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14. ABSTRACT Given the requirement of the AR activation pathway for prostate cancer growth and progression, it is necessary to identify alternative means of targeting this pathway for the treatment of prostate cancer. The work herein has examined the role of BAF57 in its ability to activate the androgen receptor (AR). The research carried out under this proposal has fine mapped the AR binding site on BAF57 to the N-terminus (proline-rich region). Furthermore, the DBD and hinge region of AR also appear to play a significant role in the ability of BAF57 to activate AR. Together, this data presents the idea of targeting the interaction between AR and BAF57 at the N-terminus for the possibility of using this as a therapeutic for prostate cancer. Additional studies will determine the actual function of BAF57 as well as the efficacy of targeting this interaction.					
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Introduction

The development and progression of prostate cancer relies on the activity of the androgen receptor (AR). AR is a hormone activated transcription factor that is necessary for normal prostate development and function. In cases where the cancer is confined to the prostate, treatment is virtually always effective. In later stages, treatment employed relies on the requirement of androgen for disease progression. Patients are subjected to hormone ablation therapy, which is generally effective. Unfortunately, this effectiveness is only temporary. After two to three years, the cancer returns and has found a means to bypass the requirement of androgen. Current research focuses on targeting the AR activation pathway, as the AR becomes re-activated and remains an essential component to the advancement of late stage prostate cancer. As a transcription factor, many additional molecules assist the AR in its ability to activate transcription. One such group is the SWI/SNF chromatin remodeling complex. SWI/SNF is composed of a core ATPase, used for remodeling chromatin to either repress or activate gene transcription, and several BRG1-associated factors (BAFs) that serve as accessory proteins to the ATPase. We found that the BAF57 subunit is an essential factor in the activation of AR. *It is our goal to determine the mechanism of BAF57 action on AR activation and examine the efficacy of targeting BAF57 as a means of treating prostate cancer.* Thus far, we have made significant progress on completing the tasks brought forth in the initial proposal. The binding region between AR and BAF57 has essentially been identified, as was the goal of task 1. Furthermore, functional studies have been utilized to confirm the importance of the AR: BAF57 interaction site. Although the projected completion of task 2 is not until next year, initial studies have been carried out that will be included in the following report. These studies will lead nicely into the final task of examining the requirement of BAF57 in early and late stage prostate cancer.

Body

Task 1: Elucidate the necessary functional domains of BAF57 involved in regulating AR activity.

Overview of Task 1:

The goal of task 1 was to essentially determine the necessary domain(s) of BAF57 in regulating activation of the androgen receptor (AR). Based on previous studies from our lab, we identified BAF57 as a critical component of the AR activation pathway. To further identify the role of BAF57 in AR activation, we have fine-mapped the region of AR binding to BAF57. Additionally, we took the proposed task one step further to complement these studies. We identified at least two regions on AR to which BAF57 binds.

Upon completion of identifying the binding region between BAF57 and AR, functional studies were initiated. Currently, much progress is being made to examine the necessary functional domain(s) for BAF57 function in the AR activation pathway. Again, to complement these studies, we are also examining a critical domain within the AR that is required for full activation by BAF57.

Accomplishments of Task 1:

BAF57 binds to DNA binding domain (DBD) and hinge region of AR

As outlined in the initial proposal, the first task was to identify the regions of binding between BAF57 and the AR. As opposed to constructing GST fusion proteins from the BAF57 mutants, we took the approach of using AR deletion mutants already fused to GST (deletion mutants of AR are necessary as attempts of cloning full length AR into the GST expression vector have proven unsuccessful). Consequently, we used the BAF57 deletion mutants (obtained from collaborator Dr. Trevor Archer (1)) for *in vitro* transcription/translation. This approach not only allowed us to determine where AR is interacting on the BAF57 surface, but given the number of GST-AR mutant constructs in our possession, it was also possible to identify (at least in part) where BAF57 is interacting on the surface of AR. In order to use our GST-AR fusion proteins, it was necessary to narrow down the region of BAF57 binding to AR. Therefore, the first step we took was to determine whether BAF57 binds to the N- or C-terminus of AR. As seen in Figure 1, strong binding occurs between GST-BAF57 and AR 506-919 indicating a C-terminal binding site.

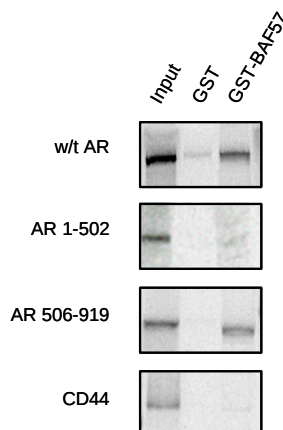


Figure 1. BAF57 bindings to C-terminus of AR. GST-BAF57 was purified and bound to glutathione agarose. Purified protein was incubated with ^[35]S-Methionine labeled wtAR, AR 1-502 (N-terminus), AR 506-919 (C-terminus), or CD44 (negative control). Binding was detected via autoradiography.

Included in the AR C-terminal region of the above construct are the well-characterized zinc finger DNA binding domain (DBD) and the hinge region. Given the significant role of these two domains in AR DNA binding and activity, we assessed the ability of BAF57 (which also harbors the ability to strongly bind DNA (2)) to bind to the AR DBD and/or hinge region. As can be seen from the GST-pulldown analysis in Figure 2, strong binding of BAF57 occurs at the DBD and hinge regions of AR.

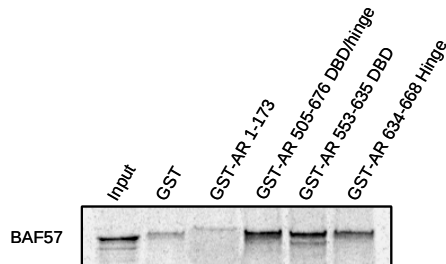


Figure 2. BAF57 binds to DBD and hinge region of AR. GST-AR fusion proteins were purified and incubated with 35 S-Methionine labeled BAF57. BAF57 binding to the GST-AR deletion mutants was detected by autoradiography.

AR hinge domain required for complete BAF57 binding and activation

To further characterize the BAF57 binding site on AR, we utilized an AR construct (AR Δ 629-636) lacking a small portion of the hinge domain. GST-BAF57 binding to this AR mutant was reduced by ~50% as determined by semi-quantitative methods (Figure 3).

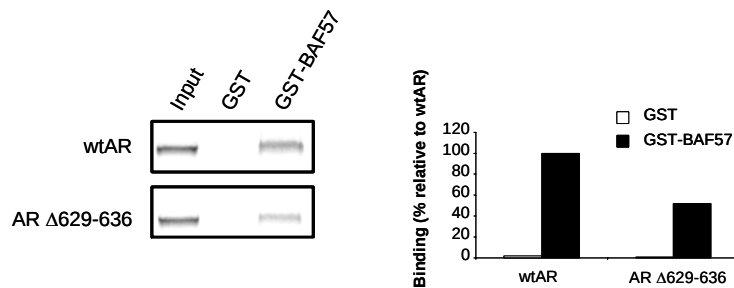


Figure 3. AR hinge domain is necessary for BAF57 binding. Purified GST or GST-BAF57 was incubated with 35 S-Methionine labeled AR or AR Δ 629-636. Binding was detected by autoradiography. GST-BAF57 binding to wtAR was set to "100."

Having established a concrete binding site for BAF57 on AR, it was then necessary to examine the ability of BAF57 to activate this AR hinge deletion mutant. Therefore, reporter analysis was carried out and AR activation by BAF57 was examined. As seen in Figure 4, the ability of BAF57 to activate the mutant AR is significantly reduced compared to the activation seen with wtAR. Together, this identifies the AR hinge region as a necessary region for the action of BAF57 in modulating AR activity.

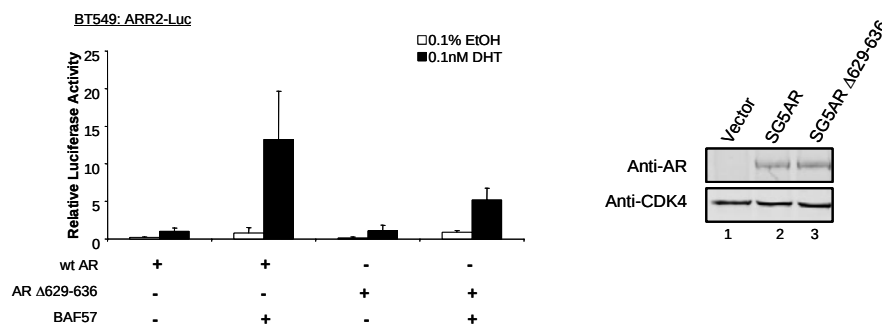
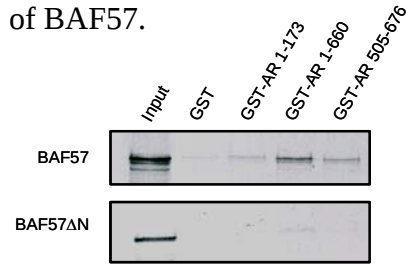


Figure 4. AR hinge region necessary for activation via BAF57. Left, BT549 cells (BAF57 null) were transfected with AR or AR Δ 629-636 and +/- BAF57. Cells were treated with 0.1nM DHT or 0.1% EtOH as indicated and AR activity was measured via the ARR2 reporter tagged to luciferase. Right, Western blot analysis showing expression of AR and mutant AR.

AR binds to proline-rich region of BAF57

Having established the DBD and hinge domains of AR as critical binding sites for BAF57, it was then possible to proceed to identify the region of BAF57 that is necessary for AR binding. Initially, several GST-AR fusion proteins were utilized to assess BAF57 binding. Additionally, an N-terminal deletion mutant of BAF57, BAF57 Δ N, was examined in its ability to bind AR. Interestingly, as seen in Figure 5, BAF57 Δ N is unable to bind to AR as compared to full length BAF57. This finding enabled us to focus the remainder of our binding studies within the N-terminus of BAF57.

Figure 5. AR is unable to bind to BAF57 N-terminal deletion mutant. GST-pulldown analysis was carried out using GST or the GST-AR fusion proteins as indicated. Binding to BAF57 or BAF57 Δ N is shown.



In order to demonstrate sufficiency of AR binding to the N-terminus of BAF57, I constructed a BAF57 deletion mutant containing only the N-terminus (amino acids 1-145). Upon successful cloning of the BAF57 1-145 construct, GST-pulldown analysis was carried out and as expected, significant binding to AR was observed (Figure 6).

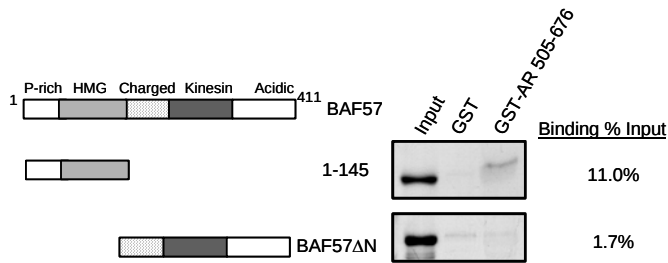


Figure 6. BAF57 N-terminus is sufficient for AR binding. GST-pulldown analysis was carried out using GST or GST-AR 505-676 as indicated. Binding to BAF57 1-145 or BAF57 Δ N is shown.

After identifying the first 145 amino acids of BAF57 as containing the primary binding site(s) for AR, the binding site was further refined so as to accomplish the overall goal of the study. After obtaining two BAF57 deletion mutants from our collaborator, GST-pulldown analysis was performed with GST or GST-AR 505-676 and full length BAF57, BAF57 Δ N, BAF57 Δ PR, or BAF57 Δ HMG. As depicted in Figure 7, AR binds strongly to the proline-rich domain of BAF57.

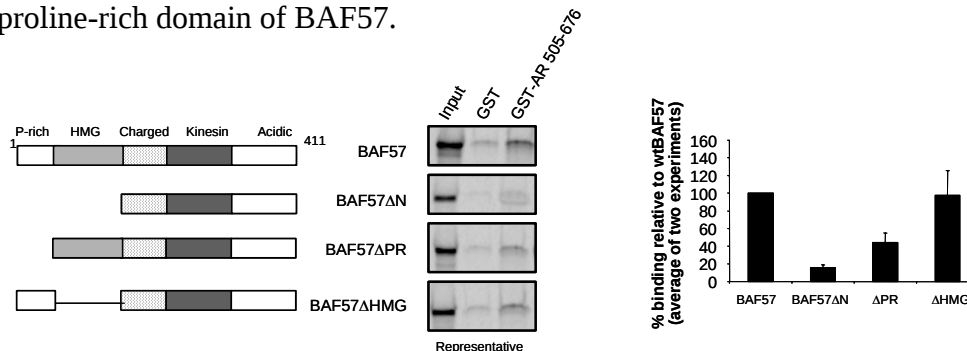
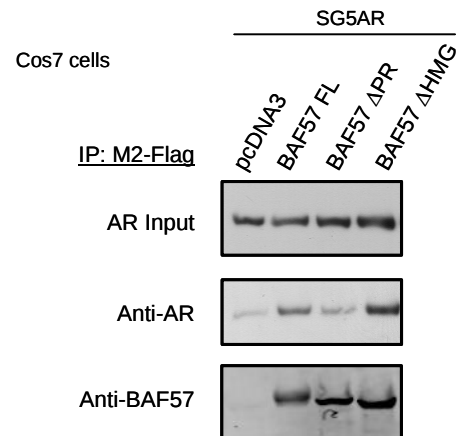


Figure 7. AR binds proline-rich region of BAF57. GST-pulldown analysis was carried out as above, and binding from two separate experiments was quantitated with AR binding to full length BAF57 set at "100."

Additionally, binding was also assessed using a cell culture model, wherein Cos7 cells were transfected with full length AR and the BAF57 mutants listed. Co-immunoprecipitation analysis was carried out and levels of AR binding were assessed through immunoblotting (Figure 8). Together, these data indicate that AR binds strongly to the proline-rich domain of BAF57.

Figure 8. AR binds BAF57 proline-rich domain in cultured cells. Cos7 cells were transfected with AR, H2B-GFP (transfection efficiency), and pcDNA3 (empty vector) or the indicated flag-tagged BAF57 constructs. Following transfection, cells were harvested and immunoprecipitation was carried out. BAF57 protein was IP'd using M2 flag resin, and AR binding was analyzed by immunoblotting. Transfected BAF57 levels are also shown.



BAF57 N-terminus blocks full-length activation of AR

In accordance with task 1, the functional impact of BAF57 was to be examined on its ability to activate AR. These experiments were carried out utilizing the BAF57 1-145 N-terminal construct. As demonstrated in the above section, AR clearly binds to the N-terminus of BAF57. Therefore, we tested the ability of BAF57 1-145 to inhibit the activity of AR elicited by wtBAF57, as this mutant is unlikely to maintain SWI/SNF association given the ability of BAF57ΔN to assemble into the SWI/SNF complex (3). Figure 9 demonstrates that increasing concentrations of BAF57 1-145 are able to significantly reduce AR activity.

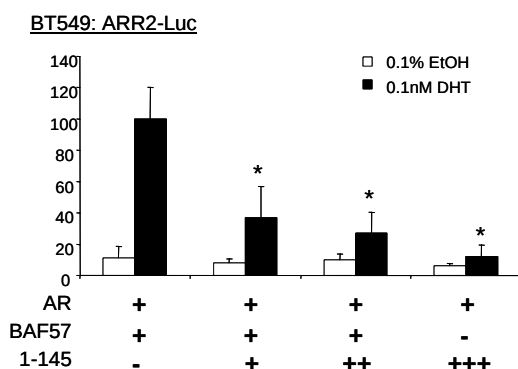


Figure 9. BAF57 1-145 inhibits wtBAF57 activation of AR. BT549 cells were transfected with AR, BAF57, or BAF57 1-145 as indicated. Cells were treated with 0.1nM DHT or 0.1% EtOH as indicated and AR activity was measured via the ARR2 reporter tagged to luciferase. AR + BAF57 + DHT is set to "100." Asterisk indicates statistical significance $p < 0.05$ based on the Student t-test.

To complement the above transcriptional studies, the N-terminus of BAF57 was tested in its ability to block endogenous AR activity. To carry out these studies, the LNCaP prostatic adenocarcinoma cell line was employed. LNCaP cells harbor endogenous AR activity, which can be monitored through the expression of the prostate specific antigen (PSA). Expression of PSA was examined upon transfection of these cells with either vector (negative control), dominant negative AR (positive control), or BAF57 1-145. As demonstrated in Figure 10, BAF57 significantly attenuates PSA expression similar to the dnAR.

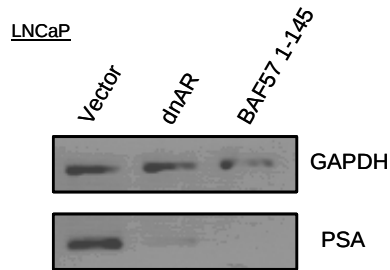


Figure 10. BAF57 1-145 inhibits PSA expression. LNCaP cells were transfected with H2B-GFP (transfection control) and the indicated expression plasmids. RNA was isolated, reverse transcriptase PCR was performed, and PSA was amplified. GAPDH was amplified a loading control.

Summary of Task 1 Accomplishments

As the goal of task 1 was to determine the necessary functional domain(s) of BAF57 in regulating AR activity, we first set out to determine the site of interaction between BAF57 and AR. Although not part of the initial proposal, it was first necessary to identify the point of BAF57 interaction on AR. Using several GST-AR deletion mutants, we were able to narrow down the binding of BAF57 to the DBD and hinge region of AR. However, our studies have not ruled out the possibility of an interaction site for BAF57 within the ligand binding domain of AR. Taking this route also highlighted the requirement of the AR hinge domain for activation by BAF57. In addition to allowing us to continue with identifying the AR interaction site(s) on BAF57, narrowing down where BAF57 interacts with the AR surface was certainly an added and exciting bonus. Given the strong ability of BAF57 to bind the DBD and hinge region of AR, the GST-AR 505-676 fusion protein containing both the DBD and hinge region was utilized to determine which region(s) of BAF57 are necessary for interacting with AR. GST-pulldown analysis confirmed by co-immunoprecipitation demonstrate that the N-terminal proline-rich region of BAF57 is the primary point of AR interaction. Furthermore, the activation of AR, elicited through BAF57, is compromised when the N-terminal BAF57 1-145 region is introduced.

Task 2: Delineate the molecular mechanism of BAF57 action on AR function, thereby establishing BAF57 as a putative therapeutic target.

Overview of Task 2:

Previously, our lab has shown the recruitment of BAF57 to the PSA enhancer element in response to ligand. Furthermore, we have clearly identified BAF57 as a critical factor in regulating AR activity (4). However, the mechanism of BAF57 action on AR is undefined. As task 1 identified the region of BAF57 and AR interaction, task 2 aims to examine the mechanism of how BAF57 elicits its function on modulating AR activity. Although task 2 has just now commenced, we have already made progress on

determining the function of BAF57 in activation of AR. Our preliminary data, as shown below, suggests that BAF57 may impact AR stabilization or recruitment of AR to androgen responsive elements.

Accomplishments of Task 2:

BAF57 provides AR stabilization on the PSA enhancer element.

As the goal of the second task is to define the mechanism of BAF57 action on AR function, we set out to examine AR recruitment in the absence and presence of BAF57 or BAF57 Δ N. In order to carry out this experiment, BARP1 cells were generated from the BAF57 null BT549 cell line using a two step stable transfection process. First, plasmid encoding SG5AR was transfected (along with pBABE-puro) and selection was carried out using puromycin. Individual clones were selected and screened for AR expression. AR expressing cells were then transfected with PSA61-luciferase (along with pcDNA3) and selected with G418. Expression was confirmed through reporter analysis with the addition of BAF57 in the presence of DHT as well as through PCR analysis (data not shown).

Once the BARP1 cell line was established, chromatin immunoprecipitation was carried out. Cells were transfected with either pcDNA3 (empty vector), wtBAF57, or BAF57 Δ N. Cells were treated with either 0.1% EtOH or 10nM DHT for 1hr. Cells were then harvested and chromatin was obtained through standard ChIP procedures. Immunoprecipitation was carried out using either anti-AR antibody or non-specific pre-bleed control. As indicated in Figure 11, background recruitment is seen in response to EtOH treatment. DHT stimulation demonstrates no recruitment of AR (above pre-bleed or EtOH background) upon pcDNA3 vector transfection; however, transfection of wtBAF57 elicits very strong AR recruitment to the PSA enhancer ARE. By comparison, transfection of BAF57 Δ N shows reduced recruitment of AR compared to wtBAF57. Interestingly, this finding correlates nicely with our findings in task1 that identify the N-terminus of BAF57 as the critical point of interaction.

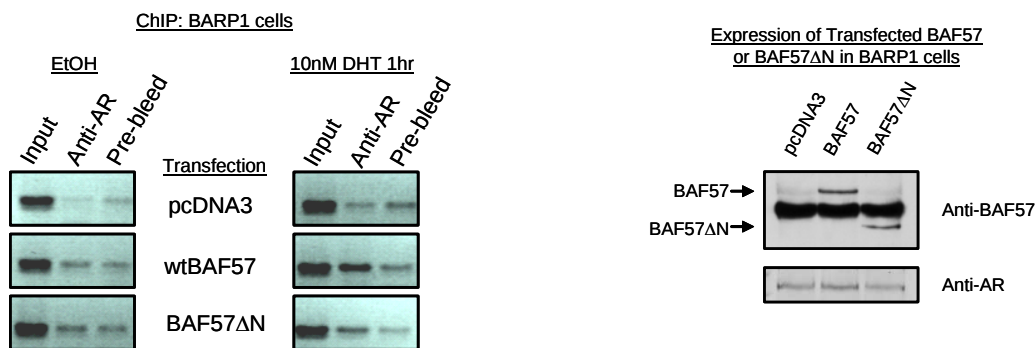


Figure 11. BAF57 enhances AR recruitment to the PSA enhancer element. BARP1 cells were transfected with either pcDNA3 (empty vector), wtBAF57, or BAF57 Δ N along with H2B-GFP (to account for transfection efficiency). Cells were stimulated with 0.1% EtOH or 10nM DHT for 1hr and ChIP analysis was performed. Anti-AR or pre-immune serum was used to examine AR recruitment. Right, Western blot demonstrating expression of BAF57 and BAF57 Δ N at the point of cell harvesting.

Summary of Task 2 Accomplishments

As task 2 is just in the initial stages, much remains to be determined as outlined in the proposal. However, we have already appeared to establish at least one role for BAF57 in mediating AR action. The above data indicates a possible role for BAF57 in stabilizing or recruiting AR to the PSA enhancer element. The remainder of task 2 must be carried out however, to draw any definite conclusions. Determining the role of BAF57 in the recruitment of SWI/SNF to AREs, in addition to examining a possible role for BAF57 in nucleosomal repositioning are two important tasks that will assist in determining the function of BAF57 in regulating AR activation.

Task 3: Examine the requirement of BAF57 for proliferation in early and late stage prostate cancer.

Overview of Task 3:

The primary drive behind task 3 is to determine the significance and ability to target the BAF57-AR interaction *in vivo*. Although not yet carried out fully, initial studies have begun examining expression in advanced prostate cancer samples. Results below show expression of BAF57 in all prostate cancer cell line and tissue examined implicating BAF57 as a good choice for therapeutic targeting.

Accomplishments of Task 3:

BAF57 is expressed in all prostate cancer cell lines and metastatic tissue examined.

Task 3 has only been briefly visited, but promising results have been uncovered. First, western blot analysis has been carried out in a multitude of prostate cancer cell lines as shown in the initial proposal. Shown below (Figure 12) is an improved version, demonstrating that BAF57 is strongly expressed in all prostate cancer cell lines examined. Additionally, new immunohistochemistry data shows strong expression of BAF57 in two prostate cancer metastases (lymph node) similar to expression of AR. Together, this data depicts BAF57 as a possible target in prostate cancer.

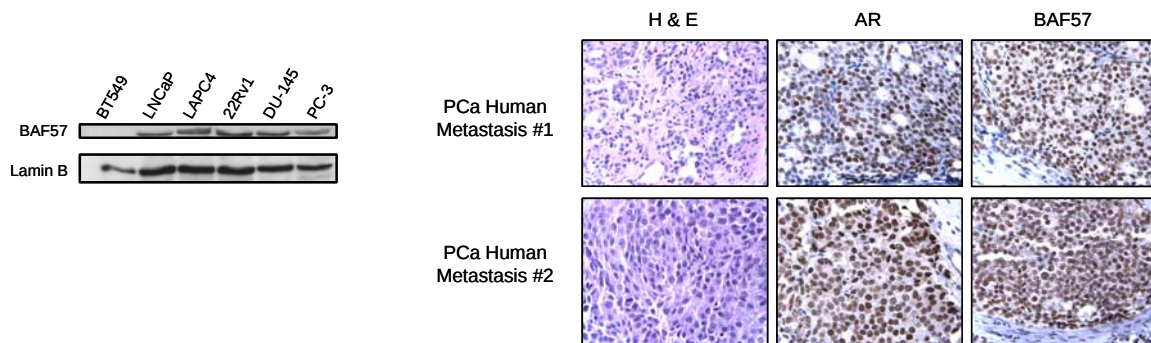


Figure 12. BAF57 is expressed in metastatic prostate cancer tissue. Left panel, Western analysis showing expression in all prostate cancer cell lines tested. BT549 cells were used as the negative control. Right panel, Immunohistochemistry analysis demonstrating expression of BAF57 in two prostate cancer metastases. Comparatively, AR is similarly robustly expressed.

Summary of Task 3 Accomplishments

Although work on task 3 is not scheduled to proceed for a few more months, progress has been made to clearly identify expression of BAF57 in human metastatic prostate tissue. Future support from the grant will be necessary in order to carry out the remaining goals of task 3.

Key Research Accomplishments

- Our studies indicate that BAF57 binds to the AR DBD as well as the hinge region. Furthermore, AR activity elicited by BAF57 requires the AR hinge domain for full activation. This finding complements the examination of the BAF57 regions extremely well, and provides one more avenue to explore in determining the signaling between BAF57 and AR.
- We demonstrate that the N-terminal region (primarily the proline-rich region) of BAF57 is necessary and sufficient for AR binding. Identification of the binding region gives tremendous rationale for investigating this region further for its functional potential, which was examined as described below.
- To complement the binding studies, we also show that expression of the N-terminus of BAF57 is able to inhibit the ability of full length BAF57 to activate the AR. This finding indicates the N-terminus of BAF57 as a critical player in activating the AR.
- We have shown that BAF57 may confer AR stabilization on the PSA enhancer region, and this may be occurring through the N-terminus as indicated above.
- Finally, we have found robust expression of BAF57 in human metastatic prostate cancer, lending to the ability of actually targeting this protein in the treatment of advanced prostate cancer.

Reportable Outcomes

Manuscripts: None as of yet...one is expected to be submitted within 2-3 months

Abstracts/Presentations:

1. Link et. al. Keystone Symposia on Nuclear Receptors. Banff, Alberta, Canada. March 2006 Poster Presentation.
2. Link et. al. Endocrine Society Annual Meeting. Boston, MA. June 2006 Poster Presentation.
3. Link et. al. University of Cincinnati Cell and Cancer Biology Seminar. Cincinnati, OH. November 2006. Oral Presentation.
4. Link et. al. AACR Innovations in Prostate Cancer Research. San Francisco, CA. December 2006 Poster Presentation (AFLAC scholar-in-training travel award).

Patents: None

Degrees obtained supported by this award: None as of yet

Development of cell lines: BARP1 cells (BT549 cells with AR and PSA61-luc integrated stably).

Informatics: None

Funding: None applied for

Employment or research opportunities: None as of yet

Conclusion

A global view of the research at hand portrays a necessity in identifying novel approaches for the treatment of prostate cancer. Therefore, much of the current research is aimed at deciphering the mechanism of androgen receptor signaling in hopes of identifying a potential target for the treatment of prostate cancer. With the closing of the first year of research under the direction of my proposal, I have made much progress in regards to determining the role of BAF57 in AR activation. As outlined in the proposal, the study includes 1) determining the necessary functional domains of BAF57 involved in regulating AR activity, 2) identifying the molecular mechanism of BAF57 action on AR function, thereby establishing BAF57 as a putative therapeutic target, and 3) examining the requirement of BAF57 for proliferation in early and late stage prostate cancer.

Currently, our progress has been on par with the statement of work. Our studies have identified at least one region of the AR necessary for binding (DBD/hinge) and activation (hinge) by BAF57. Furthermore, the AR binding domain within BAF57 has emerged as the N-terminal proline-rich domain. It also appears that BAF57 is also able to elicit its primary function through this N-terminus, as overexpression appears to inhibit full length BAF57 activation of AR.

In addition to the near completion of task 1, work has also been completed for tasks 2 and 3. First, a potential role for BAF57 has arisen in modulating AR recruitment or stability on the PSA enhancer element. Additionally, BAF57 expression has been confirmed in late stage human metastatic prostate cancer.

Changes to future studies:

Initial reporter analysis in BT549 cells with the BAF57 mutants have been inconsistent, therefore we have established alternative means (as outlined in task 1 accomplishments) of examining the impact of the N-terminus of BAF57 as this was established as the primary AR interaction site. In addition to the above experiments, the ability of the BAF57 1-145 mutant to inhibit prostate cancer cell proliferation is also being examined. More consistent reporter analysis will also be sought.

Currently, plans for the remainder of the study will be carried out as outlined in the initial proposal pending no complications.

So What?

The outcomes of the above mentioned research have quite an impact on elucidating the signaling pathway of the AR and the involvement of BAF57. Given the importance of BAF57 in AR activation and proliferation of prostate cancer cells (4), the identification of the interaction surfaces between BAF57 and AR is critical. Having identified the potential interaction surfaces, it is now possible to examine a means of disrupting this interaction with the anticipation of using this disruption as a therapeutic for the treatment of prostate cancer. Expression of BAF57 in human metastatic prostate cancer further confers the potential to target this SWI/SNF subunit in the treatment of prostate cancer.

Additionally, in outlining the mechanism of BAF57 action for the activation of AR, the proposal will also probe the dynamic action of the SWI/SNF ATPase, BRM, and the coordination it has with BAF57 in modulating AR activation. Together, completion of this proposal will result in a detailed understanding of the SWI/SNF complex (specifically BAF57) in its ability to modulate AR activity. This understanding, in turn, will result in determining the efficacy of actually targeting BAF57 as a possible prostate cancer therapeutic.

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