesis using a gonadotropin-releasing hormone (GnRH) agonist and disrupting AR ligand binding ability by administering antiandrogens, such as bicalutamide and flutamide (Reviewed in 45). However, these approaches cannot entirely block androgen signaling. A large body of evidence shows that prostatic tumors that survived androgen depletion retain the dependence of AR signaling despite of limiting serum androgens (Reviewed in 45). Thus, new strategies targeting the AR signaling pathway have been developed and many of them are being tested in clinical trials in CRPC, as shown in the Figure B below. Evolving treatments for CRPC will be discussed below.
Figure B: Therapeutic targets of the AR pathway. 1) Adrenal androgen synthesis. 2) Conversion of testosterone to dihydrotestosterone. 3) Ligand activation of AR. 4) AR-regulated gene expression. 5) HSP-mediated AR stability. 6) RTK signalings. 7) Src kinase.

**Androgen Reducing Agents**

After androgen ablation therapy, remaining serum androgens and an increased intratumoral androgen synthesis may be sufficient for CRPC growth for patients undergoing hormone-depletion therapy, especially when the pathway is under the regulation of overexpressed and mutated AR. Under these conditions, the tumor response can be described as entering the antiandrogen withdrawal stage (30). To lower androgens, new strategies that target the intracellular testosterone synthesis as well as the conversion from testosterone to dihydrotestosterone are employed, as shown Pathway 1 and 2 (Figure B).
Because the antiandrogen withdrawal results in diminution of prostate-specific antigen (PSA) in only 15% to 30% of androgen-independent prostate cancer (AiPCa) patients, ketoconazole, a nonspecific CYP17 inhibitor, is commonly utilized for the adrenal androgen ablation. 27% of patients who received ketoconazole had a markedly reduced levels of serum PSA as compared with 11% of patients underwent the antiandrogen withdrawal. However, there was no difference with or without ketoconazole in the patient survival rate (31). It should be noted that the inhibition of ketoconazole is not only on the testosterone synthesis but also cortisol levels (32).

Unlike ketoconazole, Abiraterone, a selective CYP17 inhibitor, reduces serum testosterone without affecting on cortisol levels. Abiraterone targets both adrenal and the intracellular androgen synthesis. A clinical report showed that with simultaneous hormone deprivation and abiraterone treatments, the levels of testosterone and adrenal androgens were substantially reduced (33). However, the serum testosterone level can be blocked by abiraterone in a short term but be recovered with long-term treatments, suggesting that another backup mechanism bypasses CYP17-mediated the testosterone synthesis (34).

The conversion from testosterone to dihydrotestosterone depends on two isoforms of 5α-reductase, 5αR1 and 5αR2, which are encoded by SRD5A1 and SRD5A2, respectively. Because SRD5A2 is the predominant form in the benign prostate, finasteride, a SRD5A2 specific inhibitor, can reduce PSA and the size of the prostate in the benign prostatic hyperplasia (BPH) (35). In contrast, as the prostatic disease progresses to intraepithelial neoplasia (PIN) and prostate cancer, the SRD5A1 expression increases and the SRD5A2 expression decreases (36). Dutasteride, a potent inhibitor for
SRD5A1 and SRD5A2, suppresses tumor growth in animal models (37). Surprisingly, administration of Dutasteride resulted in an increase testosterone levels. This may due to a feedback mechanism. Thus, a combination of the castration and dutasteride may enhance the efficacy against prostate cancers (38).

**Second Generation Antiandrogens**

Antiandrogens exert their effects by competing with natural androgens, resulting in the inactivation of ligand-activated AR genomic functions, (Pathway 3). Flutamide, and bicalutamide (casodex) are commonly used for this purpose (39). While these antiandrogens can prolong prostate cancer patients’ life; whereas, most of them suffer a rapid recurrence of prostatic tumors. Worst of all, these compounds can act as agonists in advanced prostate cancers (39,40). Hence, the second generation of antiandrogens is in the clinical development.

MDV-3100, developed by the Sawyers’ Laboratory showed a 10-fold higher AR binding affinity than bicalutamide. As a result, MDV-3100 inhibits AR transcriptional activity by blocking AR nuclear translocation and recruitment of AR coactivators. Importantly, in a Phase I/II clinical trial, 43% (13/30) of patients showed a greater than 50% decline of serum PSA levels (41).

BMS-641988, another antiandrogen inhibitor targeting the AR signaling, is invented by Bristol-Myers Squibb Company. This compound exhibited a higher binding affinity to AR and better reduction of AR transactivation as compared to bicalutamide. In two mouse models, BMS-641988 showed a continuous inhibition on xenograft tumors
survived from the bicalutamide treatment. It’s undergoing Phase I clinical trial in CRPC (42).

**HDAC Inhibitors**

Prior to the transcription, Histone acetyl transferases (HATs) tends to destabilize chromatin structure by adding acetyl groups to histones and turns DNA into a transcriptionally active state (43). The activator, such as AR is first recruited to the promoter region of its target gene and followed by the transcription initiation complex, resulting in initiating of the transcription. The counterpart of HAT is the histone deacetylase (HDAC), which silences DNA by removal of acetyl residues from histones. HDAC inhibitors (HDACis) were designed to reverse this DNA silencing, therefore reactivating dormant tumor suppressors, such as p53, and HSP90 (43). Recently, HDACi, such as SAHA (vorinostat) and LBH589, are being tested in clinical studies (Pathway 4).

Sawyers and colleagues exhibited that HDACi decreases AR protein levels resulting from blocking the AR transcript synthesis. Additionally, HDACi interferes with AR activity by disrupting the assembly of the transcription initiation complex after AR binds to the enhancers of target genes, leading to inhibiting of AR-regulated genes expression (43). This novel therapy is used to suppress AR transcriptional activity when conventional hormone ablation therapies fail.
**HSP90 Inhibitors**

17-Allyamino-17-demethoxygeldanamycin (17-AAG) is an inhibitor of the HSP90 chaperone protein (Pathway 5) (44). Inhibition of HSP90 by 17-AAG leads to the proteasomal degradation of multiple client proteins including HER2, Akt, and both mutant and wild-type AR as well as induces retinoblastoma (Rb)-dependent cell cycle arrest in prostate cancer xenografts (44). This approach offers an alternative to target the AR signaling in CRPC.

**Kinase Inhibitors**

The growth and metastasis of tumors are affected by multiple cellular pathways such as, VEGF and EGFR/HER2 signalings. Therefore, the therapeutics targeting EGFR/HER2 signalings, includes kinase inhibitors and monoclonal antibodies (Pathway 6) have been investigated clinically in CRPC but clinical studies did not showed a promising future (45). One of the possible mechanisms by which CRPC gaining the resistance to EGFR inhibitors is PTEN loss, which is commonly found in prostate cancer. The Loss-of-Function of PTEN leads to continuously activation of the PI3K-Akt pathway and the activation of intracellular molecular effectors independent of EGFR/HER2 (46).

SRC kinase, a nonreceptor tyrosine kinase, promotes AR transcriptional activity and invasion of the androgen-independent prostate cancer (17). Dasatinib (Pathway 7), an BCR-ABL kinase inhibitor, is approved for the treatment of chronic myelogenous leukemia (CML) (47). It is also used as SRC inhibitor in CRPC patients and is undergoing Phase II clinical trials. (45).
p53 and Carcinogenesis

p53, known as “the guardian of the genome”, plays many important roles in maintaining the integrity of DNA, which requires p53 nuclear localization (53). The p53 nucleocytoplasmic shuttling is mediated by a bipartite nuclear localization signal (NLS) located at amino acid residues 305-321 (48) and two nuclear export signal (NES) located within the 11-27 and 340-350 leucine-rich regions (49,50). Mdm2, an E3 ligase that binds to NES regions of p53, induces p53-Mdm2 nuclear export, leading to cytoplasmic degradation (51,52,53).

Under normal circumstances, p53 is maintained at low concentrations and negatively regulated by Mdm2 that mediates nuclear export and cytoplasmic degradation via the ubiquitin/proteasome pathway (54). Under stress conditions, such as exposure to carcinogens, UV light, or DNA damage, p53 is stabilized, forming a tetramer that shuttles into the nucleus and activates its downstream targets that promote DNA repair, cell cycle arrest, or apoptosis (55).

DNA damage triggers series of cell responses, such as activation of H2AX, CHK2, ATM/ATR kinase. These kinases disrupt Mdm2-mediated ubiquitination of p53, resulting in p53 nuclear accumulation (56). Hence, this stabilized p53 can induce the DNA repair machinery that includes nucleotide excision repair (NER), base excision repair (BER), nonhomologous end-joining (NHEJ) and homologous recombination, showing that p53 is important for DNA repair (57).

Cell cycle arrest occurs in response to various stress conditions and provides time for DNA repair. p21 is an important p53 target that acts as a negative regulator of the cell cycle. When undergoing cell cycle arrest, p21 inhibits the activity of CDK2 and
CDK4 complexes, which control the transition from G1 to S phase and leads to cell cycle withdrawal at the G1 checkpoint, resulting in cell cycle arrest (58,59).

p53 can also mediate apoptosis through either intrinsic (mitochondrial) or extrinsic (death-receptor) pathway (60). In the intrinsic pathway, p53 transactivates the proapoptotic members of Bcl family that facilitate cytochrome c release from mitochondria into cytosol. The cytosolic cytochrome c binds to an adaptor protein called Apaf-1, which then binds to procaspase-9. Procaspase-9 cleaves itself to become caspase-9, which triggers a caspase cascade, leading to apoptosis (61). In the extrinsic pathway, Fas/CD95, and DR5 genes are direct targets of p53 that transcriptionally controls the expression of death receptors. An activated death receptor recruits intracellular adaptor proteins that bind to procaspase-8, which cleaves and activates caspase cascade that leads to extrinsic cell program cell death (62-65).

Importantly, p53 is frequently mutated, occurring in approximately ~50% in human cancers. However, the frequency of p53 mutations is only ~20% in primary prostate cancer, but p53 mutations are commonly found in advanced prostate cancer. Importantly, while p53 deficiency is not sufficient for causing cancer, the combination of PTEN and p53 knockouts potently promote prostate tumorigenesis (66). Loss of PTEN leads to down-regulation of NKX3.1, resulting in decreased p53 protein stability, thereby increasing cell metabolism, growth and survival (67). Together, the mutual interplay of these two tumor suppressors reveals the significance of prostate carcinogenesis.

Another mechanism to inhibit p53 activity is to sequester the protein in the cytoplasm, thereby blocking its classical tumor suppressing roles in cancers (68). Furthermore, this nuclear exclusion inactivates the tumor suppressing functions of wild-
type p53 in breast cancer (69). Increasing evidence suggests that this inactivation of p53 in the cell cytoplasm may be a crucial mechanism for tumorigenesis. For example, Jun activation domain-binding protein 1 (Jab1) is a nuclear exporter and may be responsible for p53 cytoplasmic sequestration, leading to Mdm2-dependent degradation (70). Additionally, p53-associated parkin-like cytoplasmic protein (Parc) functions as a cytoplasmic anchor that sequesters p53 within the cytoplasm. Interestingly, knockdown of Parc expression using RNAi leads to nuclear translocation of p53 and apoptosis (71). Furthermore, nuclear p53 interacts with ligand-activated glucocorticoid receptor (GR), resulting in cytoplasmic redistribution. This interaction complex undergoes a Mdm2-dependent protein degradation (72). This cytoplasmic inactivation of p53 may be important for tumors that contain wild-type p53

**AR-Regulated Gene Expression**

Because AR signaling remains important and AR overexpression is frequently observed in CRPC, identification of genes that are regulated by AR may help us to understand how the AR genomic action facilitates development and progression of advanced disease. Therefore, an array of AR-regulated genes have been identified, including those genes encoding proteins involved in metabolism (*HPGD*), transport/trafficking (*FKBP5*), protease activity (*PSA* and *TMPRSS2*), transcription regulation (*NKX3.1*), signal transduction (*LIFR*), proliferation/differentiation (*MAF*) and so on (Reviewed in 73).

For example, prostatic specific antigen (*PSA*), also known as *hKLK3* is one of the classical AR-regulated genes and therefore is widely used as diagnostic marker in early
detection of prostate cancer (Reviewed in 74). Moreover, Chinnaiyan and colleagues were the first to describe the gene fusion of the 5′ untranslated region of \textit{TMPRSS2} to \textit{ERG} or \textit{ETV1} in prostate cancer tissues. Because \textit{TMPRSS2} gene is a downstream target of AR, these fusion products, especially \textit{TMPRSS2-ERG} becomes a useful molecular diagnostic marker for prostate cancer (75).

\textit{NKX3.1}, another AR-regulated gene, is selectively expressed in the prostate and in the testis. It encodes prostate-specific homeobox protein, which functions as a transcription factor. Hence, \textit{NKX3.1} has been shown to play important roles in the normal prostate development and prostatic diseases by transcription regulation. (76). Additionally, our lab has discovered several novel AR-regulated genes involved in prostate tumorigenesis. For instance, \textit{ETV1} modulates metastasis by turning on matrix metalloproteinase (MMP) genes (77) and the multidrug resistance-associated 4 (MRP4) makes prostate tumors insensitive to anti-cancer drugs (78). The most interesting AR-regulated gene is \textit{sGCα1}, which is discussed below.

\textbf{The Role of sGCα1 in Prostate Tumorigenesis}

Soluble guanylyl cyclase α1 (\textit{sGCα1}) functions as a novel AR-regulated gene involved in prostate cancer cell proliferation (79). \textit{sGC} is an α1 and β1 heterodimeric protein and the major downstream target of nitric oxide (NO), a simple diatomic gas that plays important roles in signaling transduction in both animals and plants (80). When activated by NO, \textit{sGC} catalyzes the synthesis of cGMP (3′, 5′-cyclic guanosine monophosphate) that activates a variety of proteins, including ion channels, protein kinases and phosphodiesterases in the classical NO pathway (81,82). Two reports also
suggest some novel roles for NO signaling in tumor initiation and progression (83). For example, in ovarian cancer, p53-dependent apoptosis is suppressed by endogenous sGC/cGMP activity (84). In breast cancer, oxygen-mediated regulation of cell invasiveness is associated with elevated levels of sGCα1 (85). Our recent studies have demonstrated that the disruption of sGCα1 expression significantly impedes the growth of both androgen-dependent and -independent prostate cancer cells (79). Interestingly, overexpression of sGCα1 alone is sufficient for promoting cell proliferation, suggesting that sGCβ1 is not required (79). Importantly, sGCα1 expression is highly elevated in prostate cancer in comparison to normal and benign prostatic hyperplasia (BPH) (79). This pro-proliferative action of sGCα1 in prostate cancer is distinct from the conventional NO signaling pathway, inferring that sGCα1 acts via novel interacting partners and novel signaling pathway(s). Hence, my research project is to discover such signaling pathway(s).
Materials and methods

Cell Culture and Androgen Treatment

LNCaP and PC-3 cells were grown as previously described (Cai et al., 2007b). CWR22Rv1 cells were cultured in RMPI-1640 medium with 10% FBS and 50 mg/ml Gentamicin (Gibco), and VCaP cells in the same medium without Gentamicin. For androgen (R1881) treatment, LNCaP and VCaP cells were grown in medium containing 2% FBS extracted with dextran-coated charcoal (DCC). 48 hrs later, ethanol or 1 nM R1881 was added to the cells. After an additional 48 hrs, the cells were subjected to semi-quantitative RT-PCR, real time-PCR, Western blotting, or immunocytochemistry.

siRNA Transfection

siRNA of 50 nM final concentration was transfected into LNCaP or VCaP cells using X-tremeGENE (for MTT proliferation assay) or lipofectamine 2000 (for other assays) as described previously (Cai et al., 2007b). The sGCα1 siRNA and Luciferase GL3 control siRNA (Dharmacon) are described previously. p53 siRNA was purchased from Santa Cruz.
**Reporter Assay and Plasmid Transfection**

LNCaP cells were grown to 80-90% confluence in RPMI-1640 with 10% FBS. After 48 hrs, medium was replaced with serum-free medium and the cells were transiently transfected with the p53-Luc reporter plasmid, expression plasmid for sGCα1, sGCα1-GFP, sGCα1(D531A), sGCα1(D531A)-GFP, or p53, and 0.5 µg pCH110, used to measure transfection efficiency (Shenk et al., 2001). The p53-Luc plasmid was kindly provided by Dr. Andrei Gudkov and contains three p53-responsive elements. For those experiments studying NO signaling, chemicals affecting this pathway were added 24 hours later. The transfection was performed by using Lipofectamine 2000 and luciferase assays performed as described (Cai et al., 2007b).

**Adenovirus Infection**

LNCaP cells were infected with 20 MOI adenovirus expressing rat sGCα1 protein as described (79).

**Semi-Quantitative RT-PCR and Real-Time PCR**

Total mRNA was isolated and subjected to semi-quantitative RT-PCR and real-time PCR analyses as described (79). The RT-PCR upstream and downstream primers, respectively, used for each gene were: p53AIP1, 5’- GGACTGGCC CTAACAACAAA-3’ and 5’-TTCACTGCAGGGACTTACCC-3’; Survivin, 5’-GGACCACCCGCATCTCTACAT-3’ and 5’-GACAGAAAGGAAAG CGCAAC-3’; p53, 5’-
GGCCCACTTCACCGTACTAA-3’ and 5’-GTGGTT TCAAGGCCAGATGT-3’; sGCα1, 5’-AGCAGTGTGGAGAGCTGGAT-3; and 5’-CTGATCCAGAGTGCAGTCCA-3’; PCBP4, 5’-TTTCTGGGTTATGGGAGCAG-3’ and 5’-TGGGACTCCAGGATAACAG-3’; p21, 5’-GA CACCACTGGAGGGTGACT-3’ and 5’-CAGGT CCACATGGTCTCTCCT; GAPDH, 5’-CGACCACCTTGTCAAGCTCA-3’ and 5’-AGGGGAGATTCAGTGTGG TG-3’.

**Western Blotting and Cell Fractionation**

Western blotting was performed as described (79) using primary antibodies against sGCα1 (Cayman Chemical), p53 (Santa Cruz Biotechnology), β-Actin (Abcam), hRARa (Santa Cruz Biotechnology), or MLK3 (Santa Cruz Biotechnology).

LNCaP cells from confluent 60-mm dishes were harvested and washed once with cold PBS. 10% of the cells were saved as input and the remaining portion was divided into nuclear and cytosolic fractions using Nuclear/Cytosol Fractionation Kit (MBL International). The fractions were then subjected to Western Blotting to measure sGCα1 and p53 protein levels.

**Immunoprecipitation**

IP experiments were done with either endogenous proteins in LNCaP cells or proteins expressed in E. coli. For the first, whole-cell extracts from LNCaP cells grown in 100-mm dishes were prepared and subjected to IP following a commercial protocol
(from Santa Cruz) using Protein A/G plus Agarose (Santa Cruz). IP antibodies used were the anti-p53 (Santa Cruz) and anti-sGCα1 (Cayman Chemical).

For the E. coli expression, sGCα1, sGCβ1, or p53 were expressed in BL-21 cells and individual extracts were prepared and followed the protocol from Stratagene and subjected to IP experiments either as individual extracts or mixtures using the anti-p53 (Santa Cruz), anti-sGCα1 (Cayman Chemical), or anti-sGCβ1 (Cayman Chemical) antibodies.

**Gel Filtration**

Gel filtration chromatography was utilized to purify the protein complex associated with sGCα1. Cytosolic LNCaP cell extract was fractionated through a S-300 Sephacryl column (HiPrep) using high performance liquid chromatography (HPLC) (BioRad Bio-Logic Duo Flow HPLC), following Gel Filtration, Principles and Methods (GE Healthcare). Fractions were collected and subjected to Western to detect the elution profile of sGCα1 and p53.

**cGMP Synthesis Assay**

PC-3 cells were transfected with sGCα1 or sGCα1(D531A) mutant and sGCβ1 (Origene). After 48 hrs, the cells were harvested and subjected to a cGMP assay to measure intracellular cGMP levels by following the manufacturer’s protocol (Assay Designs).
**Immunocytochemistry and Immunohistochemistry**

Immunocytochemistry and immunohistochemistry were used to study the levels and subcellular localization of sGCα1 and p53 proteins in LNCaP cells and prostate tumors obtained from the Cooperative Human Tissue Network (CHTN), respectively. FITC labeled sGCα1 antibody (1:100 dilution; Santa Cruz Biotechnology) and TRITC labeled p53 antibody (1:100; Santa Cruz Biotechnology) were used for immunocytochemistry using the Fluorescence staining protocol adapted from Vector Laboratories.

**p53 Signaling Pathway PCR Array**

LNCaP cells were cultured in a 60 mm-dish and transfected with control siRNA or sGCα1 siRNA. After two days of incubation, mRNA isolated using the Trizol reagent (Invitrogen) was subjected to human p53 Signaling Pathway PCR Array (SA Biosciences) to measure the expression of p53-regulated genes. All data analysis followed the procedure provided by SA Biosciences.
Expression of a Hyperactive Androgen Receptor Leads to Androgen-Independent Growth of Prostate Cancer Cells

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Running Head: Hyperactive AR induces androgen-independent cell growth.

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ABSTRACT

Cellular changes that affect the androgen receptor (AR) can cause prostate cancer to transition from androgen-dependent to androgen-independent, which is usually lethal. One common change in prostate tumors is over-expression of the AR, which has been shown to lead to androgen-independent growth of prostate cancer cells. This led us to hypothesize that expression of a hyperactive AR would be sufficient for androgen-independent growth of prostate cancer cells. To test this hypothesis, stable LNCaP cell lines were generated that express a VP16-AR hybrid protein, which contains full-length AR fused to the strong viral transcriptional activation domain VP16. This fusion protein elicited as much as a 20-fold stronger transcriptional activity than the natural AR. Stable expression of VP16-AR in LNCaP cells yielded androgen-independent cell proliferation, while under the same growth conditions the parental LNCaP cells exhibited only androgen-dependent growth. These results show that expression of a hyperactive AR is sufficient for androgen-independent growth of prostate cancer cells. To study the molecular basis of this enhanced growth, we measured the expression of sGCα1, a subunit of the soluble guanylyl cyclase, an androgen-regulated gene that has been shown to be involved in prostate cancer cell growth. Interestingly, the expression of sGCα1 is androgen-independent in VP16-AR-expressing cells, in contrast to its androgen-induced expression in control LNCaP cells. RNAi-dependent inhibition of sGCα1 expression resulted in significantly reduced proliferation of VP16-AR cells, implicating an important role for sGCα1 in the androgen-independent growth of these cells.
INTRODUCTION

Prostate cancer is an epithelial-derived cancer (Cussenot, et al. 1994) that involves the action of androgens and androgen receptor (AR), a ligand-dependent transcription factor (Chang, et al. 1988). This liganded AR is essential for the growth and survival of primary tumors (androgen-dependent prostate cancer cells) (reviewed in Jenster 1999). The primary treatment for prostate cancer is aimed at disrupting the androgen-AR interaction (Eder, et al. 2000; Jiang, et al. 2004). In the prostate, two main forms of androgens are secreted: testosterone and dihydrotestosterone (DHT). Although DHT is the more potent form, both forms are involved in the development of both the prostate and male secondary sex characteristics (Singh, et al. 2000). The liganded AR forms a homodimer and binds to the androgen-responsive elements (AREs) found on the promoters of target genes to regulate gene expression (reviewed in (Beato and Klug 2000; Kastner, et al. 1995; Mangelsdorf and Evans 1995; Mangelsdorf, et al. 1995; Thummel 1995).

The AR is important not only in the development of prostate cancer, but also in its progression to the usually lethal androgen-independent form (reviewed in Jenster 1999). Several mechanisms for the transition of prostate cancer from a hormone-sensitive to a hormone-refractory form have been proposed. First, the AR pathway is bypassed by activation of oncogenes and/or inactivation of tumor suppressor genes (Jenster 1999). In some cases, this can lead to the loss of AR expression by either gene deletion/mutation or DNA methylation, as exemplified by several prostate cancer cell lines (Russell and Kingsley 2003). Another mechanism is ligand-independent activation of AR, which can be mediated by several growth factors, including keratinocyte growth factor (KGF),
epidermal growth factor (EGF), and interleukin-6 (IL-6) (Roznovanu, et al. 2005; Smith, et al. 2001). AR mutations that broaden ligand specificity are common in late-stage prostate cancer (Brinkmann, et al. 1995; Marcelli, et al. 2000), and recent evidence has shown that such mutations can not only initiate prostate cancer but also cause it to progress to a hormone-refractory stage in transgenic mice (Han, et al. 2005). Lastly, AR gene amplifications are observed in 28-30% of recurring tumors following anti-androgen therapy, leading to increased levels of AR and presumably to a more sensitized and active AR pathway (Jenster 1999). In support of this, it was recently demonstrated that AR over-expression is sufficient to convert androgen-dependent prostate cancer to an androgen-independent form (Chen, et al. 2004).

Since these earlier studies demonstrate that hormone-independent prostate cancer cells exhibit increased AR expression and, thus, transcriptional activity (Kokontis, et al. 2005) and AR function has been linked to the development and progression of androgen-independent prostate cancer form, we sought to study the direct effect of AR transcriptional activity on the growth of prostate cancer cells. Hence, we hypothesized that expression of a hyperactive AR can convert the growth of prostate cancer cells from androgen-dependent to androgen-independent. Our data show that fusion of the activation function of VP16 (Tiley, et al. 1992) to the AR renders a receptor that has significantly higher transcriptional activity than the native AR. Stable expression of VP16-AR in prostate cancer cells yielded androgen-independent cell proliferation. Importantly, this androgen-independent growth correlated with androgen-independent expression of soluble guanylyl cyclase α1 (sGCα1), an AR-regulated gene that has been previously implicated in hormone-refractory prostate cancer cell growth (Cai, et al. 2005).
2007a). These data show that mechanisms that elevate AR transcriptional activity can lead to hormone-refractory growth of prostate cancer cells and suggest that androgen-independent expression of sGCα1, and perhaps other AR-regulated genes, may be responsible for the cell growth.

MATERIALS AND METHODS

Plasmids

To make VP16-AR/pCI-Neo, hAR/pSG5 (Bubulya, et al. 1996) digested with BamHI/DraI and inserted into pcDNA3.1/Zeo(+), generating hAR/pcDNA3.1/zeo(+). The VP16 transactivation domain (amino acids 414-490) (Berger, et al. 1990) was synthesized by polymerase chain reaction (PCR) and inserted into the BamHI site of AR/pcDNA3.1/zeo(+), making VP16-hAR/pcDNA3.1/zeo(+). VP16-hAR was digested out of VP16-hAR/pcDNA3.1/zeo(+) with NheI and NotI and inserted using these same restriction sites into pCI-Neo, yielding VP16-AR/pCI-Neo.

The reporter plasmids MMTV-CAT, PSA-CAT, and hKLK2CAT have been previously described (Bubulya et al. 1996). Transfection efficiency was standardized according to β-galactosidase activity, which comes from the transfected pCH110 plasmid (Shenk et al. 2001). The PSA-Luc (Shenk et al. 2001) and ARE4-Luc (Chen et al. 2006a) luciferase reporters have been previously described.

Transient Transfection and Reporter Gene Assays
Transient transfections with reporter gene plasmids of LNCaP and stable cell lines were carried out using the CaPO₄ method as described before (Chen et al. 2006b). For all transfections, cells were transfected with 2 mg pCH110 and enough pTL1 to bring the final plasmid amount to 10 mg per dish (Chen, et al. 2006b). Whole cell extracts were prepared and subjected to CAT (Bubulya et al. 2001) or luciferase assays (Cai et al, 2007a). CAT assay results were quantified using the Bio-Rad Molecular Imager FX and Quantify One software.

Generation and Growth of Stable Cell Lines

Generation of stable LNCaP cell lines using a pCI-Neo-based plasmid has been already described (Chen et al. 2006b). Several cell lines were generated that express VP16-AR, from which three lines (V94, V134, and V149) were selected for study in this work. C14 is a control LNCaP cell line stably transfected with an empty pCI-Neo vector (Chen et al. 2006b). For the experiments described here, these LNCaP stable cell lines were grown in RPMI 1640 complete medium (Sigma) containing 1-5% fetal bovine serum (FBS from Hyclone), depending on the experiment, and 0.1mg/ml neomycin.

Cellular Proliferation Assay

C14 or V94 cells were seeded at 3 x 10⁴ cells per well in 24-well plates, with 1 ml RPMI 1640 phenol-free medium containing either 1% or 5% DCC FBS (Hyclone). After two-day incubation, cells were treated with ethanol (vehicle control) or 1 nM R1881. After 0, 3, and 6 days incubation period, the MTT assay was used according to the manufacturer’s instructions (Sigma) to determine cell number. In the case of Casodex
treatment, 10 µM Casodex was together with ethanol or R1881. Note that cells with siRNA transfection were treated with R1881 24 hrs after transfection, as previously (Cai et al. 2007a). 50 nM control or sGCα1 siRNA was used (Cai et al. 2007a).

_Semi-quantitative RT-PCR and Real-Time Quantitative-PCR Analyses_

The Trizol Reagent (Invitrogen) was used to isolate total RNA from C14, V94, V134, and V149 cells and subjected to either semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) or real-time quantitative-PCR (Q-RT-PCR) analysis using Sybr Green (iSCRIPT from Bio-Rad). The PCR reactions were carried out utilizing the following upstream and downstream primers, respectively, for each gene: PSA, 5’-GCAGCATTGAACCAGAGGAG-3’ and 5’-CCCATGACGTGATACCTTGA-3’; sGCα1, 5’-AGCAGTGTGGAGAGCTGGAT-3’ and 5’-CTCATCCAGAGTGCA GTCCA-3’; TMPRSS2, 5’-CACTGTGCATCACCTTGACC-3’ and 5’-ACACACCG-ATTCTCAGCCTC-3’; and GAPDH, 5’-CGACCACCTTTGTCAAGCTCA-3’ and 5’-AGGGAGATTCCAGTGTGGT-3’. Note that GAPDH was used as a control for RNA amount.

_Western Blotting_

Cell extracts were prepared by boiling cells for 5-10 min in SDS-Sample Buffer (Chen et al. 2006). Nitrocellulose blots were probed with antibodies against sGCα1 (Cayman Chemical), AR (Upstate), VP16 (Clonotech), CDK1 (Cell Signaling), CDK2 (Santa Cruz Biotechnology), CDK4 (Santa Cruz Biotechnology), Cyclin D (Santa Cruz
Biotechnology), Rb (Cell Signaling), PSA (Biodesign), p27 (Santa Cruz Biotechnology), sGCα1 (Cayman Chemical), b-actin (Abcam), and b-tubulin (Chemicon) and then developed using the Chemiluminescence Detection system from Amersham. Cells were treated with 1 nM R1881, with or without 10 mM Casodex or 10 mg/ml cycloheximide (CHX). Note that b-actin or b-tubulin was used as a control for protein amount.

**Immunocytochemistry**

LNCaP cells were grown in 2% DCC-FBS on glass cover slips and treated with either ethanol (carrier) or 1 nM R1881 for two days. The cells were then fixed with formaldehyde and incubated with Image-iT FX signal enhancer (Molecular Probes). After rinsing with PBS, cells were incubated with an anti-AR antibody (from Santa Cruz Biotechnology). This was followed by staining with secondary antibody (Molecular Probes) and development with Antifade Reagent, proLong Gold (Molecular Probes), Images were viewed using a confocal microscope (Olympus Fluoview IX70).

**RESULTS**

*Expression of VP16-AR results in markedly higher AR transcriptional activity*

Since studies have demonstrated that hormone-independent prostate cancer cells exhibit increased AR expression and transcriptional activity (Kokontis et al. 2005) and AR function has been linked to the development and progression of androgen-independent prostate cancer form (Chen et al. 2004), we hypothesized that expression of
a hyperactive AR can convert the growth of prostate cancer cells from androgen-dependent to androgen-independent. In order to test this hypothesis, a hyperactive AR was generated through the fusion of the activation domain of VP16, a strong transcriptional activator (Tiley et al. 1992), with a full length AR protein to produce the fusion protein VP16-AR (Fig. 1A). In transient transfection experiments, this fusion protein exhibited strong androgen-dependent transcriptional activity on different promoters, 3-6-fold stronger than endogenous AR in LNCaP cells (Fig. 1B). As shown in Fig. 1C, VP16-AR protein is expressed in this experiment, but this expression is significantly weaker than the expression of endogenous AR. In addition and interestingly, androgen has no effect on the stability of this transiently expressed VP16-AR protein, while it is significant on the endogenous AR (Fig. 1C). To better compare the relative activities of VP16-AR and AR, we transfected Cos cells, which do not express endogenous receptor. This experiment showed that VP16-AR has a 4-fold higher activity than AR on PSA-Luc (Shenk et al, 2001) and nearly 20-fold higher on ARE4-Luc (Chen et al, 2006a) (Fig. 1D). The expression levels of the two proteins are comparable (Fig. 1E), arguing that increased activity of VP16-AR is not due to increased protein expression.

To study the biological effects of this hyperactive AR, stable LNCaP cell lines were generated expressing VP16-AR. All VP16-AR-expressing cell lines have elevated R1881-induced AR activity, ranging from 3- to 35-fold better than the endogenous activity found in control C14 cells (Fig. 1F). Interestingly and surprisingly, V272 cells, which exhibited the highest AR activity, grew very poorly (S Chen and L Shemshedini, unpublished results), suggesting that high AR activity that is supra-physiological can be
detrimental to the growth of prostate cancer cells. Therefore, we elected to use for the studies below cell lines with moderately elevated, and thus more physiological, AR activity.

**Expression of VP16-AR leads to androgen-independent growth of prostate cancer cells.**

The stable LNCaP cell lines were first monitored for expression of VP16-AR. RT-PCR was used to show that VP16 was expressed in VP16-AR cells (V94, V134, and V149) but not control cells (C14) (Fig. 2A). As expected, the three VP16-AR stable cell lines exhibited a markedly higher AR mRNA expression than C14 cells in either the absence or presence of R1881 (Fig. 2A). Western blotting demonstrated expression of VP16-AR protein in all three V cell lines, but not C14 cells (Fig. 2B). Additionally, R1881 increased the levels of VP16-AR and the anti-androgen Casodex (Bicalutamide) inhibited this positive androgen effect (Fig. 2B), as previously observed with endogenous AR (Bai et al, 2005). This result shows that androgen affects stably expressed VP16-AR but not transiently expressed VP16-AR (see Fig. 1C). Further analysis using different concentrations of R1881 revealed that the VP16-AR protein was equally sensitive to the androgen stabilizing effect as native AR (Fig. 2C). To directly measure the stability of these two proteins, the protein synthesis inhibitor cycloheximide was used. Cycloheximide had a similar effect AR and VP16-AR protein levels, and the kinetics of recovery of protein levels induced by R1881 was also similar for AR and VP16-AR (Fig. 2D), indicating that these two proteins do not differ significantly in stability. To examine the expression of these two proteins in cells, immunoctyochemistry was performed using
an AR antibody, which can detect both endogenous AR and VP16-AR. As shown in Fig. 2E, C14 cells express small levels of AR scattered throughout the cell with androgen. Treatment with R1881 caused increased AR levels and nuclear localization (Fig. 2E), as previously observed (Mora et al., 1996). Interestingly, the V cell lines express higher protein levels than the C14 cells, representing signal from AR and VP16-AR, and positive signal is found in the cytoplasm and nucleus (Fig. 2E). Addition of R1881 enhanced these protein levels and induced nuclear localization, just as in C14 cells. Collectively, these data suggest that in the absence of R1881 the V cells have some nuclear VP16-AR protein, which may be responsible for the androgen-independent growth of these cells (see Fig. 3 below).

To test the hypothesis that expression of a hyperactive AR can convert the growth of prostate cancer cells from androgen-dependent to androgen-independent, the VP16-AR-expressing cells were monitored for cell growth. We performed an MTT growth assay to measure the proliferation of C14, V94, and V149 cells grown in steroid-reduced culture medium (5% DCC-extracted serum). Under these growth conditions, C14 cells did not grow in the absence of R1881 (Figs. 3A and B). However and importantly, both V94 (Fig. 3A) and V149 (Fig. 3B) cells exhibited a time-dependent increase in cell number in the absence of androgens and this growth is statistically higher than the growth of C14 cells. In fact, the androgen-independent growth of V149 cells is comparable to the androgen-induced growth of C14 cells (Fig. 3B). Collectively, these data show that expression of VP16-AR in LNCaP cells leads to androgen-independent cell growth. Interestingly, despite the androgen-independent growth of V94 and V149 cells, this
growth is enhanced by R1881 treatment, and V94 cells grew statistically better than C14 cells in the presence of R1881 (Figs. 3A and B).

To demonstrate that the growth of V94 and V149 cells in the absence of exogenous androgen was indeed androgen-independent, cell growth was measured in the presence of Casodex. As shown in Fig. 3C, Casodex suppressed the R1881-induced growth of C14, V94, and V149 cells, but had no effect on the R1881-independent growth of V94 and V149 cells. Interestingly, Casodex almost completely inhibited the growth of C14 cells, while it only reduced the growth of V94 and V149 cells to the level observed in the absence of R1881 (Fig. 3C). These data show that the VP16-AR-expressing cells exhibit a growth phase that is insensitive to Casodex and thus independent of androgens.

Our data above (Figs. 3A-C) demonstrate that V94 and V149 cells grow better in the presence of R1881 than in its absence, suggesting that their growth is androgen inducible. To get a relative measure of the androgen sensitivity of these cells, their growth was compared to that of C14 cells under very low serum (1% DCC-extracted serum) conditions and different concentrations of R1881. As shown in Fig. 3B, the growth profiles of V94 and V149 cells were similar to C14 cells. All three cell lines exhibited a growth response at an R1881 concentration of 0.01 to 0.1 nM and optimal growth at 1 nM (Fig. 3D). Interestingly, the V149 cells elicited the highest growth response to R1881 treatment and C14 cells the lowest (Fig. 3D). Collectively, these data show that V94 and V149 cells exhibit androgen-independent proliferation that is androgen inducible.
VP16-AR-expressing cells display altered expression of cell cycle regulatory proteins.

To begin to understand the molecular mechanism responsible for the androgen-independent growth of VP16-AR-expressing cells, we used Western blotting to measure the expression of several cell cycle regulatory proteins (Fig. 4). Retinoblastoma (Rb) protein expression was repressed by androgen in C14 cells, as previously shown (Taneja et al, 2002). Interestingly, Rb expression was significantly reduced in V94 cells, but only in the absence of androgen, as compared to C14 cells. By contrast to C14 cells, however, R1881 induced Rb expression in V94 cells. V94 cells expressed reduced levels of p27, as compared to C14 control cells, in both the presence and absence of R1881. As shown in Fig. 4, cyclin-dependent kinase 1 (CDK1) and CDK2 are expressed each as one protein band in C14 cells, while in V94 cells there is a second, more slowly migrating band that appears for both proteins. On the other hand, CDK4 expression is the same in C14 and V94 cells.

Expression of sGCα1 is androgen-independent in VP16-AR-expressing prostate cancer cells.

To understand the molecular basis of the androgen-independent growth of the VP16-AR cells, the expression of several androgen-regulated genes was measured. Since our previous data indicate that sGCα1 is important in the growth of both androgen-dependent and androgen–independent LNCaP cells (Cai et al. 2007a), we first measured sGCα1 expression in our VP16-AR-expressing cells. Interestingly, the expression of sGCα1, as measured by semi-quantitative RT-PCR (Figs. 5A-B), QRT-PCR (Fig. 5C),
and Western blotting (Fig. 5D), is androgen-independent in V94, V134, and V149 cells, mimicking what has observed in the other androgen-independent prostate cancer cells (Cai et al. 2007a; C Cai and L Shemshedini, unpublished results). This androgen-independent expression is not a common feature of all androgen-regulated genes, since TMPRSS2 (Lin, et al. 1999) is androgen-induced in both C14 and the VP16-AR cell lines (Figs. 5A-C). We have also confirmed androgen regulation in V94 cells of the PSA and hKLK2 genes (Fig. 5A; C Cai and L Shemshedini, unpublished results). These findings, together with our data from other androgen-independent prostate cancer cells (Cai et al. 2007a; C Cai and L Shemshedini, unpublished results), strongly argue that androgen-unresponsive expression of sGCα1 may be a common property of hormone-refractory prostate cancer cells.

Previous studies have shown that Casodex can induce expression of PSA in prostate cancer cells that over-express AR (Chen et al. 2004). To determine if this is also true for our VP16-AR-expressing cells, PSA expression was measured by semi-quantitative RT-PCR in cells treated with Casodex. As shown in Fig. 5E, Casodex had a weak negative, not positive, effect on PSA expression in V94 and V149 cells, the same as C14 control cells. Interestingly, however, the basal expression of PSA is higher in the VP16-AR cells than in the control cells. In contrast, R1881 was able to induce PSA expression in all three cell lines (Fig. 5E). Similar results were obtained for PSA protein expression with R1881 and Casodex, although the protein levels varied in the different cell lines (Fig. 5F). These results demonstrate that Casodex does not have agonistic activity in VP16-AR-expressing cells.
**sGCα1 is involved in the androgen-independent growth of VP16-AR-expressing prostate cancer cells.**

To directly test if sGCα1 is involved in V94 cell proliferation, a growth assay was performed in which sGCα1 expression was diminished by siRNA transfection. Transfection of sGCα1 siRNA markedly down-regulates the endogenous expression of sGCα1 mRNA in V94 cells, as compared to control siRNA (Fig. 6A), as it has been observed in C14 cells (Cai et al. 2007a). The same effect was observed on sGCα1 protein levels in V94 and C14 cells (Fig. 6B). This reduction in sGCα1 expression results in significantly decreased growth of C14 cells (Fig. 6C), as previously shown (Cai et al. 2007a). Most significantly, sGCα1 siRNA also inhibited the growth of V94 cells both in the absence and presence of androgens (Fig. 6D). These findings strongly suggest that sGCα1 is required for the androgen-independent growth of V94 cells.

**DISCUSSION**

Present in all forms of prostate cancer is a transcriptionally active AR (Meehan and Sadar 2003; Trapman and Cleutjens 1997). Among the multiple mechanisms implicated in the conversion of prostate cancer growth from androgen-sensitive to an androgen-insensitive (reviewed in Jenster 1999), over-expression of AR has been recently shown to be sufficient for this transition (Chen et al. 2004). In support of this, several earlier studies have demonstrated that hormone-independent prostate cancer cell lines exhibit increased AR expression and transcriptional activity (Culig, et al. 1999; Gao, et al. 1999; Kokontis, et al. 1998; Lu, et al. 1999; Thalmann, et al. 2000). In addition,
clinical studies have shown that about 30% of advanced prostate tumors exhibit AR over-expression (Koivisto, et al. 1997; Palmberg, et al. 1997; Visakorpi, et al. 1995). These studies collectively led us to hypothesize that increased AR transcriptional activity, like increased AR expression, can promote androgen-independent growth of prostate cancer cells. This hypothesis was directly tested by expression in LNCaP cells of VP16-AR, a fusion protein that harbors androgen-inducible transcriptional activity that is 10-fold higher than the endogenous AR. We demonstrate in this study that cells expressing VP16-AR do indeed exhibit androgen-independent growth, under the same culture conditions that yield only androgen-dependent growth for parental LNCaP cells (see Fig. 3). Interestingly however, the growth of VP16-AR cells is higher in the presence of R1881 than in the absence, demonstrating that, while these cells can proliferate in an androgen-independent manner, the presence of this androgen can further accelerate their growth. Thus, our VP16-AR cells are both androgen-independent and androgen-inducible, suggesting that the VP16-AR fusion protein has two growth-promoting functions, one that depends on androgens and another that does not. This conclusion is supported by our experiments with Casodex, since this anti-androgen only partially inhibited the growth of VP16-AR cells. Indeed, Casodex reduces the growth of VP16-AR cells to a level that is observed in the absence of androgens, strongly implying that there is an androgen-independent component and an androgen-dependent component to the growth of VP16-AR cells.

In view of the recent study reporting that increased AR expression is sufficient to convert prostate cancer cell growth from hormone-sensitive to hormone-refractory (Chen et al. 2004), it is possible that our VP16-AR cells simply represent another example of
androgen-independent growth in response to AR over-expression. However, the data provided argue against this and suggest that our cells may represent another cellular state of hormone-refractory prostate cancer. First and most importantly, we have no evidence that the antagonist Casodex has any agonistic activity in VP16-AR cells, as observed in AR-over-expressing cells (Chen et al. 2004). Indeed, the VP16-AR cells exhibited no detectable Casodex-induced expression of PSA (see Figs. 5E and 5F), or of several other androgen-regulated genes (C-L Hsieh and L Shemshedini, unpublished results). And secondly, AR over-expression in LNCaP cells increased their sensitivity to R1881-induced growth (Chen et al. 2004), while expression of VP16-AR had no effect (see Fig. 3D). Thus, our VP16-AR cells likely represent a mechanism of androgen independence that depends more on elevated AR transcriptional activity than expression level. Since no more than 30% of advanced prostate tumors exhibit increased AR expression (Koivisto et al. 1997; Palmberg et al. 1997; Visakorpi et al. 1995), it is possible that our VP16-AR cells may mimic one cancer cellular state that is found among the remaining 70% of tumors lacking increased AR expression. In addition to AR gene amplification, what other mechanisms can lead to AR over-expression? One can speculate that mutations in the AR gene promoter disrupting the activities of silencer elements can lead to higher AR expression. It is also possible that mutations that disrupt the activity of transcriptional repressors that act on the AR promoter can have the same effect. Perhaps these mechanisms are operating in some prostate tumors, but empirical evidence needs to be obtained in the future.

The molecular changes responsible for the hormone-refractory cancer state in tumors expressing normal AR protein levels are poorly understood. Several mechanisms
have been suggested (reviewed in Jenster 1999), including AR mutations that broaden ligand-specificity, generation of signaling pathways that lead to ligand-independent activation of AR, and over-expression of AR coactivators. All these provide means by which to activate AR, but they do not address the issue of AR-regulated gene expression that may be responsible for the hormone-refractory cancer state. Our VP16-AR cells provide some insight into this, with our discovery that these cells express the sGCα1 gene, an AR-regulated gene (Cai et al. 2007a), in a hormone-independent manner (see Figs. 5A, 5B, and 5C). sGCα1 is a component of sGC (soluble guanylyl cyclase), the enzyme that catalyzes cGMP synthesis in response to nitric oxide (reviewed in Hanafy, et al. 2001). Our earlier study showed that sGCα1 expression is androgen-independent in C81 cells (Cai et al. 2007a), a hormone-refractory LNCaP cell line whose AR expression is similar to that of the androgen-dependent parental cells (Igawa, et al. 2002). Furthermore, we demonstrated that sGCα1 protein expression levels are directly related to the growth capacity of LNCaP cells (Cai et al. 2007a). While the molecular mechanism responsible for the sGCα1 role in cell proliferation is not yet known, it is clear from our data thus far that the sGCα1 protein levels are directly proportional to the growth capacity of prostate cancer cells (Cai et al. 2007a). As expected, we find in this study that endogenous sGCα1 expression is also required for the androgen-independent growth of VP16-AR cells (see Fig. 6D), suggesting that androgen-independent expression of sGCα1 may be responsible for the hormone-refractory growth of LNCaP cells. Importantly, the transition to androgen-independent expression of sGCα1 is not unique to LNCaP cells, but may be a common property of prostate cancer cells since we have observed a similar

It is interesting to note that androgen-independent expression is not observed globally on AR-regulated genes in hormone-refractory cells, but in fact is target-gene specific. Our VP16-AR cells here (see Figs. 5A, and 5B) and C81 cells (Igawa et al. 2002) exhibit androgen-induced expression of two hallmark AR-regulated genes, PSA and TMPRSS2. The contrasting expression patterns of PSA and TMPRSS2, as compared to sGCα1, suggest that genes important in prostate cancer biology are selected for hormone-independent expression. This is supported by another novel AR-regulated gene, ETV1, also elicits androgen-independent expression in VP16-AR (C Cai and L Shemshedini, unpublished results) and C81 cells (Cai, et al. 2007b). ETV1 is involved in prostate cancer cell invasiveness (Cai et al. 2007b) and sGCα1 in cell proliferation (Cai et al. 2007a), both processes essential to the progression of prostate cancer. For now, we do not know how many more AR-regulated genes transition to androgen-independent expression or the molecular basis of the gene selection process for such expression. These will be two important objectives of future work.

Previous work has suggested that reduced Rb protein expression may be responsible for androgen-induced proliferation of LNCaP cells (Taneja et al, 2002). In support of this, we observed that R1881 repressed Rb expression in LNCaP cells (C14 cells) (see Fig. 4). Interestingly, Rb expression is even lower in the V94 cells without androgen than C14 cells with androgen (see Fig. 4), suggesting that reduced levels of Rb in V cells may be involved in their androgen-independent growth. Surprisingly, however, Rb levels are higher in V94 cells treated with androgen than without, indicating a
complex function of Rb in prostate cancer cell growth. On the other hand, protein levels of p27 (Kip1) are consistently and significantly lower in V94 than C14 cells (see Fig. 4). In view of previous findings showing that increased levels of p27 are associated with decreased prostate cancer cell growth and increased cell cycle arrest (Deep et al, 2007), our finding suggests that decreased p27 expression in V cells may be in part responsible for the increased growth capacity of these cells. Supporting this conclusion is the previous finding that decreased expression of p27 is associated with androgen-independent proliferation of LNCaP cells (Murillo et al, 2001). Previous data also suggest that increased levels of CDKs and cyclins are found in proliferating LNCaP cells (Deep et al, 2007). While we observed no significant change in CDK expression between C14 and V94 cells, a second larger band was detected for both CDK1 and CDK2 in V94 cells (see Fig. 4). These larger proteins may represent phosphorylated forms of CDK1 and CDK2 that may be involved in androgen-independent growth of our LNCaP cell lines. Collectively, these data, together with the surprising finding that cyclin D1 expression is reduced in V94 as compared to C14 cells (see Fig. 4), show the complex nature of cell cycle regulatory proteins in LNCaP cells. Future work will be directed at a biochemical analysis of the larger CDK proteins and determining what roles these proteins, as well as Rb and p27, play in cell cycle progression of VP16-AR-expressing cells.
ACKNOWLEDGMENTS

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FIGURE LEGENDS

Fig. 1. VP16-AR has strong androgen-dependent transcriptional activity in prostate cancer cells.

(A). This diagram shows the schematic structure of the fusion protein VP16-AR, which contains the VP16 transactivation domain (amino acids 414-490) fused to the N-terminus of full-length AR. (B) LNCaP cells were transfected with 1 mg MMTV-CAT, 5 mg hKLK2-CAT, or 5 mg PSA-CAT with or without 1 mg AR or VP16-AR. 1 nM R1881 was used as indicated. (C) LNCaP cells were transiently transfected with AR or VP16-AR and treated with different concentration of R1881, as indicated, for 2 days, and subjected to Western blotting using an anti-AR or anti-VP16 antibody. The two right arrows on the AR Western blot point to AR (a) and VP16-AR (b) proteins. (D) Cos cells were transfected with 1 mg of PSA-Luc or ARE4-Luc with or without 1 mg AR or VP16AR. 1 nM R1881 was used, as indicated. Asterisks indicate statistical significance (p < 0.05), as indicated. (E) Western blotting show similar expression of AR and VP16-AR proteins in transfected Cos cells. (F) LNCaP stable cell lines expressing VP16-AR were selected on neomycin-containing medium and screened for androgen-dependent transactivation by transiently transfecting 1 mg MMTV-CAT and treated with R1881. Note that C14 cell line represents a stable transfection with an empty pCI-Neo vector and V lines represent VP16-AR-expressing cells, with those V lines in bold-face selected for further study in this report. All CAT activities are relative to the activity of C14 cells, and this activity was set to 1. 1 nM R1881 was used in B, D, and F.
Fig. 2. The VP16-AR protein in LNCaP stable cell lines exhibits similar stability to endogenous AR.

(A) Semi-quantitative RT-PCR was used to measure the expression of AR or VP16 in C14, V94, V134, and V149 cells in the presence or absence of 1 nM R1881, as indicated. (B, C, D) Western blotting was used to show that (B) Casodex can reduce VP16-AR protein levels in all three cell lines, (C) R1881 has a similar stabilizing effect on the AR and VP16-AR proteins, and (D) the cycloheximide (CHX) effect indicates a similar stability for the AR and VP16-AR proteins. (E) Immunocytochemistry was used to show that VP16-AR expressing cell lines express higher total AR protein levels than control C14 cells.
Fig. 2.
Fig. 3. Expression of VP16-AR leads to androgen-independent growth of prostate cancer cells.

(A) V94 or (B) V149 cells were measured for proliferation in the presence (solid dark line) or absence (broken dark line) of 1 nM R1881 for 0-6 days in medium containing 5% DCC-extracted serum, and compared to the proliferation of C14 cells (solid light line, with R1881; broken light line, without R1881). (C) C14, V94, or V149 cells were measured for proliferation under the same conditions as above, with or without Casodex. Bar graphs represent the average of three independent experiments plus the standard deviation. (D) The same three cell lines were grown for six days in medium containing 1% DCC-extracted serum, with increasing concentrations of R1881, as indicated. Each point in A, B, and D represents the average of three independent experiments plus/minus the standard deviation. Asterisks indicate statistical significance (p < 0.05) on the effect of Casodex on cell growth.
Fig. 3.
Fig. 4. VP16-AR-expressing prostate cancer cells exhibit differential expression of cell cycle proteins.

C14 and V94 cells were grown for two days in the presence or absence of 1 nM R1881, as indicated, and subjected to Western blotting to measure the expression of VP16-AR, AR, Rb, p27, CDK1, CDK2, CDK4, Cyclin D1, and b-tubulin. Note that the V94 cells express two proteins detected by the anti-AR antibody, endogenous AR and VP16-AR (upper band).
Fig. 4.
Fig. 5. The expression of sGCα1 is constitutive in VP16-AR-expressing prostate cancer cells.

The expression of sGCα1, PSA, and/or TMPRSS2 in C14, V94, V134, and V149 cells treated with or without 1 nM R1881 or 10 mM Casodex, as indicated, was measured by (A, B, E) semi-quantitative RT-PCR, (C) Q-RT-PCR, or (D, F) Western blotting. Note that mRNA levels measured by Q-RT-PCR are represented relative to GAPDH expression (C). Note that the PSA protein in F is the lower band. Asterisks indicate statistical significance (p < 0.05) on the sGCα1 expression in V94 and V149 cells as compared to C14 cells. Note that there is no statistically significant DHT effect on sGCα1 expression in either V94 or V149 cells (C).
Fig. 5.
Fig. 6. sGCα1 is involved in the androgen-independent growth of V94 cells.

(A) V94 cells were transfected with sGCα1 siRNA or an unrelated control (Ctrl) siRNA and expression of sGCα1 and sGCβ1 was measured by semi-quantitative RT-PCR. (B) Western blotting shows reduced sGCα1 protein expression in C14 or V94 cells transfected with sGCα1 siRNA. (C) C14 or (D) V94 cells were transfected with sGCα1 siRNA or control siRNA and grown for 0-6 days in the presence or absence of 1 nM R1881, as indicated, and measured for cell number. Bar graphs represent the average of three independent experiments plus the standard deviation. Asterisks indicate statistical significance (p < 0.05) on the effect of sGCα1 siRNA on cell growth.
Fig. 6.
Manuscript II

Soluble Guanylyl Cyclase α1 and p53 Cytoplasmic Sequestration: A Novel Mechanism for p53 Down-Regulation in Prostate Cancer

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Running Title: sGCα1 inhibits p53 activity in prostate cancer.

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Our lab has previously identified soluble guanylyl cyclase α1 (sGCα1) as a novel androgen-regulated gene essential for prostate cancer cell proliferation. sGCα1 expression is highly elevated in prostate tumors, contrasting with the low expression of sGCβ1, with which sGCα1 dimerizes to mediate nitric oxide (NO) signaling. In studying its mechanism of action, we have discovered that sGCα1 can inhibit the transcriptional activity of p53 in prostate cancer cells independent of either classical mediators of NO signaling or the guanylyl cyclase activity of sGCα1. Interestingly, sGCα1 inhibition of p53-regulated gene expression was gene-specific, targeting genes involved in apoptosis/cell survival. Consistent with this, over-expression of sGCα1 makes prostate cancer cells more resistant to etoposide, a chemotherapeutic and apoptosis-inducing drug. Immunoprecipitation and immunocytochemistry assays show a physical and direct interaction between sGCα1 and p53 in prostate cancer cells. Interestingly, sGCα1 induces p53 cytoplasmic sequestration, representing a new mechanism of p53 inactivation in prostate cancer. Analysis of prostate tumors has shown a direct expression correlation between sGCα1 and p53. Collectively, these data suggest that sGCα1 regulation of p53 activity is important in prostate cancer biology and may represent an important mechanism of p53 down-regulation in those prostate cancers that express significant levels of p53.
INTRODUCTION

Tumor suppressors, such as PTEN and p53, play important roles in prostate cancer initiation and progression. While knocking-out p53 in the mouse prostate fails to develop prostate cancer, complete inactivation of PTEN triggers non-lethal invasive prostate cancer after long latency (Chen et al., 2005). More interestingly, double knock-out of p53 and PTEN can lead to lethal prostate cancer (Chen et al., 2005). These findings suggest that PTEN has a role in prostate cancer initiation and p53 is essential for tumor progression. p53 plays a key role in regulating several critical pathways, including cell cycle arrest, apoptosis, DNA repair, and cellular senescence, all of which are essential for normal cellular homeostasis and maintaining genomic integrity (Reviewed by Hussain and Harris, 2006). p53 is found frequently mutated in a variety of human cancers and these mutations are the most common genetic alteration found in human cancers (Reviewed by Levine et al., 1991; Hollstein et al., 1991, and Greenblatt et al., 1994). However, p53 mutations are found almost exclusively in advanced prostate cancers (Qian et al., 2002; Navone et al., 1999). In response to DNA damage, p53 expression is induced and the protein is translocated into the nucleus to directly regulate gene expression (Fritsch et al., 1993). Several p53 downstream targets are known, including the pro-apoptotic Bax (Miyashita et al., 1995), the cell cycle inhibitor p21/CIP1 (Brugarolas et al., 1995) and its auto-regulator Mdm2 (Barak et al., 1993).

Cytoplasmic sequestration of p53 has been proposed as an important mechanism to disrupt its function as a tumor suppressor (Steele et al., 1998). In 37% of breast cancers, p53 has been found localized in the cytoplasm, suggesting a mechanism of inhibiting p53 function via nuclear exclusion (Moll et al., 1992). A good example is the
interaction between glucocorticoid receptor (GR) and p53, which results in cytoplasmic sequestration of both p53 and GR (Sengupta et al., 2000). Another factor that induces p53 cytoplasmic localization is Jab1, which was shown to facilitate p53 nuclear exclusion and degradation (Oh et al., 2006). More recently, Parc, a parkin-like ubiquitin ligase, was shown to function as cytoplasmic anchor protein of p53 (Nikolaev et al., 2003).

Androgen-regulated genes are critical for prostate cancer. This is supported by the recent findings of androgen regulation of TMPRSS2:ERG fusion gene expression (Tomlins et al., 2005), chromosomal rearrangement (Tomlins et al., 2007), and ETV1 expression (Cai et al., 2007a). However, how androgens regulate tumor suppressors is not clear. The androgen-induced protein NKX3.1 was shown recently to increase p53 acetylation and thus half-life through interference of Mdm2-dependent mechanism (Lei et al., 2006). NKX3.1-deficient mice develop prostatic hyperplasia, which, however, failed to progress to metastatic cancers (Bhatia-Gaur et al., 1999; Abdulkadida et al., 2002), suggesting that NKX3.1 inactivation is involved in prostate cancer initiation. While NKX3.1 can positively regulate p53, no androgen-induced protein has been identified to repress p53 function in prostate cancer development and progression, where AR signaling is hyperactivated. Here, we report that soluble guanylyl cyclase 1 (sGCα1) can interact with cytoplasmic p53 and negatively regulate its transcriptional activity. We have previously shown that sGCα1 is androgen-regulated gene and plays an important role in prostate cancer cell proliferation (Cai et al., 2007b). In contrast to NKX3.1, sGCα1 is involved in both androgen-dependent and androgen-independent prostate cancer cell proliferation and its expression is significantly increased in higher stages of metastatic prostate cancers (Cai et al., 2007b). Our findings here support a novel mechanism for
p53 down-regulation, via an sGCα1-dependent cytoplasmic sequestration of p53, which may be important in the development and progression of prostate cancer.

RESULTS

sGCα1 inhibits p53 transcriptional activity

p53 transcriptional activity was measured using a luciferase reporter plasmid containing p53-responsive elements. Over-expressing p53 in LNCaP cells increased p53 transcriptional activity about 2.5-fold (Fig. S1A), while disruption of endogenous p53 expression by siRNA (Fig. S1B) led to an 80% decrease of p53 activity (Fig. S1C), confirming the responsiveness of the reporter gene assay to p53 expression. We used this reporter to study the effect of sGCα1 on p53. Transient transfection of sGCα1 in LNCaP cells led to a dose-dependent inhibition of p53 transactivation (Fig. 1A), but had no effect on endogenous AR transcriptional activity (Fig. S1E). Diminution of endogenous sGCα1 expression (Fig. 1C) resulted in a small, but reproducible and statistically significant, increase in p53 activity (Fig. 1B). The exogenous expression of sGCα1 was confirmed using Western blotting, as shown in Fig. S1D. Together, these results show that both endogenous and exogenous sGCα1 can inhibit p53 activity.

VCaP cells, another AR-positive prostate cancer cells, express wild-type AR, PSA, and p53 (Fig. S2A; Korenchuch et al., 2001). Recently, VCaP cells were found to carry the TMPRSS2:ERG fusion gene, which is detected in more than 40% prostate cancer cases and is believed to play a key role in prostate cancer (Tomlins et al., 2005). Consistent with the expression in LNCaP cells, sGCα1 is also androgen-induced in VCaP cells (Fig. S2A). More importantly, down-regulating sGCα1 expression (Fig. S2B) also
resulted in the inhibition of VCaP cell growth (Fig. S2C), suggesting the same pro-proliferative role for sGCα1 as in LNCaP cells (Cai et al., 2007b). Transfected sGCα1 can significantly repress exogenous p53 activity in VCaP cells (Fig. 1D), similar to what was observed in LNCaP cells (see Fig. 1A).

To determine if the sGCα1 inhibition can occur in hormone-refractory prostate cancer, we utilized two hormone-independent prostate cancer cell lines, CWR-22Rv1 (Kim et al., 2002) and C81 cells (Igawa et al., 2002), both of which interestingly exhibit hormone-independent sGCα1 expression (Fig. 1G; Cai et al., 2007b). While the effect is weaker than in either LNCaP or VCaP cells, sGCα1 shows a significant inhibitory effect in both C81 (Fig. 1E) and CWR-22Rv1 cells (Fig. 1F). In contrast, exogenous sGCα1 has no effect on exogenous p53 in the AR-negative PC-3 cells (Fig. 1H), which do not express either sGCα1 (Cai et al., 2007b) or p53 (Bataller and Porgtugal, 2005) and do not have a functional sGC/cGMP pathway (Chen et al., 2002). All these data together suggest that sGCα1 represses p53 activity in prostate cancer cells that harbor a functional AR pathway, and this activity does not depend on mutated AR.

*The negative effect of sGCα1 on p53 is independent of mediators of NO signaling*

NO interfering drugs were used to determine if nitric oxide (NO) signaling is involved in sGCα1-mediated repression of p53 transcriptional activity. As shown in Fig. 2A, ODQ, which inhibits sGC enzyme activity (Schrammel et al., 1996), had no effect on sGCα1-mediated repression of p53, even though these concentrations strongly inhibited sGC-catalyzed cGMP synthesis (data not shown), suggesting that sGCα1 inhibits p53 activity independent of sGC enzyme activity. C-PTIO is an NO scavenger (Haruyama et
al., 1998) and thus would be expected to enhance p53 activity if sGCα1 requires NO for its inhibitory activity; contrary to this, c-PTIO inhibited p53 activity (Fig. 2B), suggesting that NO is not involved in the sGCα1 inhibition. Supporting this conclusion, the NO donor SNP had no effect (Fig. 2C). 8-Br-cGMP did not repress, but in fact weakly enhanced p53 transcriptional activity (Fig. 2D), implying that the second messenger of NO signaling is not involved in sGCα1 repression of p53. Collectively, these results argue that the sGCα1 effect on p53 occurs independent of NO signaling. Most importantly, the mutant sGCα1(D531A) (Fig. 2G), which has greatly reduced cyclase activity (Fig. 2F) at equal expression levels as wild-type protein (Fig. 2G), is fully able to inhibit p53 transcriptional activity (Fig. 2E), demonstrating that sGCα1 activity on p53 does not depend on its guanylyl cyclase activity.

**sGCα1 influences the expression of p53-regulated genes involved in apoptosis/survival**

To determine if sGCα1-mediated inhibition of p53 affects p53-regulated gene expression, a PCR array analysis was performed using a p53 Signaling Array (from SA Biosciences). LNCaP cells were transfected with sGCα1 siRNA (Fig. 3A), which results in enhanced p53 transactivation (see Fig. 1B above), and subjected to PCR array analysis. Among the 84 genes on the array, only three exhibited differential expression by more than 3.5-fold in response to siRNA-mediated diminution of sGCα1, showing that the sGCα1 role on p53-regulated gene expression is gene-specific, not global. Knockdown of sGCα1 led to increased expression of p53AIP1 (Wang et al., 2006) and PCBP4 (Pio et al., 2004), two p53-induced genes mediating apoptosis, and decreased expression of Survivin (or BIRC5), a p53-repressed gene that protects cells from apoptosis and
mediates cell survival (Knauer et al., 2007) (Fig. S3A). The PCR array data were verified by quantitative real-time-PCR (QRT-PCR) (Fig. 3A). Interestingly, p53-regulated genes involved in other p53-mediated pathways, including cell proliferation, were not significantly affected by sGCα1 siRNA, and this is shown for p21 (Fig. S3B), suggesting that sGCα1 may be a specific inhibitor of p53 activity involved in apoptosis/cell survival. Supporting these data, knocking-down sGCα1 expression enhanced serum starvation-triggered cell death of LNCaP cells (Fig. 3B). To monitor apoptosis, Caspase 3/7 activity was measured in LNCaP cells transfected with siRNA against sGCα1. As shown in Fig. 3C, down-regulation of sGCα1 increased Caspase activity almost 2-fold, and thus resulted in enhanced apoptosis. To bolster these data, we used the anti-cancer drug etoposide, which is known to induce p53-dependent apoptosis of prostate cancer cells (Salido et al., 2004). Over-expressing sGCα1 by adenovirus infection significantly reduced the cytotoxic effect of Etoposide (Fig. 3D), while diminishing sGCα1 had the opposite effect, enhancing etoposide-induced cytotoxicity (Fig. 3E). These results show that the sensitivity of LNCaP cells to Etoposide-induced apoptosis is inversely related to sGCα1 protein levels and are consistent with our finding that sGCα1 interferes with p53-regulated pro-apoptotic gene expression.

sGCα1 mediates cytoplasmic localization of p53

To determine if there is a physical association between sGCα1 and p53, IP experiments were performed with endogenous proteins found in LNCaP cells. When p53 was immunoprecipitated, endogenous sGCα1 protein was co-purified which was not seen
with a negative control immunoprecipitation (IP) (nonspecific IgG) (Fig. 4A). A complementary IP showed that endogenous p53 co-purified with endogenous sGCα1 (Fig. 4A). Interestingly, when an LNCaP cytoplasmic extract was run on a gel filtration FPLC column, sGCα1 and p53 were found in the same elution fractions (Fig. 4B), suggesting the existence of a cytoplasmic pool of associated sGCα1 and p53 in LNCaP cells. To confirm these data, immunocytochemistry was used to visualize sGCα1 and p53. This study showed that endogenous p53 is localized in both the nucleus and cytoplasm, while sGCα1 is exclusively cytoplasmic (Fig. 4C). Interestingly, cytoplasmic p53 is co-localized with sGCα1 (Fig. 4C). Importantly, this cytoplasmic co-localization between sGCα1 and p53 was also observed in the hormone-refractory prostate cancer cells CWR22Rv1 (Fig. 4C). Collectively, these data show that endogenous sGCα1 and p53 co-associate in the cytoplasm of both hormone-dependent and hormone-refractory prostate cancer cells.

To determine if the sGCα1-p53 interaction is direct, we expressed the proteins in E. coli and carried out immunoprecipitation (IP) experiments (Fig. 4D). As shown, the anti-p53 antibody can pull-down p53 from E. coli extract. More importantly, when p53 and sGCα1 extracts are mixed and an IP is performed with the anti-sGCα1 antibody, p53 is co-purified, suggesting that E. coli-expressed p53 and sGCα1 associate with one another. As a control, p53 and sGCβ1 extracts were mixed and subjected to an IP with an anti-sGCβ1 antibody, yielding substantially less co-purified p53. When sGCα1 was measured, the results show that sGCα1 can come down, as expected, with the anti-sGCα1 IP and, more importantly, with the anti-p53 IP, confirming the sGCα1-p53 interaction. The anti-sGCβ1 IP can pull-down substantial levels of sGCα1, as expected.
Lastly, monitoring sGCβ1 levels showed that this protein can co-IP with sGCα1 very strongly, and only weakly with p53, demonstrating, as expected, that sGCβ1 associates much more strongly with sGCα1 than with p53. In showing that E. coli-expressed p53 and sGCα1 can interact with one another, these data confirm our experiments in LNCaP cells and thus suggest that this interaction is direct.

The data above showing sGCα1 and p53 interaction and cytoplasmic co-localization suggest that sGCα1 may inhibit p53 transcriptional activity by mediating cytoplasmic sequestration of this tumor suppressor. To obtain evidence for this, immunocytochemistry and Western blotting were used to monitor protein expression and sub-cellular localization of sGCα1 and p53 in LNCaP cells. As observed previously (see Fig. 4C above), endogenous sGCα1 is exclusively cytoplasmic in LNCaP cells and co-localizes with cytoplasmic p53 (Fig. 4E). These sGCα1 levels are greatly diminished following siRNA transfection, and importantly, this coincides with an almost complete elimination of cytoplasmic p53 (Fig. 4E). To complement this experiment, transient transfection was used to express a GFP-tagged sGCα1 in LNCaP cells, which can be seen in Fig. 4F as a green cytoplasmic signal. Remarkably, we observed markedly elevated levels of cytoplasmic and sGCα1-co-localized p53 only in the cells over-expressing sGCα1-GFP (Fig. 4F). Importantly, the same result was obtained with the cyclase-deficient mutant sGCα1(D531A)-GFP (Fig. 4F). It should be noted that addition of GFP did not affect the ability of either wild-type sGCα1 or the D531A mutant to inhibit p53 transcriptional activity (Fig. S4A). These results strongly demonstrate that the cytoplasmic levels of p53 protein are directly correlated with sGCα1 protein levels.
As a more quantitative measure of p53 subcellular localization, Western blotting of cell fractions was used in cells having altered levels of sGCα1. Reducing endogenous sGCα1 protein levels by siRNA resulted in significantly less cytosolic and more nuclear p53 protein levels, as compared to control siRNA transfection (Fig. 4G), confirming the immunocytochemistry data (see Fig. 4E). In contrast, adenovirus-mediated expression of sGCα1, which results in high sGCα1 expression (Cai et al., 2007b), yield substantially elevated cytosolic p53 protein levels and reduced nuclear levels (Fig. 4H), in complete agreement with the immunocytochemistry findings (see Fig. 4E). Note that Western blotting for MLK3 (cytosolic) and RARα (nuclear) confirmed the purity of the two cell fractions (Fig. S4B). These data collectively show that sGCα1 can influence the p53 subcellular localization in prostate cancer cells and strongly suggest that inhibition of p53 is mediated by sGCα1-induced cytoplasmic sequestration of p53.

*sGCa1 is co-expressed and co-localizes with cytoplasmic p53 in prostate tumors*

Our data in prostate cancer cells reveal a biological interaction between sGCα1 and p53. To determine if such an interaction exists in prostate tumors, sGCα1 and p53 expression was measured by semi-quantitative RT-PCR and protein subcellular expression by immunohistochemistry. Significant p53 and sGCα1 mRNA expression was found widely in the prostate cancer tissues (Fig. 5A). Among the 30 tumors examined, 23 (77%) exhibited comparable p53 and sGCα1 expression levels, differing by less than 1.4-fold. Interestingly, one of these tumors, C25, expresses no detectable p53 or sGCα1. The remaining seven tumors express p53 and sGCα1 mRNA levels that differ by more than 1.4-fold, including four that express proportionately higher p53 (C6, C7, C17, C20).
and three higher sG\(\alpha\)1 (C10, C19, C26). Collectively, these data demonstrate a direct correlation in mRNA expression between p53 and sG\(\alpha\)1 and strongly suggest high sG\(\alpha\)1 levels are selected in those tumors that have high p53 expression.

We used immunohistochemistry to measure protein expression and subcellular localization in prostate cancer. This analysis showed p53 and sG\(\alpha\)1 expression in both basal and luminal epithelial cells. Interestingly, only cytoplasmic p53 protein was detected and this protein co-localized with sG\(\alpha\)1 (Fig. 5B), mimicking what was observed in prostate cancer cell lines (see Fig. 4C).

DISCUSSION

Disruption of normal p53 function is necessary for tumorigenesis. As a result, tumor cells usually harbor multiple mechanisms for p53 disruption (reviewed in Tomkova et al., 2008). Perhaps the most common mechanism is loss-of-function mutations, which can lead to diminished or no p53 expression or expression of an altered p53 protein that aids, rather than harms, tumor cells. In prostate cancer, this strategy is utilized by about 50% of late-stage tumors (reviewed in Heidenberg et al, 1995; Dong, 2006). For the remaining tumors, strategies must exist that lead to inactivation of the wild-type p53 protein when it is expressed. We provide in this paper a novel mechanism for p53 inactivation in prostate cancer, cytoplasmic sequestration mediated by sG\(\alpha\)1.

Originally identified as a component of the NO receptor, sG\(\alpha\)1 appears to have multiple functions in prostate cancer that are distinct from its role in NO signaling. One such function is mediating prostate cancer cell proliferation. We have recently published that sG\(\alpha\)1 is a novel androgen-regulated gene mediating prostate cancer cell
proliferation independent of classical mediators of NO signaling and, thus, leading to hormone-independent proliferation (Cai et al., 2007b). The inhibition of p53 represents a second novel function of sGCα1 in prostate cancer. This sGCα1 activity does not depend on either its cyclase activity or mediators of NO signaling, mimicking its role in cell proliferation (Cai et al., 2007b). Interestingly, however, the inhibition of p53 is unlikely to be involved in cell proliferation, since knock-down of p53 expression does not affect LNCaP proliferation (see Fig. S1F). This is consistent with our finding here that sGCα1 does not affect the expression of p21, a p53-induced gene important for cell-cycle progression (Barley et al., 1998), and an earlier study suggesting that p53 disruption does not initiate prostate cancer, but is an important step in late-stage disease (Chen et al., 2005).

Any novel mechanism of p53 regulation would be expected to influence the expression of a wide diversity of genes, since p53-regulated gene expression is involved in cell-cycle progression, apoptosis, senescence, and genomic stability (reviewed in Tomkova et al., 2008). Interestingly, our PCR array analysis identified only three p53-regulated genes to be affected by sGCα1, and all three genes function in apoptosis. Two genes, p53AIP1 (Wang et al., 2006) and PCBP4 (Pio et al., 2004), are p53-inducible and promote apoptosis and one, Survivin (Knauer et al., 2007), is p53-repressible and antagonizes apoptosis. Our data show that sGCα1 knock-down leads to increased apoptosis, an expected outcome in view of our finding that the knockdown results in expression that is higher for p53AIP1 and PCBP4 (pro-apoptotic) and lower for Survivin (anti-apoptotic). Among these three genes, Survivin is perhaps the most interesting because of its importance in prostate cancer. Survivin is over-expressed in prostate
cancer and confers drug resistance to cancer cells (Zhang et al, 2005a; Zhang et al, 2005b). As a p53-repressed gene, Survivin expression is decreased with sGCα1 knockdown, resulting from the increased p53 activity. And consistent with Survivin’s role in drug resistance, sGCα1 knock-down results in greater sensitivity of prostate cancer cells to the cytotoxic effects of etoposide. Conversely, over-expressing sGCα1 decreases the sensitivity. Thus, these results demonstrate a direct link between sGCα1 control of p53-regulated genes and sensitivity of prostate cancer cells to apoptosis-inducing drugs.

Among other p53-regulated genes involved in apoptosis, we observed no expression changes in response to sGCα1 siRNA. This includes Bax (Miyashita and Reed, 1995), Bcl2 (Findley et al, 1997), Puma (Villunger et al, 2003), and several other genes. There are several possible reasons for why sGCα1 does not have a global effect on p53-regulated gene expression. First, the PCR array experiment was done with siRNA-knockdown of sGCα1, which resulted in more nuclear p53. Since LNCaP cells express significant levels of nuclear p53, this may be sufficient for expression of many genes and thus increasing these nuclear levels by transfection of sGCα1 siRNA will not affect these genes. Secondly, it should be remembered that sGCα1 over-expression reduced, but did not eliminate, nuclear p53 protein. And lastly, it has been shown that not all p53-regulated genes are involved in apoptosis in a given cell context. For example, it was recently shown that Puma does not play a role in p53-triggered apoptosis of non-small-cell lung cell lines, even though PUMA is highly expressed (Gu et al, 2004). Thus, it is possible that sGCα1 activity on p53-regulated genes is targeted to those genes having important roles in prostate cancer, and arguably survivin and p53AIP1 fit this
description. The molecular basis of this gene-selective activity is not known, but it is possible that it may reflect promoter-specific activities of the p53 protein, which, interestingly, has been shown have lower binding affinities for DNA sites on apoptosis target genes than on cell-cycle target genes (reviewed in Riley et al, 2008).

It is interesting to note that sGCα1 is expressed in prostate cancer cell lines that express p53 (LNCaP, VCaP, and CWR-22Rv1 cells), but not in a cell line that does not (PC-3 cells). Indeed, sGCα1 is not expressed in another prostate cancer cell line, DU145, which expresses a mutant p53 protein (Gurova et al, 2003). These data suggest that sGCα1 over-expression offers a selective advantage to prostate cancer cells that have to contend with the anti-cancer activities of p53. This co-expression in cell lines is supported by our tumor expression study here, which shows a strong correlation in mRNA expression between p53 and sGCα1, making it possible that the two proteins are also coordinately expressed in prostate tumors, as they are in the cell lines. Future work will analyze the p53 protein expression and status of the p53 gene (wild-type or mutant).

Many p53-interacting proteins have been identified, most of which have role in cancer, underscoring the importance of p53 regulation in this disease. Some of these proteins, including GR (Sengupta et al., 2000), PARC (Nikolaev et al., 2003), and Jab1 (Lee et al., 2006), have been implicated in mediating nuclear export of p53. Among these, Jab1 is most interesting because of its elevated expression in LNCaP cells and physical interaction with sGCα1 (data not shown). Indeed, we have shown by IP and co-purification that sGCα1 associates with Jab1 (CSN5), CSN7, and CSN4 (data not shown), all subunits of the COP9 Signalosome (reviewed in Kato and Yoneda-Kato 2009). Interestingly, however, the reported Jab1 interaction with p53 leads to p53 protein
degradation (Lee et al, 2006; reviewed in Zhang et al, 2008), which is not the case with the sGCα1 interaction. In fact, over-expression of sGCα1 appears to result in higher p53 protein level, which is likely a consequence of decreased protein degradation since sGCα1 has no effect on p53 mRNA levels (data not shown). Regardless of what role Jab1 or other proteins may play, this sGCα1 activity is probably mediated via a direct interaction with p53, as the two proteins exhibit a physical association when expressed in E. coli. Thus, we propose a novel mechanism of p53 regulation, in which sGCα1 over-expression leads to nuclear export and cytoplasmic sequestration of p53, resulting in p53 inactivation (Fig. 5C). Decreased protein degradation may also contribute to the elevated cytoplasmic levels of p53. This model provides an active mechanism for p53 down-regulation, in which sGCα1 over-expression, as observed in the progression of prostate cancer (Cai et al., 2007b), would lead to p53 inactivation. This contrasts from the passive role of NKX3.1, in which decreased expression of this protein in prostate cancer results in decreased p53 protein stability (Lei et al., 2006) and thus inactivation. Hence, prostate cancer cells have at least two mechanisms for inactivation of the p53 protein, and the importance of the sGCα1-dependent mechanism will be a focus of our future work.
MATERIALS/SUBJECTS AND METHODS

Cell Culture and Androgen Treatment

LNCaP and PC-3 cells were grown as previously described (Cai et al., 2007b). CWR22Rv1 cells were cultured in RPMI-1640 medium with 10% FBS and 50 μg/ml Gentamicin (Gibco), and VCaP cells in the same medium without Gentamicin. For androgen (R1881) treatment, LNCaP and VCaP cells were grown in medium containing 2% FBS extracted with dextran-coated charcoal (DCC). 48 hrs later, ethanol or 1 nM R1881 was added to the cells. After an additional 48 hrs, the cells were subjected to semi-quantitative RT-PCR, real time-PCR, Western blotting, or immunocytochemistry.

siRNA Transfection

siRNA of 50 nM final concentration was transfected into LNCaP or VCaP cells using X-tremeGENE (for MTT proliferation assay) or lipofectamine 2000 (for other assays) as described previously (Cai et al., 2007b). The sGCα1 siRNA and Luciferase GL3 control siRNA (Dharmacon) are described previously. p53 siRNA was purchased from Santa Cruz.

Reporter Assay and Plasmid Transfection

LNCaP cells were grown to 80-90% confluence in RPMI-1640 with 10% FBS. After 48 hrs, medium was replaced with serum-free medium and the cells were transiently transfected with the p53-Luc reporter plasmid, expression plasmid for sGCα1, sGCα1-GFP, sGCα1(D531A), sGCα1(D531A)-GFP, or p53, and 0.5 µg pCH110, used to
measure transfection efficiency (Shenk et al., 2001). The p53-Luc plasmid was kindly provided by Dr. Andrei Gudkov and contains three p53-responsive elements. For those experiments studying NO signaling, chemicals affecting this pathway were added 24 hours later. The transfection was performed by using Lipofectamine 2000 and luciferase assays performed as described (Cai et al., 2007b).

**Adenovirus Infection**

LNCaP cells were infected with 20 MOI adenovirus expressing rat sGCα1 protein as described (Cai et al., 2007b).

**Semi-Quantitative RT-PCR and Real-Time PCR**

Total mRNA was isolated and subjected to semi-quantitative RT-PCR and real-time PCR analyses as described (Cai et al., 2007a). The RT-PCR upstream and downstream primers, respectively, used for each gene were: p53AIP1, 5’-GGACTGGCCCTAACAACAAA-3’ and 5’-TTCACTGCAGGGACTTACCC-3’; Survivin, 5’-GGACCACCAGCATCTACAT-3’ and 5’-GACAGAAAGGAAAGCGCAAC-3’; p53, 5’-GGCCCACTTCACCAGTCTACAA-3’ and 5’-GTGGTTTCAAGGCCAGATGT-3’; sGCα1, 5’-AGCAGTGATGGAGAGCTGGAT-3; and 5’-CTGATCCAGAGTGCAGTCCA-3’; PCBP4, 5’-TTTCTGGGTTATGGAGCAG-3’ and 5’-TTGGGACTCCAGGATAACAG-3’; p21, 5’-GA

CACCAGTGAGGATGACT-3’ and 5’-CAGGTCCACATGGTCTTTCTCT; GAPDH, 5’-CGACCACCTTTGTCAAGCTCA-3’ and 5’-AGGGGAGATTCAGTGTG-3’.

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**Western Blotting and Cell Fractionation**

Western blotting was performed as described (Cai *et al.*, 2007b) using primary antibodies against sGCα1 (Cayman Chemical), p53 (Santa Cruz Biotechnology), β-Actin (Abcam), hRARα (Santa Cruz Biotechnology), or MLK3 (Santa Cruz Biotechnology).

LNCaP cells from confluent 60-mm dishes were harvested and washed once with cold PBS. 10% of the cells were saved as input and the remaining portion was divided into nuclear and cytosolic fractions using Nuclear/Cytosol Fractionation Kit (MBL International). The fractions were then subjected to Western Blotting to measure sGCα1 and p53 protein levels.

**Immunoprecipitation**

IP experiments were done with either endogenous proteins in LNCaP cells or proteins expressed in E. coli. For the first, whole-cell extracts from LNCaP cells grown in 100-mm dishes were prepared and subjected to IP following a commercial protocol (from Santa Cruz) using Protein A/G plus Agarose (Santa Cruz). IP antibodies used were the anti-p53 (Santa Cruz) and anti-sGCα1 (Cayman Chemical).

For the E. coli expression, sGCα1, sGCβ1, or p53 were expressed in BL-21 cells and individual extracts were prepared and followed the protocol from Stratagene and subjected to IP experiments either as individual extracts or mixtures using the anti-p53 (Santa Cruz), anti-sGCα1 (Cayman Chemical), or anti-sGCβ1 (Cayman Chemical) antibodies.
Gel Filtration

Gel filtration chromatography was utilized to purify the protein complex associated with sGCα1. Cytosolic LNCaP cell extract was fractionated through a S-300 Sephacryl column (HiPrep) using high performance liquid chromatography (HPLC) (BioRad Bio-Logic Duo Flow HPLC), following Gel Filtration, Principles and Methods (GE Healthcare). Fractions were collected and subjected to Western to detect the elution profile of sGCα1 and p53.

cGMP Synthesis Assay

PC-3 cells were transfected with sGCα1 or sGCα1(D531A) mutant and sGCβ1 (Origene). After 48 hrs, the cells were harvested and subjected to a cGMP assay to measure intracellular cGMP levels by following the manufacturer’s protocol (Assay Designs).

Immunocytochemistry and Immunohistochemistry

Immunocytochemistry and immunohistochemistry were used to study the levels and subcellular localization of sGCα1 and p53 proteins in LNCaP cells and prostate tumors obtained from the Cooperative Human Tissue Network (CTHN), respectively. FITC labeled sGCα1 antibody (1:100 dilution; Santa Cruz Biotechnology) and TRITC labeled p53 antibody (1:100; Santa Cruz Biotechnology) were used for immunocytochemistry as previously described (ETV1-c-Jun paper) and immunohistochemistry using the Fluorescence staining protocol adapted from Vector Laboratories.
**p53 Signaling Pathway PCR Array**

LNCaP cells were cultured in a 60 mm-dish and transfected with control siRNA or sGCα1 siRNA. After two days of incubation, mRNA isolated using the Trizol reagent (Invitrogen) was subjected to human p53 Signaling Pathway PCR Array (SA Biosciences) to measure the expression of p53-regulated genes. All data analysis followed the procedure provided by SA Biosciences.

**CONFLICTS OF INTEREST**

All authors declare that there is no competing financial interest in relation to the work described in this paper.

**ACKNOWLEDGMENTS**

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LNCaP cells are associated with a protective effect of neuropeptides on etoposide-induced apoptosis. *Cell Biol Int* **28**: 397-402.


FIGURE LEGENDS

Figure 1. sGCα1 inhibits p53 transcriptional activity in sGCα1- and p53-positive prostate cancer cells.

(A) LNCaP, (D) VCaP, (E) C81, (F) CWR-22Rv1, and (H) PC-3 cells were transfected with 0.1 µg p53-Luc and 0.1 or 0.5 µg sGCα1, with or without 0.5 µg p53. (B) LNCaP cells were transfected with control (-) or sGCα1 (+) siRNA. p53 activity was quantified by measuring luciferase activity. All activities are relative to the first condition, and this activity was set to 1. Bar graphs represent averages of three independent experiments plus standard deviations. Asterisks indicate statistical significance (P<0.05). Western blotting was used to measure the expression of sGCα1 and p53 in LNCaP cells transfected with control (-) or sGCα1 (+) siRNA (C) or sGCα1 expression in CWR22-Rv1 cells treated with ethanol (-) or 1 nM R1881 (+) (G). β-actin expression was used to standardize the Western blot.
Fig. 1.
Figure 2. sGCα1-mediated repression of p53 is independent of NO signaling.

LNCaP cells were grown in the presence of (A) 5 or 50 μM ODQ, (A) 10 or 100 μM C-PTIO, (C) 10 or 100 μM SNP, or (D) 1, 10 or 100 μM 8-Br-cGMP and monitored for p53 transcriptional activity by measuring luciferase activity. In A and E, cells were transfected with 0.1 μg p53-Luc and (A) 0.5 μg p53 and 0.5 μg sGCα1 or (E) 0.5 and 1 μg sGCα1 or sGCα1(D531A) mutant. (F) PC-3 cells were transfected with 1 μg sGCβ1 and 1 μg sGCα1 or sGCα1(D531A) and measured for cGMP levels using a cGMP ELISA assay. All activities are relative to the first condition, and this activity was set to 1. Bar graphs represent averages of three independent experiments plus standard deviations. Asterisks indicate statistical significance (P<0.05). (G) Western blotting was used to measure the expression of sGCα1 or the sGCα1(D531A) mutant, which is shown in the schematic above.
Fig. 2.
Figure 3. sGCα1 affects p53-regulated gene expression involved in apoptosis/cell survival.

LNCaP cells were transfected with control siRNA and sGCα1 siRNA and subjected to (A) to real-time RT-PCR to measure the expression of p53-regulated genes, (B, E) MTT assay to measure cell viability (B) in serum-depleted medium or (E) in the presence of etoposide, as indicated, or (C) caspase assay to measure apoptosis. In (D), LNCaP cells were infected with control empty adenovirus (20 MOI) or sGCα1-expressing adenovirus (2 or 20 MOI) and treated with 10 µM etoposide, as shown, and subjected to MTT assay to measure cell viability. Bar graphs represent averages of three independent experiments plus standard deviations. All activities are relative to the first condition, and this activity was set to either 1 or 100%. Asterisks indicate statistical significance (P<0.05).
Fig. 3.
Figure 4. sGCα1 interacts with p53 and mediates p53 cytoplasmic localization.

LNCaP cytosolic extracts were subjected to (A) immunoprecipitation (IP) using antibodies against p53 (Left) or sGCα1 (Right) or (B) to fractionation through a Sephacryl S300 gel filtration column using HPLC. In either case, Western blotting was used to measure the (A) co-purification or (B) co-elution of sGCα1 and p53. (C) LNCaP cells were subjected to immunocytochemistry using anti-sGCα1 or anti-p53 antibody to measure subcellular localization of endogenous proteins in LNCaP (Upper) and CWR22-Rv1 (Lower) cells. (D) sGCα1, sGCβ1, and p53 were expressed in BL-21 cells and subjected to an IP using an antibody against sGCα1, sGCβ1, or p53 and Western blotting was used to measure co-IP. (E) LNCaP cells were transfected with sGCα1 siRNA and subjected to immunocytochemistry using anti-sGCα1 or anti-p53 antibody to measure subcellular localization of endogenous proteins. (F) LNCaP cells were transfected with sGCα1-GFP (Left) or sGCα1(D531A)-GFP (Right) and monitored for expression and subcellular localization of GFP-tagged sGCα1 protein and p53 by immunocytochemistry. In C, E, and F, sGCα1 protein is green and p53 is red, colocalization is shown in yellow in the Merge image, and nuclei are blue from DAPI staining. All images were viewed by confocal microscopy. (G, H) LNCaP cells were (G) transfected with sGCα1 siRNA or (H) infected with a sGCα1 adenovirus and subjected to cell fractionation, followed by Western blotting to measure the cytosolic (C) or nuclear (N) levels of sGCα1 and p53. Note the numbers above the lanes represent percentage of p53 protein levels in sGCα1 siRNA-transfected cells as compared to control siRNA, sGCα1 adenovirus-infected cells as compared to control virus, or in C or N cell fractions as compared to the sum of C and N fractions.
Figure 5. sGCα1 and p53 are co-expressed and colocalized in primary prostate tumors.

(A) The mRNAs isolated from 30 different prostate tumors were subjected to semi-quantitative RT-PCR to measure the gene expression of p53, sGCα1 and GAPDH, which served as a loading control. All bar graphs represent expression levels of either p53 or sGCα1 relative to GAPDH levels. (B) Prostate cancer tissue was subjected to immunohistochemistry using anti-sGCα1 or anti-p53 antibody to measure subcellular localization of endogenous proteins sGCα1 protein is green (i) and p53 is red (ii), colocalization is shown in yellow in the Merge image (iii), and nuclei are blue from DAPI staining (iv). All images were viewed by confocal microscopy. (C) Model of sGCα1 action on p53 in prostate cancer cells. Over-expression of sGCα1 results in p53 cytoplasmic accumulation, which can result from reduced nuclear import, enhanced nuclear export, and perhaps decreased proteosomal degradation.
Fig. 5
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Endogenous levels of p53 can be altered in LNCaP cells, but result in no change in cell proliferation.

LNCaP cells were transfected with 0.1 µg p53-Luc and (A) 0.1 or 0.5 µg p53 or (C) 50 nM p53 siRNA and monitored for p53 transcriptional activity. (B, F) LNCaP cells were transfected with control or sGCα1 siRNA and measured for (B) p53 protein expression by Western blotting and (F) cell proliferation by MTT assay. (D, E) LNCaP cells were transfected with (D) 0.5 µg sGCα1 and protein expression was measured by Western blotting or (E) 0.5 µg sGCα1 and 0.1 µg ARE4-Luc and AR transcriptional activity was monitored. (A, C) p53 and (E) AR transcriptional activity was quantified by measuring luciferase activity. All activities are relative to the first condition, and this activity was set to 1. Bar graphs represent averages of three independent experiments plus standard deviations. Asterisks indicate statistical significance (P<0.05).
Fig. S1
Supplementary Figure 2. Knock-down of sGCα1 blocks the proliferation of VCaP cells.

(A) VCaP cells were grown in the absence (-) or presence (+) of 1nM R1881, as indicated, measured for expression of sGCα1 and p53 by Western blotting. (B, C) VCaP cells grown in the presence of 1nM R1881 were transfected with 50 nM control or sGCα1 siRNA and measured for (A) sGCα1 protein expression by Western blotting or (C) cell proliferation by MTT assay. β-actin expression was used to standardize as a loading control.
Figure S2

(A) Western blot analysis of sGCα1 and β-Actin in R1881 and VCaP cells. 

(B) Immunoblot analysis of sGCα1 and β-Actin in control siRNA and sGCα1 siRNA-transfected VCaP cells.

(C) Graph showing cell density (A595 units) over days 0, 3, and 6 for control siRNA and sGCα1 siRNA-transfected VCaP cells.
Supplementary Figure 3. Knock-down of sGCα1 affects p53-regulated gene expression.

(A) mRNAs were prepared from LNCaP cells transfected with control siRNA and sGCα1 siRNA and were subjected into two p53 signaling pathway RT² profiler™ PCR array modules. Gene profiles from two treatments were compared and only those changed were selected. (B) The cDNAs from above was subjected to real-time RT-PCR to measure p21 and sGCα1 gene expression. Asterisks indicate statistical significance (P<0.05).
### A.

<table>
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<td>p53-regulated apoptosis-inducing protein 1</td>
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<td>D (4.42)</td>
<td>Baculoviral IAP repeat-containing 5 (Survivin)</td>
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</tbody>
</table>

### B.

![Graphs showing relative mRNA expression](Fig_S3)

Fig. S3
Supplementary Figure 4. Addition of GFP tag does not affect the sGCα1 inhibition of p53 transcriptional activity.

(A) LNCaP cells were transfected with 0.1 µg p53-Luc and 0.5 µg sGCα1-GFP or sGCα1(D531A)-GFP and p53 activity was quantified by measuring luciferase activity. All activities are relative to the first condition, and this activity was set to 1. Bar graphs represent averages of three independent experiments plus standard deviations. Asterisks indicate statistical significance (P<0.05). (B) LNCaP cell fractions were analyzed by Western blotting for presence of p53, sGCα1, MLK3 (a cytosolic marker), and hRARα (a nuclear marker). Note that C represents the Cytosolic Fraction and N the Nuclear Fraction.
Fig. S4
Additional results

The Region of sGCα1 is Essential for Inhibition of p53 Transcriptional Activity

Our previous data (see Fig. 1-II) show that sGCα1 can block p53 transcriptional activity. To analyze which region(s) of sGCα1 is/are required for p53 inhibition, sGCα1 truncation analysis was employed. Full-Length sGCα1, together with two sGCα1 C-terminal deletions (1-366) and (1-460) and two N-terminal deletions (367-690) and (132-690), were expressed using the pcDNA3.1 His/Myc expression plasmid (Fig.1A-III). A luciferase reporter plasmid containing p53-responsive elements was used to measure p53 transcriptional activity.

As shown in Fig.1B-III, full-length sGCα1 repressed 60% of p53 activity (Lane 1,2), as shown earlier (see Fig. xxx). Surprisingly, none of the four sGCα1 deletion mutants exhibited a comparable negative effect on p53 activity. sGCα1(132-690) inhibited p53 activity by only 20%, while, sGCα1(1-366) actually induced p53 activity by 40%; the other truncations had no significant effect. The expression levels of full-length sGCα1 and four truncations were tested using transfected Cos cells. Western blotting detected protein expression for all mutants except sGCα1(132-690), suggesting that this plasmid is nonfunctional.
**sGCα1 Physically Interacts with the N-terminus of p53**

Unlike sGCα1, p53 has a well-characterized structure and functional domains. Since sGCα1 was shown to interact with p53 *in vitro* and *in vivo* in Fig.4-II, a mammalian two-hybrid system was used to study which region(s) of p53 is/are responsible for this interaction. sGCα1 was expressed as a fusion protein with the VP16 activation domain, giving VP16-sGCα1. Full-length p53 and three p53 truncations, which were obtained from Dr. Spinella, were expressed as fusions with the GAL4 DNA-binding domain, yielding GAL4-p53 (Fig.2A-III). Protein interactions between sGCα1 and p53 were measured using the pGL5-luciferase reporter gene in transfected Cos cells. The VP16-GAL4 fusion protein was used as a positive control for the reporter plasmid (Lane 11).

Because p53 is a transcriptional activator, GAL4-p53 alone should be able to turn on luciferase activity without a VP16 fusion protein, which was what we observed (Fig.2B-III). The truncation mutants GAL4-p53(Δ102-393) and GAL4-p53(Δ117-274) had 493- and 7.1-fold increase in activity, respectively, as compared to GAL4 vector (Lane 1 and 3 or 5 or 6). Importantly, when co-transfecting VP16-sGCα1 with these GAL4-p53 constructs, luciferase activity was significantly enhanced for GAL4-p53 and GAL4-p53(Δ102-393) (Lane3, 7 and 5, 9), suggesting that the N-terminus of p53 may be responsible for its interaction with sGCα1. Western blotting of sGCα1 and VP16-sGCα1 protein levels were detected by the antibody against sGCα1 and VP16, respectively, indicating the VP16-sGCα1 is expressed (Fig.2C-III).
Studying the Role of sGCα1 in p53 Cytoplasmic Sequestration

sGCα1 can influence p53 subcellular localization by mediating p53 cytoplasmic sequestration in prostate cancer cells (Fig. 3-III). Because p53 has multiple regulatory roles and dynamically shuttles between the cell nuclei and cytoplasm, we wanted to determine the subcellular source of p53 cytoplasmic sequestration mediated by sGCα1. Nutlin3, a potent p53-Mdm2 inhibitor, was introduced to block p53 nuclear export, resulting in an increase in p53 transcriptional activity (92). If sGCα1 mediates p53 cytoplasmic sequestration by inducing nuclear export, then Nutlin3 should disrupt this activity and thus block sGCα1 inhibition of p53 transcriptional activity. As shown in Fig.3-III, sGCα1 from two different expression plasmids suppresses p53 activity by up to 60% (Lane 1 and 3 or 5). Addition of Nutlin3 led to 40% induction of p53 activity. (Lane 1,2), consistent with previous work (92). Nutlin3 was able to partially, but not fully, alleviate the sGCα1-mediated inhibition of p53 transcriptional activity (Lane 3,4 and Lane 5,6) (Lane 1 and 4 or 6). These data suggest that the sGCα1 negative effect on p53 probably not mediated by enhanced p53 nuclear export.

The Biological Significance of sGCα1 Overexpression in Prostate Cancer

To study the biological relevance of sGCα1, we generated LNCaP cell lines that stably overexpress sGCα1. Western blotting was used to show different sGCα1 protein expression among 11 different sGCα1 stable cell lines, designated LNα1-1~11, all higher than the control line LN (Fig.4A-III). LNα1-6 expressed the highest sGCα1 protein
levels, LNα1-3 and LNα1-5 had the second highest expression, and LNα1-4 had the lowest sGCα1 expression. Interestingly, androgen induction of sGCα1 mRNA expression was observed in both LN and LNα1-4 cells, but not LNα1-6 cells (Fig. 4B-III). Similar results were obtained with sGCα1 protein (Fig. 4C-III).

To determine if sGCα1 overexpression affected the AR signaling, we measured the expression of AR and an AR target, PSA. As shown in Fig. 4C-III, sGCα1 overexpression did not significantly affect AR protein levels in either the absence or presence of R1881. The same result was obtained with PSA, an AR target gene, since the same androgen induction was observed at both the mRNA (Fig. 4B-III) and protein (Fig. 4C-III) levels in sGCα1-overexpressing lines as in the control LN line. These results clearly demonstrate that sGCα1 overexpression does not affect the AR signaling.

To confirm the cytoplasmic localization of overexpressed sGCα1 in the LNCaP cell lines, immunocytochemistry was used. As shown in Fig.5-III, sGCα1 protein (green) was exclusively cytoplasmic and the expression was significantly higher in LNα1-6 and LNα1-4 than in control LN cells, consistent with RT-PCR and Western blotting results (see Fig. 4B,C-III). Interestingly, endogenous p53 protein levels (red) increased in sGCα1-overexpressing lines. More importantly, the increased p53 was colocalized with sGCα1 in the cytoplasm of LNα1 cells, implying that overexpressed sGCα1 mediates p53 cytoplasmic accumulation in LNα1 cells (Fig.5-III).

To obtain a quantitative measure of sGCα1 and p53 subcellular distributions, Western blotting was performed on nuclear and cytoplasmic cell fractions. Total sGCα1 and p53 protein of LNα1-6 and LN are shown in the left panel of Fig.6A-III. sGCα1
overexpression in LNα1-6 cells led to significantly less nuclear and more cytosolic p53 protein levels, as compared to LN cells (Fig. 6A-III), confirming the immunocytochemistry data. The data above led us to hypothesize that p53 transcriptional activity is lower in LNα1-6 than LN cells. Surprisingly, however, a luciferase assay measuring p53 activity indicated that p53 activity in LNα1-6 was not diminished as compared to LN cells (Fig. 6B-III), suggesting that the effect of overexpressed sGCα1 on p53 transcriptional activity is complex.

**sGCα1 Overexpression Sensitizes LNCaP Cells to Low Levels of Androgens**

To examine the requirement of androgens in the cellular growth of sGCα1-overexpressing cells, the effect of a wide range of androgen concentrations was studied. As shown in Fig. 7A-III, over a 6-day period, the growth of control LN cells was fully activated at 10^{-10} M of R1881. In contrast, under the same conditions, LNα1-4 and -6 cells were more sensitive to a lower amount of R1881 (10^{-11} M). It is should be noted that LNα1-4 and -6 cells exhibited small and positive effects on cell growth even in the vehicle and 10^{-12} M of R1881 (Fig. 7B-III). Taken together, this data suggested that overexpressed sGCα1 made LNCaP cells more sensitive to low androgen conditions and more importantly, promoted them to become hormone-refractory prostate cancer cells.

**LNα1 Cells Activate PI3K-Akt Signaling Pathway**

To understand which signaling pathway(s) is/are responsible for sGCα1-mediated cell proliferation, we used inhibitors of PI3K and MAPK signaling, two important proliferation pathways. As shown in Fig. 8A-III, the LY294002, a PI3K inhibitor,
dramatically inhibited the viability of both LN and LNα1-6 cells in a dose-dependent manner, while the MAPK inhibitor UO126 had only a modest negative effect. These data suggest that PI3K-Akt signaling plays a crucial role in the cell proliferation of the sGCα1-overexpressing LNCaP cells. Western blotting was used to examine the status of the PI3K-Akt signaling components. Interestingly, the levels of Phospho-Akt (Ser473) and Phospho-Akt (Thr308) were significantly higher in LNα1-4 and LNα1-6 cells than LN cells, even when taking into account the small increase of total Akt in the sGCα1-overexpressing cells (Fig.8B-III). This markedly elevated Akt activation may result from significantly enhanced levels of phosphorylated PDK1 and subsequently induced activation of GSK-3β in LNα1 cells, whereas control LN cells exhibited no/low levels of these phospho-proteins (Fig.8B-III). These results indicate that the overexpressed sGCα1 provides LNα1 cells elevated Akt activation and suggest that this may be responsible for their higher cell proliferation rates.

**Peptides Mimicking the sGCβ1 Domains that Interact with sGCα1**

sGC enzyme activity requires the sGCα1/sGCβ1 heterodimer. However, in prostate cancer endogenous sGCβ1 expression is significantly lower than sGCα1 (79), suggesting an independent role for sGCα1 in prostate cancer. Transient transfection in LNCaP cells was used to study a potential role for sGCβ1 in sGCα1-mediated repression of p53. Exogenous p53 yielded significant transcriptional activity by over 2-fold (Fig. 9A-III). Transfection of sGCα1 inhibited p53 activity to below the basal level. Interestingly, co-transfection of sGCβ1 alleviated significantly, but not completely, the sGCα1 inhibition, suggesting that the sGCβ1 dimerization with sGCα1 intereferes with
the latter protein’s ability to block p53. Therefore, we designed peptides mimicking known sGCβ1 interaction domains with sGCα1 to attempt to impede sGCα1-mediated p53 repression. We hypothesized that this would result in p53 release from sGCα1 inhibition, and thus resumption of its tumor-suppressing functions. Four peptides designated as A, B, C, and D were made (Fig.9B-III), all attached to eight arginines residues (8R) at the C-terminus to facilitate membrane translocation.

**Peptide A-8R is Cytotoxic to LNCaP Cells**

Peptides A-8R, B-8R, C-8R and D-8R were tested at increasing concentrations from 1 nM to 50 μM over 6 day-period of time. As shown in Fig. 10A-III, Peptide A-8R and Peptide B-8R significantly reduced cell viability at 10 μM (blue line) by day 6, while vehicle had no effect (brown line). In contrast, there was no cytotoxic effect by either Peptide C-8R and Peptide D-8R. It should be noted that the drop in a cell number found throughout four peptide treatments at day 6 may be due to the cell growth having reached a saturation point, therefore triggering the cell death (Fig.10A-III).

To study further the peptide cytotoxic effect on LNCaP cells, we increased the peptide concentration up to 50 μM. Fig.10B-III shows that Peptide A-8R inhibited cell growth at 10 or 25 μM, resulting in nearly complete cell death at 50 μM. Peptide B-8R stopped cell proliferation but did not cause cell number to decrease. On the other hand, Peptide C-8R and Peptide D-8R had no significant cytotoxic effect. Taken together, this data indicated that Peptide A-8R was cytotoxic to LNCaP cells in a dose-dependent manner.
**Peptide A-8R Associates with sGCα1**

To determine whether Peptide A-8R interacts with sGCα1 in the cytoplasm of the cells, Peptide A-8R tagged with Biotin was used. The Biotin allowed us to carry out immunocytochemistry and pull-down experiments. Using an anti-Biotin antibody, immunocytochemistry analysis showed a cytoplasmic colocalization of Peptide A-8R and sGCα1 (yellow) (Fig.11A-III). These results suggest that Peptide A-8R associates with sGCα1 in the cytoplasm of LNCaP cells. To confirm this, a pull-down experiment using streptavidin-agarose was done with LNCaP cell extracts preincubated with Peptide A-8R-Biotin. As shown in Fig.11B-III, streptavidin-agarose beads only pulled down sGCα1 with Peptide A-8R-Biotin, suggesting that Peptide A-8R interacts with sGCα1. Importantly, the cytotoxic effect of Peptide A-8R-Biotin was comparable to Peptide A-8R (Fig.11C-III). These data show that Peptide A-8R can enter prostate cancer cells and interact with endogenous sGCα1.

**The Cytotoxic Activity of Peptide A-8R Requires the Membrane-Translocation Signal**

To examine whether the 8 arginines (8R) at the C-terminus of Peptide A-8R was essential for its cytotoxicity, we tested Peptide A without the arginines. As shown in Fig.12A-III, elimination of the arginines greatly reduced the cytotoxic activity of Peptide A, strongly suggesting that cell internalization is required for cytotoxicity.

**Peptide A-8R is Cytotoxic to Hormone-Refractory Prostate Cancer Cells**
sGCα1 is constitutively expressed in hormone-refractory prostate cancer cells, such as CWR22-Rv1 cells; in contrast, sGCα1 expression is inducible by androgens (R1881) in hormone-dependent LNCaP cells (Fig.13A-III). Since all earlier studies were done with hormone-dependent LNCaP cells, we wanted to test Peptide A-8R activity in hormone-refractory cells. Peptide A-8R had weak cytotoxicity on CWR22-Rv1 cells at 10 and 25 µM, whereas cell growth was stopped at 50 µM, importantly showing that Peptide A-8 was cytotoxic also to hormone-refractory prostate cancer cells.

**Peptide A-8R is Weakly Cytotoxic to sGCα1-Deficient Cancer Cells**

To address whether Peptide A-8R was cytotoxic to sGCα1-negative cells, PC-3, a prostate cancer cell line and Cos, a transformed monkey kidney cell line, were used. Western blotting showed that no detectable sGCα1 protein in PC-3 and Cos cells (Fig.14A-III). Interestingly, the MTT assay indicated that Peptide A-8R had a modest negative effect on either PC-3 (Fig.14B-III) or Cos (Fig.14C-III) cells, suggesting that Peptide A-8R cytotoxicity depends on endogenous sGCα1.

**Peptide A-8R Induces Apoptosis in LNCaP Cells**

Since cell death can be induced by apoptosis and/or necrosis, we wanted to know which death pathway was triggered by Peptide A-8R. LNCaP, PC-3 and Cos cells were selected for a fluorescent-based assay, which detects apoptosis by measuring Caspase (3/7) activity. Fig.15A-III shows that the apoptotic activity was induced by Peptide A-8R (gray bar) after only one of treatment and reached a peak at 8 hours. In contrast, Peptide A-8R did not induce apoptosis in either PC-3 or Cos cells, two sGCα1-deficient cell lines.
Interestingly, Etoposide (black bar), a known inducer of apoptosis, reached apoptosis levels comparable to Peptide A-8R only after 24 hours (Fig. 15-III). These data show that the apoptosis induced by Peptide A-8R is rapid (within 8 hours) and only found in sGCα1-positive LNCaP cells.

**Peptide A-8R-Induced Apoptosis Does Not Require Endogenous p53**

To determine whether p53 plays a role in mediating Peptide A-8R-induced apoptosis, we used siRNA to knock-down endogenous p53. As shown in Fig.16A-III, transfection of p53 siRNA had no effect on Peptide A-8R cytotoxicity on LNCaP cells. Western blotting showed that p53 protein levels were reduced while transfected with p53 siRNA (Fig.16B-III). These data clearly demonstrate that endogenous p53 is not required for Peptide A-8R-induced cytotoxicity.

**The Effect of Peptide A-8R on LNCaP Mouse Xenograft Tumor**

A LNCaP mouse xenograft model was used to study a potential anti-tumor activity of Peptide A-8R. LNCaP cells were injected into both flanks of a nude mouse. When the two tumors reached a size of ~500 mm³, Peptide A-8R treatment began. Peptide A-8R (8mg/kg) and vehicle were injected into the Left and Right tumor, respectively, every two days. As shown in Fig. 17-III, the size of the Peptide A-8R-treated tumor (red) began to slow down after 4 days of treatment and remained smaller throughout the entire period, when compared to vehicle treatment (black), suggesting that Peptide A-8R can hinder prostate tumor growth.
Figure 1. The Region of sGCα1 is Essential for Inhibition of p53 Transcriptional Activity.

(A) A schematic structure of sGCα1: the regulatory domain is at the N-terminus flanked by dimerization domains and catalytic domain is at C-terminus. Full-length sGCα1 and sGCα1 truncations including (1-366), (1-460), (367-690), and (132-690) were subcloned to a His/Myc expression plasmid. (B) Western blotting was used to measure the expression of these proteins in Cos cells. The projected molecular weight is indicated left. (C) p53 transcriptional activity was measured by a luciferase assay. LNCaP cells were transfected with 0.25 µg PCH110, 0.25 µg p53-Luc and 0.5 µg full-length sGCα1 or sGCα1 truncations. All results are corrected for β-galactosidase activity and related to the first condition, and this activity was set to 1. Bar graphs represent averages of three independent experiments plus standard deviations. Asterisks indicate statistical significance (P<0.05).
Dissertation additional data V7 (3/25/10)

A. Structure of sGCα1

B. Projected MW (kDa)

FL-sGCα1: 72
(367-690): 34
(1-460): 48
(1-366): 38
(132-690): 58

WB: α-His

C. Relative p53 Activity

Fig. 1-III
Figure 2. sGCα1 Physically Interacts with the N-terminus of p53.

(A) A schematic of p53 deletion mutants: p53 domains are indicated and include the transactivation (TA), proline rich (PXXP), DNA binding (DBD), nuclear localization (NL), oligomerization (Oligo), and regulatory (Basic) domains. (B) Cos cells were cotransfected with 0.25 µg of PCH110, GAL4-Luc reporter with or without VP16-sGCα1 and with or without GAL4-p53 (full length), GAL4-p53 deletion mutants. (C) Western blotting using sGCα1 and VP16 antibodies were used to measure the VP16-sGCα1 fusion protein expression in Cos cells. All luciferase results are corrected for β-galactosidase activity and related to the first condition, and this activity was set to 1. Bar graphs represent averages of three independent experiments plus standard deviations. Asterisks indicate statistical significance (P<0.05).
A. Schematic GAL4-p53 structures

B. Bar chart showing relative luciferase activity for different constructs.

C. Western blot (WB) showing 
- α-sGCα1
- α-VP16
Figure 3. Studying the Role of sGCα1 in p53 Cytoplasmic Sequestration.

LNCaP cells were transfected with 0.25 µg PCH110, 0.25 µg p53-Luc and 0.5 µg sGCα1/pCI-Neo or sGCα1/pLenti6 with or without Nutlin3 (10µM) treatment. All results are corrected for β-galactosidase activity and related to the first condition, and this activity was set to 1. Bar graphs represent averages of three independent experiments plus standard deviations. Asterisks indicate statistical significance (P<0.05).
Figure 4. Generation of LNCaP Cells that Stably Overexpress sGCα1.

(A) Western blotting was used to measure the sGCα1 expression from LNCaP cells that are stably overexpressed sGCα1 (LNα1). LN represents parental/control LNCaP cells. Pooled is the pooled population of stable cells and different numbers above represent different colonial lines. LN, LNα1-4 and LNα1-6 cells were treated with ethanol (vehicle), 0.1 nM and 1 nM of R1881, respectively and then subjected to (B) semi-quantitative RT-PCR to measure sGCα1 and PSA expression and (C) Western blotting of sGCα1, AR and PSA. GAPDH and β-actin were used as a loading control for semi-quantitative RT-PCR and Western blotting, respectively.
Figure 5. The Subcellular Localization of sGCα1 and p53 in LNα1 Cells.

LN, LNα1-4 and LNα1-6 cells were subjected to immunocytochemistry using anti-sGCα1 or anti-p53 antibody to measure subcellular localization of endogenous protein levels. sGCα1 protein is green and p53 is red, colocalization is shown in yellow in the Merge image, and nuclei are blue from DAPI staining. All images were viewed by confocal microscopy.
Figure 6. sGCα1 Mediates p53 Subcellular Redistribution Not p53 Activity.

(A) LN and LNα1-6 cells were subjected to Western Blotting (Total) or cell fractionation, followed by Western blotting to measure the cytosolic (C) or nuclear (N) levels of sGCα1 and p53. β-actin was used as a loading input. (B) LN and LNα1-6 cells were transfected with 0.5 mg PCH110, 0.5 mg p53-Luc. All results are corrected for β-galactosidase activity and related to the first condition, and this activity was set to 1.
A.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Total</th>
<th>Cytoplasm</th>
<th>Nuclear</th>
</tr>
</thead>
<tbody>
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<td>p53</td>
<td></td>
<td></td>
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<tr>
<td>sGCα1</td>
<td></td>
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<tr>
<td>β-actin</td>
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</tbody>
</table>

B.

Relative p53 Activity

- p53-Luc
- LN
- LNar1-6
Figure 7. sGCα1 Overexpression Sensitizes LNCaP Cells to Low Levels of Androgens.

LN, LNα1-4 and LNα1-6 cells were treated with ethanol (vehicle) or R1881 (A) ranging from $10^{-8}$ to $10^{-12}$ M and were monitored cell growth at day 0, 3, 6; (B) ranging from $10^{-9}$ to $10^{-12}$ and were monitored at day 6.
A.

B.
Figure 8. sGCα1 Overexpression Induces Activation of Akt signaling pathway.

(A) LN and LNα1-6 cells were treated with LY294002 (25 or 100 mM) and UO-126 (5 or 20 mM) and were subjected to a cell viability (MTT) assay after a 2-day treatment. (B) Western blotting of LN LNα1-4 and LNα1-6 cells was used to measure total-Akt, p-Akt(T308), p-Akt(S473), p-PDK1, sGCα1 and β-actin, a total protein loading control.
Figure 9. Peptides Mimicking the sGCβ1 Domains that Interact with sGCα1.

(A) LNCaP cells were transfected with 0.25 mg PCH110, 0.25 mg p53-Luc and/or 0.25 mg p53 and/or sGCα1 and/or sGCβ1. All results are corrected for β-galactosidase activity and related to the first condition, and this activity was set to 1. Bar graphs represent averages of three independent experiments plus standard deviations. Asterisks indicate statistical significance (P<0.05). (B) A schematic structure of sGCα1: the regulatory domain is at the N-terminus flanked by dimerization domains and catalytic domain is at C-terminus. A, B, C, and D are the peptides that mimic sGCα1 interacting domains found on sGCβ1. Additional 8 arginines (8R) were attached at the C-terminus of the peptides to facilitate membrane translocation.
Fig. 9-III
Figure 10. Peptide A-8R is Cytotoxic to LNCaP Cells.

LNCaP cells were treated with vehicle (DMSO) and Peptide A-, B-, C- and D-8R with the concentration ranging (A) from $10^{-9}$ to $10^{-5}$ M over a 6-day period. (B) 10, 25 and 50 mM over a 4-day period. Asterisks indicate statistical significance (P<0.05).
Fig. 10-III
Figure 11. Peptide A-8R Associates with sGCα1.

LNCaP cells were treated with Peptide A-8R-biotin and then subjected to (A) immunocytochemistry, (B) western blotting of biotin-streptavidin pull-down and (C) cell viability (MTT) assay. (A) Antibodies against sGCα1 or biotin were used to measure subcellular localization of Peptide A-8R-biotin and endogenous sGCα1. sGCα1 protein is green and Peptide A-8R-biotin is red, colocalization is shown in yellow in the Merge image, and nuclei are blue from DAPI staining. All images were viewed by fluorescent microscopy. (B) Western blotting was used to measure endogenous or co-purified sGCα1. (C) Cells were assayed at beginning of the assay, 12 and 48 hours post-treatment using different concentrations of peptides as indicated. Asterisks indicate statistical significance (P<0.05).
A.

\[ \alpha\text{-sGC}\alpha1 \quad \text{A-8R-Biotin} \quad \text{Merge} \quad \text{Merge + DAPI} \]

B.

- Peptide A-8R-Biotin
  - +
  - -
- Beads Pulldown
  - - +
- \[ \alpha\text{-sGC}\alpha1 \]

1 2 3

C.

![Graphs showing cell density over hours of treatment for A-8R and A-8R-Biotin with different peptide treatments.](Fig.11-III)
Figure 12. The Cytotoxic Activity of Peptide A-8R Requires the Membrane-Translocation Signal.

MTT assay was used to measure the cell viability in response to Peptide A-8R and Peptide A in LNCaP cells. Cells were assayed at beginning and 2, 4 days post-treatment using different concentrations as indicated. Asterisks indicate statistical significance (P<0.05).
Fig. 12-III

The graphs show the effect of different concentrations of Peptide A and Peptide A-8R on cell density over a period of 4 days. The x-axis represents the number of days, while the y-axis represents cell density (A₂₅₀ units).

- **Peptide A-8R**
  - **Vehicle**
  - **10μM**
  - **25μM**
  - **50μM**

- **Peptide A**
  - **Vehicle**
  - **10μM**
  - **25μM**
  - **50μM**

The graphs indicate a trend where higher concentrations of Peptide A-8R generally result in lower cell density compared to Peptide A, especially at later time points.
Figure 13. Peptide A-8R is Cytotoxic to Hormone-Refractory Prostate Cancer Cells.

(A) Western blotting was used to measure sGCα1 protein expression with ethanol or R1881 (1nM) treatment in LNCaP or CWR22-Rv1 cells. (B) MTT assay was used to measure cytotoxicity of Peptide A-8R to CWR22-Rv1 cells. Cells were assayed at beginning and 2, 4 days post-treatment using different concentrations as indicated.
A. sGCx1

R1881

LNCaP CWR22-Rv1

B. CWR22-Rv1

Peptide A-8R

- vehicle
- 10μM
- 25μM
- 50μM

Days of Treatment

Cell Density (A105g/mL)

Fig. 13-III
Figure 14. Peptide A-8R is Weakly Cytotoxic to sGCα1-Deficient Cancer Cells.

(A) Western blotting was used to measure sGCα1 protein expression in LNCaP, PC-3 and Cos cells. (B) MTT assay was used to measure cytotoxicity of Peptide A-8R to PC-3 and Cos cells. Cells were assayed at different time points and various concentrations as indicated.
Figure 15. Peptide A-8R Induces Apoptosis in LNCaP Cells.

A caspase-based assay was used to monitor the toxic effect of vehicle (DMSO), Peptide A-8R and etoposide in (A) LNCaP, (B) PC-3 and (C) Cos cells at different time points as indicated.
Figure 16. Peptide A-8R-Induced Apoptosis Does Not Require Endogenous p53.

LNCaP cells were transfected with control siRNA and p53 siRNA in the absence or presence of Peptide A-8R and subsequently were subjected to (A) MTT assay to measure cell viability and (B) western blotting. (A) Cells were assayed in a time-dependent and dose-dependent manner. (B) Western blotting was used to measure p53 protein expression and b-actin serving as a loading control.
Fig. 16-III

A.

Control siRNA

Cell Density (A₅₇₀ units)

Hours of Treatment

p53 siRNA

Peptide A-8R

- vehicle
- 10μM
- 25μM
- 50μM

B.

p53

β-actin

Ctrl

siRNA

p53

siRNA
Figure 17. The Effect of A-8R on LNCaP mouse Xenograft tumor.

LNCaP xenograft tumors were developed on both flank of the mouse. When the tumors reached ~500 mm³, injections of vehicle (DMSO) at right flank and Peptide A-8R (8mg/kg) were taken place every 2 days. The tumor volume was monitored at different time points as indicated.
Fig. 17-III

Tumor Volume (mm³) vs. Treatment (Days)

- **Vehicle**
- **Peptide A-8R (8 mg/kg)**

Right Flank
Left Flank

$n=1$
Discussion

The Role of AR in Hormone-Dependent Prostate Cancer and Castration-Resistant Prostate Cancer

When normal epithelial prostate cells transform to prostatic adenocarcinomas, a number of molecular changes take place. These include uncontrolled cell division, escape from apoptosis, sustained angiogenesis, metastasis/aggressiveness, and resistance to chemotherapeutics (86). Importantly, these malignant cellular changes require the action of AR, which needs to be activated by androgens. This stage of the disease that depends on ligand-activated AR, is known as the hormone-dependent prostate cancer. Thus, the current strategy for this disease aims to reduce/block serum androgens and impede AR activity. To achieve this objective, current therapies include the orchiectomy, a physical castration and injection of GnRH agonist, a chemical castration (androgen ablation), and treatment with antiandrogens, such as flutamide and bicalutamide, to compete with natural androgen binding (antiandrogen therapy). Also, a combination of these two treatments, called maximal androgen blockade (MAB) (87), is used.

Despite the initial efficacy of such treatments, most tumors eventually relapse and regrow in a hormone-refractory manner, better known as castration-resistant prostate cancer (CRPC). Importantly, the AR protein still is expressed in most of tumors, even after androgen deprivation and the appearance of CRPC (88). Moreover, PSA, a classical
AR-regulated gene, has been detected in majority of CRPC patients, indicating that AR signaling remains functional. Several possible mechanisms have been proposed to explain the transition of prostate cancer from the initial hormone-dependent stage to the hormone-independent CRPC stage: AR gene mutation, AR amplification/overexpression, overexpression of AR coactivators, adrenal androgen synthesis, or AR cross-talk with cytokines and growth factors (45).

**The Hyperactive AR (VP16) Transforms LNCaP Cells to CRPC**

A large body of evidence has demonstrated that AR is involved in the development of CRPC. One common scenario is AR overexpression, which is observed in 22%~30% of CRPC (Reviewed in 88). Sawyers and colleagues reported that AR overexpression is sufficient for converting prostate cancer from hormone-dependent to hormone-refractory and converting antagonists, such as bicalutamide, to agonists (10). Thus, we postulated that expression of a hyperactive AR would be sufficient to drive hormone-dependent prostate cancer cells to hormone-independent. To test this hypothesis, stable LNCaP cell lines were generated that express a VP16-AR hybrid protein, which contains full-length AR fused to the strong viral transcriptional activation domain VP16 (97). Importantly, casodex only had an inhibitory effect on VP16-AR cell growth under the androgen treatment (Fig.3C-I), indicating that the hyperactive AR offers an androgen-independent growth that is also inducible by androgens. Interestingly, our data exhibited that bicalutamide did not affect the PSA gene expression in VP16-AR cells and seem not reconciled with previous study, (10) showing that higher AR levels convert bicalutamide to against. Sawyers and colleagues found that an increase level of
AR protein can alter the assembly and recruitment of coactivators or corepressors on the promoters of AR-regulated genes (10). In contrast to previous work (10), VP16-AR offers AR a hyper genomic activity resulting in a high and constitutive PSA gene expression as compared to C14, a parental LNCaP cells (see Fig.5E-I). Collectively, this data imply that hyper AR transcriptional activity can mediate prostate cancer progression and likely underlies the involvement of AR-regulated genes in this process.

**sGCα1 is a Onco-protein and Plays Important Roles in CRPC**

To identify novel AR-regulated genes, a gene microarray approach was utilized. Our laboratory has discovered that the alpha 1 subunit of soluble guanylyl cyclase (sGCα1, also known as GUCY1A3) is one such gene (79). Our published work has demonstrated that sGCα1 plays an essential role in prostate cancer growth (79), independent of the of sGCα1 conventional role in NO signaling (81,82). Indeed, disruption of sGCα1 expression severely compromised the growth of both androgen-dependent and androgen-independent AR-positive prostate cancer cells, including VP16-AR stable cells (See Manuscript I). Overexpression of sGCα1 alone is sufficient for stimulating prostate cancer cell proliferation in the absence of androgens (79). In fact, the gene expression data obtained from Oncomine (http://www.oncomine.org) indicating that sGCα1 expression is markedly elevated as prostate cancer progresses. More importantly, sGCα1 protein expression from a prostate tissue array exhibited an increasing trend of sGCα1 expression, consistent with sGCα1 transcript levels (79).

To study the biological relevance of sGCα1, LNCaP cells that stably overexpressed sGCα1 (LNα1 cells) were generated. Interestingly, the same growth of
LNα1 cells can be achieved at a 10-fold lower amount of androgens than LN, a parental LNCaP cell line (Fig.7B-III). Moreover, LNα1 exhibited a modest positive cell growth in very low androgen condition (10^{-12} M), indicating that the overexpression of sGCα1 may promote hormone-refractory prostate cancer cell growth. Taken together, our results suggest that sGCα1 may function as a potent oncoprotein that mediates the prostate cancer progression.

**Mechanisms by which sGCα1 Modulates Prostate Tumorigenesis**

**sGCα1-Mediated p53 Inhibition**

One recent study showed that basal sGC reduces p53 protein levels and p53-dependent apoptosis (84). It was shown that treating cells with OQD, a specific inhibitor of sGC enzyme activity, resulted in increased p53 content and enhanced apoptosis, suggesting that this p53 inhibition depended on sGC enzymatic activity (84). In contrast to this, we demonstrate here that sGCα1, a component of the sGC enzyme, blocks p53 transcriptional activity independent of NO signaling. This was shown by using chemical inhibitors of NO signaling, as well as a guanylate cyclase-deficient mutant of sGCα1. Collectively, these data reveal a novel mechanism of sGCα1 action on p53 activity that is distinct from this protein’s guanylate cyclase activity and NO signaling.

To determine if sGCα1 affected gene expression, we used a PCR array that profiles 84 p53-regulated genes involved in apoptosis, cell cycle regulation, cell proliferation/differentiation and DNA repair. We focused on genes whose expression was changed greater than 2-fold. Interestingly, expression changed the most for genes associated with apoptosis, including p53AIP1 and PCBP4, p53-inducible genes, and
**Survivin** (also known as **BIRC5**), a p53-repressed gene (Fig.3-II). Among these genes, **Survivin** may be the most interesting. Indeed, Survivin makes prostate cancer cells insensitive to chemotherapy drugs used in prostate cancer (93). More importantly, Survivin expression is positively correlated with proliferative activity in prostate tumors, suggesting that the progression and aggressiveness of prostate cancer is aided by Survivin (94). Interestingly, RNAi silencing sGCα1 induced apoptosis and sensitized prostate cancer cells to Etoposide, a drug that can induce p53-dependent apoptosis (Fig.3-II). Collectively, sGCα1 selectively affects p53 genomic activity involved in cell survival.

Because p53 has multiple roles in maintaining normal cellular processes, mutation of p53 and disruption of its shuttling between cytoplasm and nucleus can be predispositions to malignancies. For example, germline mutations in the **TP53** gene may lead to Li-Fraumeni syndrome, a hereditary cancer predisposition disorder (89). Surprisingly, the frequency of p53 mutations in prostate cancer is low as compared to other cancers. p53 mutations are found in only ~20% in primary prostate cancer, but this frequency rises in advanced prostate cancer (90). Another mechanism for p53 inactivation is p53 nuclear exclusion, as proposed by Moll et al. (69). This redistribution of p53 can be regulated in an Mdm2-dependent or –independent manner. For example, Jab1 and glucocorticoid receptor (GR) facilitate Mdm2-dependent p53 nuclear exclusion, followed by proteasomal degradation (70,72). Additionally, p53-associated parkin-like cytoplasmic protein (Parc) functions as a cytoplasmic anchor that sequesters p53 within the cytoplasm, independent of Mdm2 regulation (71).

Our data show that endogenous sGCα1 interacts with endogenous p53 in the LNCaP cell cytoplasm (Fig.4-II) and this interaction depends on the N-terminus of p53.
Knockdown of sGCα1 using siRNA resulted in diminution of cytoplasmic p53, while overexpression of sGCα1 led to cytoplasmic p53 accumulation, suggesting sGCα1 mediates p53 cytosolic retention (Fig.5-II). It should be noted that LNCaP cells exhibit a wild-type p53 and Pten-minus genotype. Importantly, Pandolfi and colleagues (63) described that conditional inactivation of Trp53 in mouse prostate did not form tumors. However, a mouse having a combined Pten and Trp53 knockout develops advanced prostatic malignancy, suggesting that the absence of both Pten and p53 is important in prostate tumor initiation and development (66). In view of this report (66), we would like to propose that sGCα1 over-expression in tumors, which will result in suppression of wild-type p53, may collaborate with a Pten-loss to promote prostate cancer initiation and progression.

To study the clinical relevance of sGCα1 and p53, we analyzed gene expression from 28 primary prostate tumors. sGCα1 expression is highly correlated to p53 expression ($R^2=0.6121$) as shown in Fig.6A-II. Immunohistochemical analysis showed p53 and sGCα1 expression in both basal and luminal epithelial cells. Importantly, only cytoplasmic p53 protein was detected and this protein co-localized with sGCα1 (Fig.6B-II), mimicking what was observed in prostate cancer cell lines (see Fig.4C-II) and suggesting that the sGCα1-p53 interaction occurs in prostate tumors.

**sGCα1 Overexpression Induces Akt Activation**

After having generated the stable cell lines over-expressing sGCα1 (LNα1), we were interested to see if p53 activity was suppressed. Surprisingly, p53 transcriptional activity, as measured by reporter gene assay, was elevated, not repressed, in LNα1 cells
(Fig.6B-III). This unexpected difference between transiently and stably transfected sGCα1 may be explainable by cellular expression differences between the two systems, with transient transfection generally yielding higher protein expression than stable transfection. To determine if endogenous promoters may respond differently to sGCα1 stable over-expression than a promoter on a reporter gene plasmid, the expression of endogenous p53-regulated genes, particularly p53AIP1, PCBP4, and Survivin, will be measured in LNα1 cells and compared to control cells. Irrespective of these results, we already know that p53 does not play a direct role in LNCaP cell proliferation (see Fig.S1-II), making it unlikely that sGCα1 down-regulation of p53 activity is involved in the sGCα1 pro-proliferative activity in LNCaP cells. Thus, we opted to study other signaling pathways that sGCα1 may affect.

Interestingly, Akt signaling seems to be associated with sGCα1 in prostate cancer cells. As shown in Fig. 8B-III, the two LNα1 lines express strongly enhanced levels of phosphorylated and activated Akt, implying that over-expression of sGCα1 results in activation of Akt. Our results also show significantly increased levels of GSK-3β (see Fig.8B-III), which is known to modulate cyclin D1 gene expression via the Wnt/β-catenin signaling pathway, as well as cyclin D1 protein stability (26). Additionally, the cyclin D1/Cdk protein complex that is involved in cell cycle progression sequesters the CDK-inhibitory proteins p21Cip and p27Kip; resulting in the stimulation cell proliferation (26). This finding may shed some light on the mechanism by which sGCα1 over-expression offers castration-resistance for prostate cancer cell growth. Thus, we will further analyze the role of p21, p27 and cyclin D1 in sGCα1 over-expressing cells.
Since Phosphatase and tensin homolog (PTEN) is a negative regulator of PI3K-Akt signaling, PTEN loss is linked to a number of human cancers, including prostate cancer (27). Recently, Sawyers and colleagues developed a mouse model, in which transgenic TMPRSS2-ERG mice and PTEN+/− mice were crossed, and resulting in PIN prostate lesions (29). These data suggest a cooperativity between the Akt pathway and ERG aberration in prostate tumor initiation (29). It should be noted that LNα1 cells were derived from LNCaP cells, a PTEN-null cell line. Thus, we postulate that PTEN-loss and overexpression of sGCα1 results in hyperactivation of Akt kinase cascades and promotes cell cycle progression under an extra low androgen condition, implying that this synergistic effect is essential for developing CRPC.

Taken together, our preliminary data demonstrate that two pathways might be involved in this sGCα1 effect in prostate tumorigenesis. Firstly, sGCα1 induces the activation of Akt and its downstream targets that are associated with the cell proliferation (pro-proliferative) and important for prostate tumor initiation. Secondly, sGCα1 can associates with p53 in the cytoplasm and inhibits p53 transcriptional activity (anti-apoptosis) essential for prostate tumor progression.

**sGCα1 is a Novel Therapeutic Target in CRPC**

Several treatments are available for prostate cancer. The most common approach is to reduce testosterone to castration levels. Roehl et al., conducted such survey (see Table 1 below) to evaluate the death rate in patients subjected to different treatments. A cohort of 981 men diagnosed with prostate cancer from 1989 to 2001 was screened for the 10-year probability of death from prostate cancer (PCa) versus other causes under the
treatment of surgery (radical prostatectomy), radiation, watchful waiting (active surveillance), and hormonal therapy (91). Surprisingly, patients with hormonal therapy had the highest rate among prostate cancer deaths (18.5%) and the second highest rate among other-cause deaths (45.3%) (91).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Died of Prostate Cancer (%)</th>
<th>Died of Other Causes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery</td>
<td>2.4</td>
<td>14.2</td>
</tr>
<tr>
<td>Radiation</td>
<td>4.0</td>
<td>31.6</td>
</tr>
<tr>
<td>Watchful Waiting</td>
<td>5.5</td>
<td>52.7</td>
</tr>
<tr>
<td>Hormonal Therapy</td>
<td>18.5</td>
<td>45.3</td>
</tr>
</tbody>
</table>

Another approach for cancer treatment is to utilize antibodies that target cancer cell surface receptors, such as anti-EGFR or anti-HER2/neu antibodies to block the pro-mitogenic function of circulating growth factors (Review in 95). However, using these cancer-targeting antibodies is often problematic because a large antibody size makes it difficult to penetrate the entire tumor mass (96). Additionally, a non-specific binding may lead to high toxicity (96). Therefore, peptides, peptidomimetics, or small molecules, have been developed as alternatives that are more effective at targeting cancer. Indeed, peptides are small, easy to synthesize, and can be N- and C-terminally blocked or cyclized to further enhance their stability (Review in 96).

After reviewing the prostate cancer mortality rate under current hormonal therapy and cancer-targeting agents, we used a novel strategy for prostate cancer treatment by targeting an AR-regulated gene that is important in tumorigenesis: sGCα1. Hence, we designed 4 distinct peptides aimed at disrupting the sGCα1 pro-cancer activity, such as
p53 inhibition or Akt activation. Interestingly, Peptide A-8R, which is able to enter the cell and associate with sGCα1, shows a marked cytotoxicity on both hormone-sensitive and hormone-refractory prostate cancer cells, which are sGCα1-positive (Fig.13B-III). However, the cytotoxicity mediated by Peptide A-8R (Fig.15A-III) appears to occur independent of p53- (Fig.16-III). As seen in Fig.15A-III, etoposide induces apoptosis occurring at 24-hour post-treatment. In contrast, Peptide A-8R induces rapid and strong cytotoxic effects beginning at 1-hour post-treatment and reaching a peak after 8 hours, as seen in Fig.15A-III. We will further investigate the mechanism of Peptide A-8R induced cytotoxicity. Importantly, a xenograft mouse model shows that the treatment with Peptide A-8R (8 mg/kg) slowed down but not completely stopped the tumor growth (Fig.17-III), despite of a relatively lower concentration than others (~100 mg/kg). We will continue to characterize Peptide A-8R and improve its efficacy on prostate tumors.
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