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TITLE: Chemical Suppression of the Reactivated Androgen Signaling Pathway in Androgen-Independent Prostate Cancer

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14. ABSTRACT The project studies the role of Hedgehog/Gli signaling in generating the androgen growth-independent behavior of castration resistant prostate cancer and will test the ability of drugs that target Hedgehog or Gli as a means to suppress this behavior. The work has 3 Aims. Work in the first Aim will determine the specific roles of Smoothened or Gli2 in generating this behavior; work in Aim 2 will characterize the interaction between the AR and Gli2 to identify mechanisms of Gli2 support of AR activity in an androgen-deprived environment; work in Aim 3 will determine the extent to which Gli activity is involved in intratumoral steroidogenesis that supports androgen growth independence of advanced prostate cancer. The ultimate goal of the project is to define the mechanisms by which Hedgehog signaling molecules support aggressive cancer behaviors and to identify treatments that target the specific mechanisms as preclinical testing for potential use in advanced (metastatic) prostate cancer patients.					
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ANNUAL REPORT

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INTRODUCTION

The project studies the role of components of the Hedgehog signaling pathway and the Gli transcription factors that are activated by Hedgehog signaling in supporting the development of “androgen growth-independent” prostate cancer cells. More importantly, the project will test various chemical smoothened (Smo) or Gli inhibitors to determine a strategy to block the development of castration resistant prostate cancers in patients treated by hormonal therapies. The project Aims were modified at the start of this period to accommodate the PI’s move from the original site (the Ordway Research Institute) to a new site (the Vancouver Prostate Centre of the University of British Columbia, Canada). There are now three Specific Aims: Work in the first Aim will characterize the interaction between Gli2 and the androgen receptor (AR) protein to identify how this interaction enables androgen signaling in an androgen-free environment. Work in the second Aim will use Chromatin Immunoprecipitation procedures to demonstrate the interaction of Glis with AR at androgen response elements that regulate androgen dependent genes in prostate cancer cells. Work in the third Aim will test the relative ability of different Smo and/or Gli inhibitors in blocking the interaction of Gli with AR in prostate cancer cells as a means to devise best treatment strategies that may ultimately be used in hormone-treated prostate cancer patients or in patients with overt castration resistant prostate cancers. Our work in the reported period describes our completion of all Tasks in Aim 1 and one Task in Aim2.

BODY

Key Research Accomplishments During this Period

During the current year, we completed all Tasks under Specific Aim 1 and one Task under Specific Aim 2:

- 1) *Identified the specific binding domains on the Gli2 protein that are required for interaction with the AR protein as well as the singular binding domain on the AR protein that is required for Gli2 binding (Aim1).* This work was completed using Glutathione-S-Transferase- (GST-) pulldown assays that allow one to show a direct interaction between two different polypeptides(1). This assay required us to “tag” defined subfragments (peptides) of the Gli2 protein with GST and then to purify them from bacterial lysates by GST-capture beads. Purified Gli2 peptide subfragments were then mixed with polyhistidine- (his-) tagged AR in vitro and the Gli2 fragment was re-captured on GST affinity beads. Those fragments that bind to AR will be captured, along with AR, onto the bead and the interaction is shown by Western blotting. By this means, we were able to show that two distinct peptides within the Gli2 C-terminal domain (amino acid [aa] 628-897 and 987-1091) were able to bind to AR protein. The aa628-897 appeared to have higher affinity than the 987-1091.
- 2) *Defined all elements on Gli2 required for co-activation of AR in prostate cancer cells (Aim1).* Using an androgen-dependent reporter construct in which the PB-ARR2 promoter drives expression of a luciferase reporter gene, we tested the ability of our Gli2 subfragments to increase expression of the reporter on co-transfection of 293T cells (that lack AR expression) with a wildtype AR expression vector. In our assays, we showed that the co-activation of the luciferase reporter requires the two AR binding sites described above, as well as the Gli2 activation domain that lies at the far C-terminus of Gli2. In summary, our work showed that Gli2 coactivation of AR requires binding of the Gli2 protein to AR as well as a functional activation domain on Gli2. All of these activities are found on the C-terminal domain of Gli2 so it does not require full-length Gli2 for AR coactivation.
- 3) *Identified the specific binding domain on the AR that interacts with Gli2 (Aim1).* Using a similar approach with polyhistidine- (his-) tagged AR protein subfragments, we were able to localize Gli2 binding to the N-terminal domain of AR and then specifically to the AR tau5/AF5 domain (aa 392-558) within the N-terminus(2). This was a very remarkable observation since the tau5/AF5

domain of AR is specifically referred to as the “ligand-independent” activation domain that appears to support hyperactive AR function in the absence of androgens. This observation then is consistent with our previous demonstration that Gli2 is a co-activator of wildtype AR in the presence or in the absence of added androgen (3). Indeed, when we mutated the critical WxxLF motif within the tau5/AF5 domain, we drastically reduced the ability of Gli2 to bind to tau5/AF5 and this was a functional demonstration that tau5/AF5 is involved in Gli2 co-activation. More important, the tau5/AF5 domain is critical to the ability of natural truncated ARs to function when the ligand-binding domain is lacking (due to an alternate splicing) and these naturally-truncated AR variants are believed to support the development of castration resistant prostate cancers (4). Indeed, we have also demonstrated during this period that the Gli2 C-terminal domain is able to co-activate the V7 natural truncation variant of AR, and collectively, our data supports our idea that Gli2 upregulation in prostate cancer cells following androgen ablation promotes the development of castration resistant disease in conjunction with the truncated receptors.

- 4) *Demonstrated interaction of Gli2 with AR at androgen response elements (AREs) on DNA near to androgen-regulated genes (Aim2).* Here we used a chromatin immunoprecipitation (ChIP) assay using our AR+ LNCaP cells that were genetically engineered to express a myc-tagged variant of Gli2 conditionally expressed when the cells were treated with doxycycline (i.e. a tetracycline) (LNCaP-dox-Gli2). We performed ChIP assays on both doxycycline-unstimulated and stimulated cells using either anti-myc antibody (that recognizes the tagged Gli2) or a non-immune isotype control antibody to immunoprecipitate sheared fixed chromatin from the cells. The outcome of this experiment showed significant enrichment of the ARE within the PSA enhancer element as well as the region containing an ARE in the promoter of the PGC gene by the myc antibody only when the cells were stimulated by doxycycline. This work then demonstrates that Gli2 functionally interacts with AR at AREs on chromatin and supports the idea that this interaction increases the production of androgen-dependent gene expressions.

Reportable Outcomes During this Period

- We have identified 2 Gli2 binding sites for AR within the C-terminus of Gli2 protein.
- We have shown that the C-terminus of Gli2 is sufficient for co-activation of ligand-independent AR activity and that the activity of this polypeptide requires the 2 AR binding sites as well as the Gli2 co-activation domains.
- We have identified that Gli2 binds specifically at the tau5/AF5 domain of the AR.
- We have shown that the WxxLF motif within tau5/AF5 of the AR is critical for Gli2 binding activity.
- We have shown that Gli2 co-activates naturally truncated (splice variant-generated) AR receptors.
- We have shown functional association of Gli2 at cis-acting androgen response elements of androgen-regulated genes.

Our work during the last year of the project resulted in the submission/acceptance of 3 different abstracts, one that has already been presented (for the Society of Basic Urologic Research conference, November 2012, the American Association of Cancer Research in April 2013 and the American Urological Association Annual Conference in May 2013).

Li N, Chen M, Buttyan R. Fine mapping of the interaction domains between Gli2 and the androgen receptor. (Presented at the SBUR annual conference, November 2012).

Li N, Chen M, Buttyan R. Identification of the binding domains that enable the ligand-independent co-activation of androgen receptor by Gli2 (for presentation at the AUA conference, May 2013)

Li N, Chen M, Buttyan R. Identification of the binding domains that allow ligand-independent co-activation of androgen receptor by Gli2 (for presentation at the AACR conference, April 2013).

We are currently preparing a manuscript for submission to the journal *Oncogene* as well and expect to have it submitted within the first week of December 2012.

Chen M, Li N, Yan C, Hongbo W and Buttyan R. Mapping the elements that enable Gli2 co-activation of wildtype and truncated androgen receptors. (In Preparation). ,

Conclusions

Gli2 is a co-activator of wildtype and truncated androgen receptors that supports acquired growth of prostate cancer cells in an androgen-free environment. The important elements of the co-activation activity are the two AR binding sites and the activation domain within the Gli2 C-terminal domain in addition to the tau5/AF5 domain within the N-terminal portion of the AR. The interaction between Gli2 and AR in the ligand-independent growth of prostate cancer cells was functionally demonstrated by chromatin immunoprecipitation studies that showed the co-localization of these two proteins at the cis-acting androgen response elements within the promoters or enhancers of androgen regulated genes. The nature of the Gli2-AR interaction allows co-activation of a naturally truncated AR variant (V7) that is thought to be active in castration-resistant prostate cancer and we propose that this interaction contributes to progression to castration resistant disease in prostate cancer patients.

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FIGURES

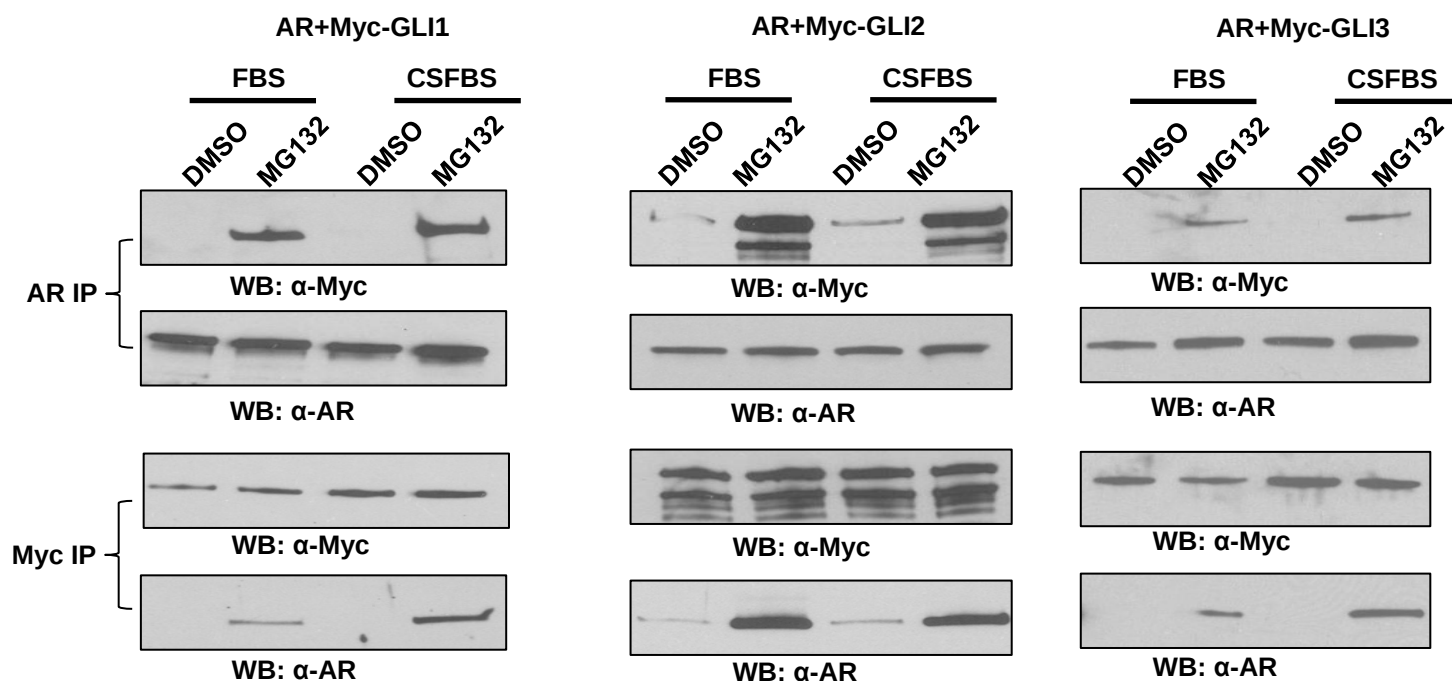


Figure 1. All Gli variants (1,2 and 3) co-immunoprecipitate with wildtype AR. Full length Gli1, 2 or 3 were co-transfected into LNCaP cells and 72 hours later, lysates from these cells (with or without the proteasome inhibitor MG132) were immunoprecipitated (IP) with an anti-myc antibody (to pull down the tagged Gli protein) or anti-AR antibody. Immunoprecipitates were electrophoresed and transferred to Western blots (WB) then probed with the indicated antibody. Results show that all 3 Gli variants co-immunoprecipitate with AR and vice versa.

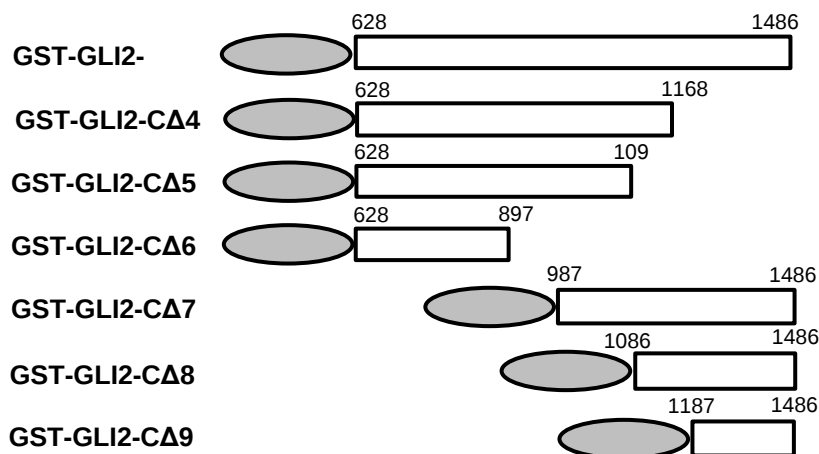


Figure 2. Description of GST-tagged Gli2 subfragments generated for use in the GST pulldown assays. The GST tag is indicated by the grey oval. Numbers describe the Gli2 amino acid parameters of the fragment. The use of C-terminal only subfragments is based on other evidence showing that the C-terminus of Gli2 is the only part of Gli2 that can bind to AR.

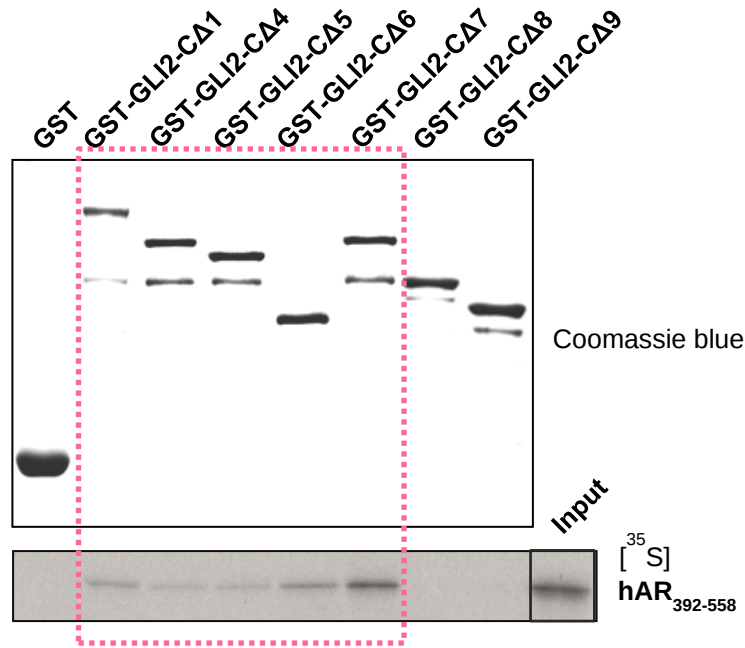


Figure 3. Acrylamide gel (top) shows semi-purified GST-tagged subfragments of the Gli2 C-terminus described in Figure 2 (from bacterial lysates). Bottom panel identifies the ability of each of the subfragments to retain the Tau5/AF5 domain of the androgen receptor protein. The red box identifies those subfragments that were able to bind to the AR domain. The outcomes show effective binding to the aa628-897 and aa987-1486 fragment. Binding ability was lost beyond aa 1086.

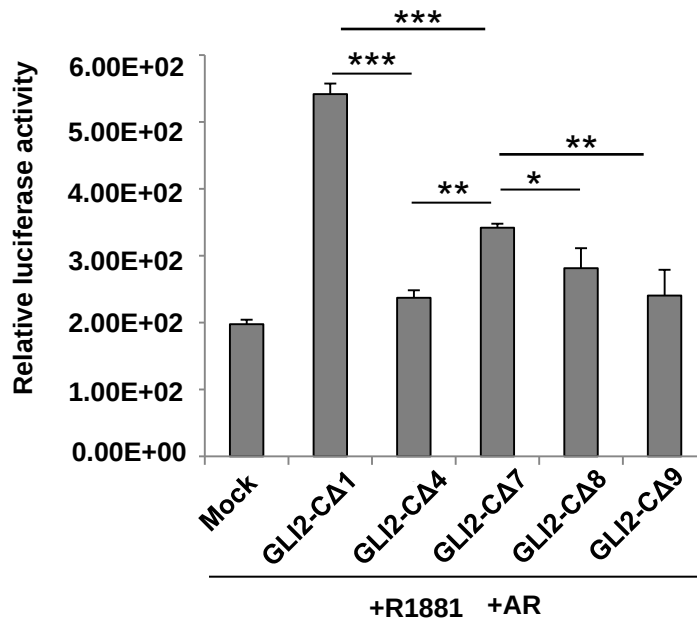


Figure 4. The Gli2 activation domain (aa 1168 – aa 1468) is required for co-activation of AR in transient transfection assay using the PGC-promoter/luciferase reporter system.

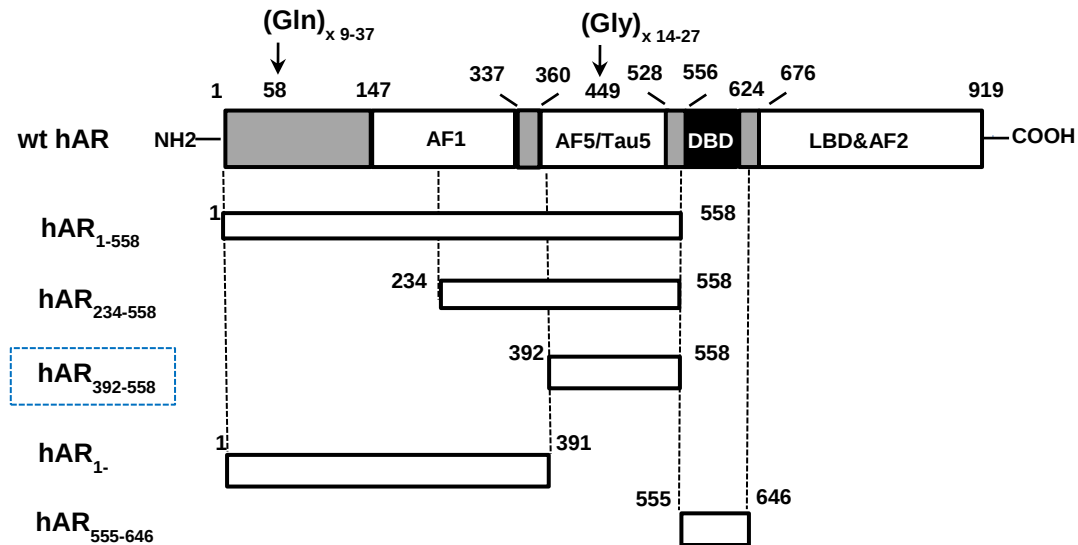


Figure 5. Subfragments of the AR N-terminal/DNA binding domains used to fine map the Gli2 binding site on AR. Numbers (left) identify the corresponding amino acids that define the different subfragments.

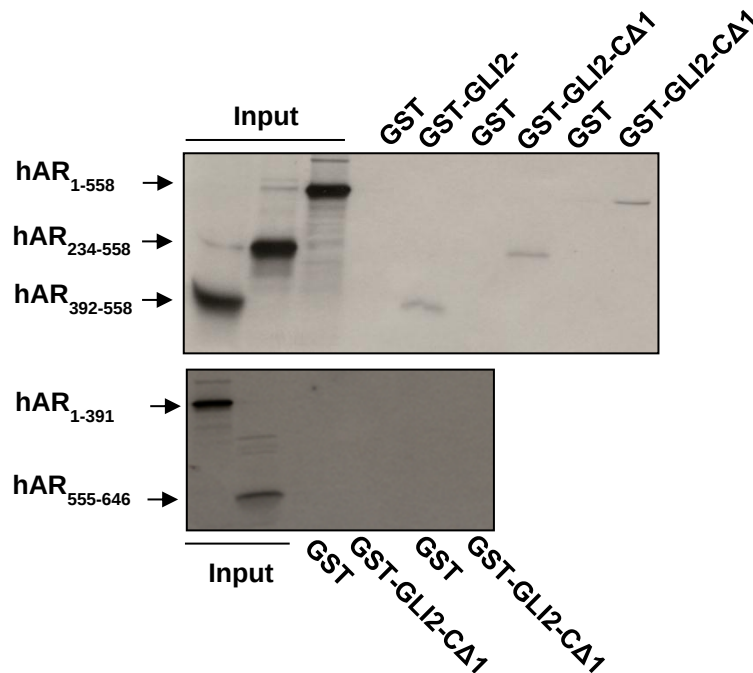


Figure 6. GST pulldown assay shows that the hAR 392-558 is sufficient to bind to the C-terminal domain of Gli2 (Top). hAR subfragments 1-391 and 555-646 do not bind to the Gli2 C-terminus (Bottom).

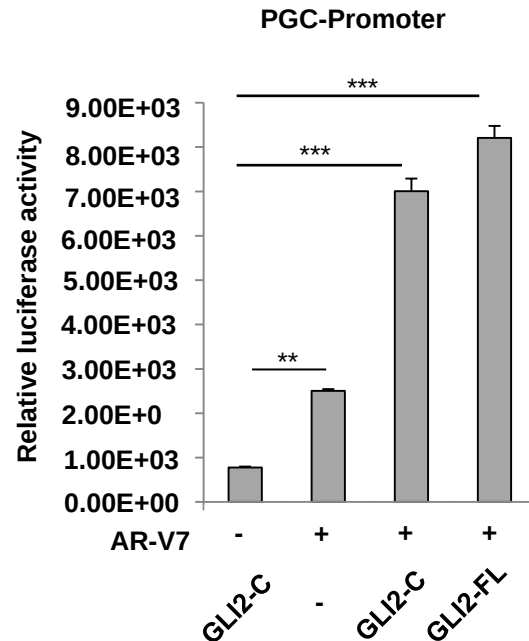


Figure 7. Full length (FL) or the C-terminus (C) of Gli2 co-activate the natural AR truncation variant, V7 and this is consistent with the ability of Gli2 to bind to the AR tau5/AF5 domain involved in ligand –independent co-activation of AR and its truncated variants.

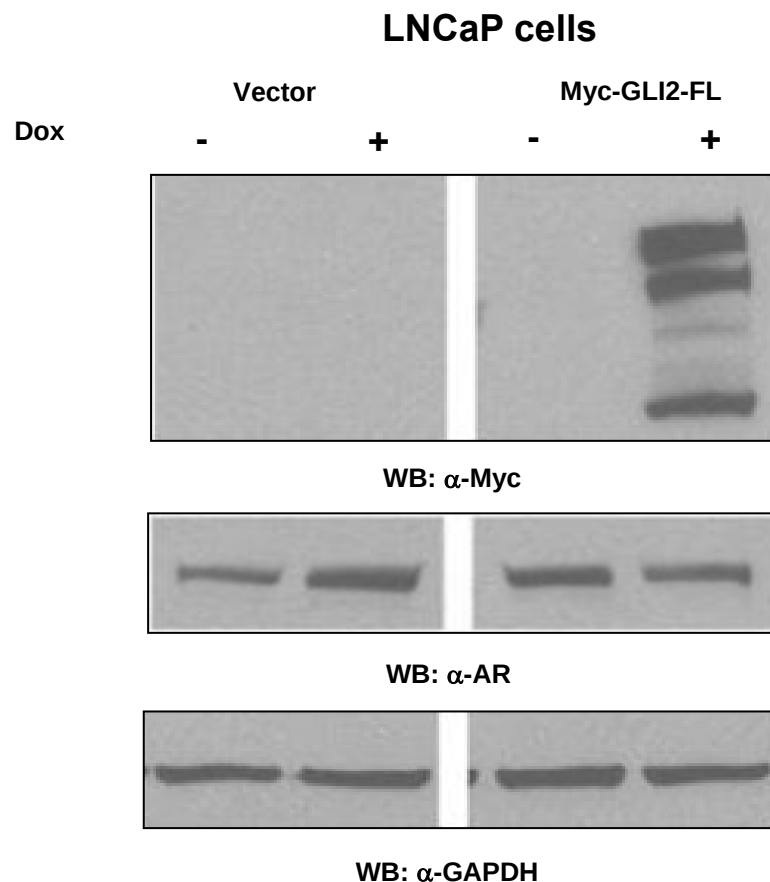


Figure 8 (Previous Page). Doxycycline treatment induces expression of myc-tagged Gli2 protein in the LNCaP-Myc-Gli2-FL cells but not in control-transduced (vector) LNCaP Cells.

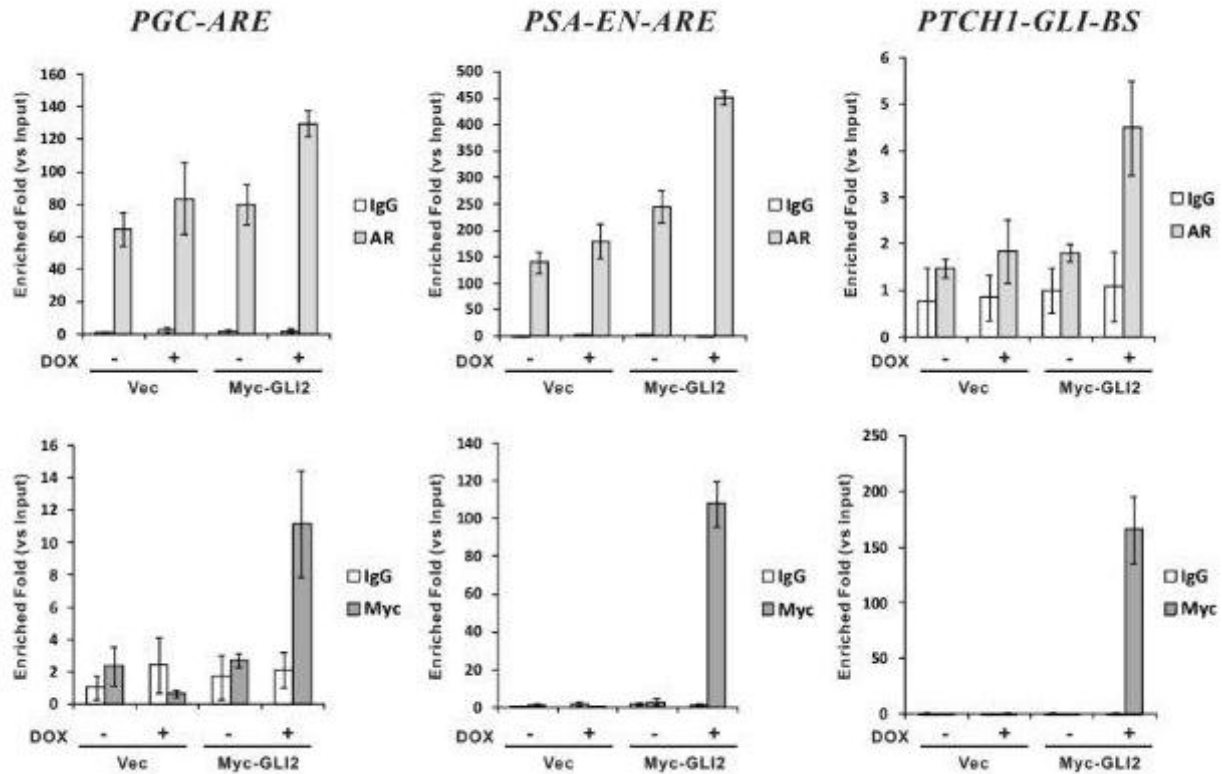


Figure 9. Gli2 associates with androgen binding sites (ARE) on fixed chromatin from Dox-treated LNCaP-Myc-Gli2-FL cells and AR associates with Gli2 binding sites in the same cells. Association is shown by enrichment of a short region of chromatin containing an ARE in the PGC gene promoter and the PSA enhancer (EN) when immunoprecipitated by anti-myc tag (bottom panel) as well as by increased enrichment of the Gli2 binding element in the human Ptch1 gene when immunoprecipitated by anti-AR (top panel).