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TITLE: FGFR4 Downregulation of Cell Adhesion in Prostate Cancer

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We have been successful in creating the necessary constructs for generating prostate cancer cells inducible for FGFR4. Despite our best efforts, however, we have not been able to successfully incorporate the pVgRXR regulatory plasmid into prostate cancer cells. We proceeded by transiently transfecting PC3 prostate cancer cells to look at effects on downstream signaling components and found no significant differences in MAPK activity, NCAM expression or STAT1 and STAT5 localization when comparing the various FGFR4 constructs, including FL-FGFR4 and PTD-FGFR4. We have also made a very exciting discovery recently that may be important for understanding the role of FGFR4 in prostate cancer progression. Using a yeast-two-hybrid assay, we found that FGFR4 interacts with IKKbeta and leads to its tyrosine phosphorylation. We are currently investigating if there are any differences in binding tyrosine phosphorylation with the G388R polymorphism. Though not specifically relating to cell adhesion, understanding the role of FGFR4 in altering IKKbeta activity may be important for our understanding of prostate cancer progression.
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Introduction

Prostate cancer is currently the second most common type of cancer resulting in male mortality in the U.S. A recent publication demonstrates the importance of the common FGFR4 polymorphism Gly388Arg for the initiation and progression of prostate cancer (1). We set out to understand the role for this polymorphism in the proliferation and cellular adhesion of cells of prostatic origin. Previous investigations by our lab into differences between WT and G388R FGFR4 rendered few significant differences. This was possibly due to low transfection efficiencies, resulting in very small differences in endogenous signaling pathways. We set out to create prostate cancer cells inducible for FGFR4 expression to discover whether this method would make it easier to see slight differences in endogenous signaling. We were also interested in determining whether a truncated form of FGFR4 arising from alternative splicing, first identified in pituitary tumors (2-4), would increase the effects of the FGFR4 polymorphism. We have since been unsuccessful in creating these inducible prostate cell lines; however, we have found other interesting avenues of research relating to the G388R polymorphism and its role in prostate cancer.

Body

The first task was to prepare prostate cell lines inducible for expression of various FGFR4 genes. During the first month, we constructed the proper FGFR4 clones for this system, including FL-FGFR4-WT, FL-FGFR4-G388R, PTD-FGFR4-WT, and PTD-FGFR4-G388R. The first step was creating the PTD-FGFR4-WT and PTD-FGFR4-G388R in a pcDNA3 background using our FL constructs also in a pcDNA3 background. The FL-FGFR4 gene was inserted in a pcDNA3 expression plasmid using a HindIII site at the start of the gene and an XhoI site at the end of the gene. Using site-directed mutagenesis, we generated an additional HindIII site just before the alternate start site that has been previously shown to lead to transcription of the PTD-FGFR4 product (2). 10% of the PCR mix was used to transform C600 bacteria and colonies were selected using ampicillin. After sequencing these clones to verify whether they were correct, we restriction digested these constructs with the HindIII enzyme. This generated two fragments: A vector fragment containing the HindIII site immediately before the alternative PTD start site, as well as a small fragment containing the 5’ region of the FGFR4 gene with the normal start site to the alternative start site. The vector fragment was isolated from an agarose gel and ligated back together using T4 DNA ligase overnight at 16°C. C600 bacteria were transformed with half of the ligated mixture and colonies were selected using ampicillin. Miniprep DNA was sequenced and positive clones identified. We now had the FL-FGFR4-WT and FL-FGFR4-G388R, as well as the PTD-FGFR4-WT and PTD-FGFR4-G388R in the pcDNA3 expression vector.

The next step was moving each of these DNA constructs to the pIND plasmid for use in developing cell lines for inducible expression. Using the restriction sites HindIII and XhoI, which flank the FGFR4 gene, we double digested the pcDNA3 constructs using HindIII and XhoI. The FL-FGFR4-WT, FL-FGFR4-G388R, PTD-FGFR4-WT, and PTD-FGFR4-G388R genes were cut out of the pcDNA3 background and ligated into the pIND plasmid, which contain HindIII and XhoI sites in the multiple cloning region. C600 bacteria were transformed with half of the ligation mixture, and colonies were selected using ampicillin. Clones were confirmed to be correct by both restriction digest and DNA sequencing using the UCSD Moores Cancer Center DNA sequencing core facility.

The next step in creating the inducible cell lines was the introduction of the regulatory plasmid pVgRXR into prostatic cell lines and selection for plasmid retention using the antibiotic zeocin. During month 2, an antibiotic killing curve was created using zeocin on the DU145 cells to determine the optimal concentration in order to select for positively transfected clones. We tested a range of 0-750μg/ml zeocin and found that 250μg/ml was the concentration that destroyed all the non-transfected cells after 7 days.

During months 2-3, the DU145 prostate cancer cell line was next tested for optimal means of transfection. We initially tried using calcium-phosphate precipitation but found this had very low transfection efficiency,
prohibiting us from detecting any protein expression by Western Blot when transfecting pcDNA3-FGFR4-WT as a control. We tried using FuGENE 6 (Roche) and found that this transfected with much higher efficiency by testing for expression of GFP. We used 18μl of FuGENE 6 and 6μg of pVgRXR DNA for each 10cm plate, and the DU145 cells were plated at approximately 95% confluency. Cells were transfected for 24 hours in 0% serum with antibiotics, and the media was changed to RPMI with 10% FBS plus pen/strep for 24 hours. The following day, the media was replaced with RPMI supplemented with 10% FBS, pen/strep, and 250μg/ml zeocin. The media was changed on these plates every 3 days for 2 weeks. After this time period, we found no living cells remaining.

During months 4-5, we attempted a second transfection in the pVgRXR plasmid using the FuGENE 6 transfection agent in the presence of serum and absence of pen/strep to reduce cell death. Two 10cm plates of DU145 cells were split at approximately 50% confluence: one plate was transfected the next day using 5μg pVgRXR with 18μl FuGENE 6, while the other plate was left untransfected. This was done in RPMI supplemented with 10% FBS with no pen/strep added. The next day, the media was replaced with fresh media and the cells were allowed to recover overnight. The following day, the media was replaced with media containing 250μg/ml zeocin, and this was replaced every three days for two weeks.

At the same time, we tried using an alternate transfection reagent, Lipofectamine 2000 (Invitrogen). Cells were split at approximately 50% confluence. The next day they were transfected using 2.5μg pVgRXR, 30μl lipofectamine, and 20μl Plus reagent in RPMI supplemented with 10% FBS and no pen/strep. The media was changed the following day to fresh RPMI with 10% FBS and pen/strep. The next day media was replaced with media containing 250μg/ml zeocin. Every three days, media was replaced with fresh antibiotics for two weeks. Colonies began to appear at this time and were isolated using glass cloning rings. Five colonies were selected and grown on 6-well plates in media containing 250μg/ml zeocin. After 20 days, three of the clones had grown enough to split onto 10 cm plates. After two more weeks, two of the initial five clones had grown enough to test for inducibility.

To test whether the pVgRXR plasmid had been stably incorporated into the cells, we split two 6cm plates of each of the clones plus two 6cm plates of normal DU145 cells at about 50% confluence. Each plate was transfected with 6μl FuGENE 6 and 2μg of pIND-FGFR4-WT in RPMI containing 10% FBS without pen/strep. The next day, 25μl of 95% ethanol was added to one plate of each clone plus one plate of DU145 cells, while 25μl of Ponasterone A at 1mM (final concentration of 5μM) was added to the second plate of each clone and the second plate of DU145 cells. Cells were induced for 24 hours, lysed, and expression of pIND-FGFR4-WT was checked by Western Blot. No expression was detected, but it appeared this might be due to low transfection efficiency, thereby allowing us to see inducibility only when we stably incorporated the pIND-FGFR4-WT plasmid into the pVgRXR clones.

An antibiotic killing curve was generated using G418 in order to select for the expression of the pIND plasmid. A range of 0 to 750μg/ml was tested and we determined that 400μg/ml was the optimal concentration to kill all of the non-transfected cells. During months 6-8, each of the two possible DU145-RXR clones was split at 50% confluence on 10cm plates and transfected with 5μg pIND-FGFR4-WT and 18μl FuGENE 6 reagent, as per previous protocols. After one day of transfection and one day of recovery, media containing 250μg/ml zeocin plus 400μg/ml G418 was replaced every 3 days for 2 weeks. Colonies then began to appear and were isolated using glass cloning rings. Colonies were grown in 6-well plates until reaching a higher confluence, at which point they were moved to 10cm plates (about 4 weeks). Twelve colonies were chosen and tested for inducibility as before (addition of 5μM Ponasterone A to 6cm plate of cells and induced for 24 hours). None of the twelve clones tested were inducible for FGFR4-WT, indicating either the pVgRXR had not been stably incorporated into the cells, or the pIND-FGFR4-WT DNA had not been incorporated. The fact that we had tested twelve
different colonies led us to believe that the pVgRXR DNA had not been stably incorporated into the original two DU145-RXR colonies.

Simultaneously, we had been working up DU145-RXR clones from the second FuGENE 6 transfection. After two months of selecting colonies, growing them in 6-well plates, and working them up to grow on 10cm plates, we had 6 potential DU145-RXR clones with which to work. These were again tested for inducible expression of pIND-FGFR4-WT by transient transfection, however, no clones appeared positive for expression of pVgRXR. After consulting with technical service at Invitrogen, they suggested a Western Blot of the lysates from each clone and immunoblotting with an antibody against RXRalpha might elucidate positive clones. Over two weeks, potential positive clones were Western-blotted with an RXR antibody, as suggested by Invitrogen, to determine incorporation of the pVgRXR plasmid. However, even the use of positive cell lines created earlier in our lab for inducible expression of other proteins (5) failed to show RXR expression by Western blot, indicating the antibody was not specific enough to detect the RXR protein in our samples. Invitrogen sent us several other lots of antibody in the event we had received a bad batch, but unfortunately each lot of antibody produced the same, negative results.

We proceeded to review and troubleshoot possible conflicts with our process. We felt the need to verify that the pIND-FGFR4 plasmids we created during months 1-2 were indeed inducible by the Invitrogen system. During months 9-11, 293-RXR cells purchased from Invitrogen that were already stably expressing the pVgRXR plasmid were transfected with the various pIND-FGFR4 constructs. Clones were selected by addition of G418 antibiotic at 400μg/ml with zeocin at 250μg/ml, and expression was examined by induction with Ponasterone A at 5μM. As seen in Figure 1, all constructs were stably incorporated into 293-RXR cells and were inducible upon treatment with Ponasterone A. Though we had created 293 cells inducible for expression of the FGFR4 constructs, this was not the primary goal of this project, and served only to verify that the pIND-constructs were working properly and that production of an inducible system using this method was possible. Our goal continued to be creation of a prostate cancer cell line inducible for expression of FGFR4 constructs.

![Figure 1. pIND-FGFR4 constructs are inducible by ponasterone A.](image)

After some more troubleshooting with Invitrogen’s technical service, we learned that linearizing the pVgRXR plasmid in a region that will not disrupt the necessary components often helps in generating a stable cell line. Often, cells splice the DNA in the middle of the gene of interest when incorporating into the genome, and if this was occurring, the cells would not be translating the RXR gene necessary for induction of our pIND plasmids. During months 12-14, we linearized the pVgRXR plasmid using the restriction enzyme MluI. This site is present only once in a nonsignificant region of the vector backbone. The cut DNA was isolated from an agarose gel and transfected into DU145 cells as before, using 18ul FuGENE 6 plus about 5ug linearized pVgRXR for 24 hours. Cells were allowed to recover for an additional day, and the media was changed to media containing 300μg/ml zeocin that was changed every three days for 2 weeks.
Once colonies began to appear, six were isolated using glass cloning rings and grown in the presence of zeocin for another two weeks. Each clone was then transfected with pIND-FGFR4-WT and selected with 400μg/ml G418 in addition to the zeocin. Once colonies began to form, the cells were collected into a polyclonal culture, allowing us to examine many colonies at once to see if some percentage were inducible for FGFR4. We tested as before by addition of Ponasterone A to determine if the polyclonal culture was inducible for FGFR4-WT. None of the 6 polyclonal cultures was inducible for expression of FGFR4, indicating none had stably incorporated the pVgRXR, despite linearizing the DNA first.

During month 15, we obtained PC3 prostate cells from Dr. Len Deftos at UCSD. These cells express low levels of FGFR4 and we thought they might be a better choice to create inducible cell lines. A zeocin killing curve was generated for the PC3 cells using a range from 0 to 1000μg/ml to determine the optimal concentration of antibiotic to select for cells positively expressing pVgRXR. The concentration found to destroy all the non-transfected cells after 7 days was 200μg/ml zeocin.

We transfected 5μg pVgRXR using FuGENE 6 into PC3 cells during months 16-17 and selected for colonies using zeocin. No colonies formed and all the cells died. We spoke with Dr. Len Deftos who told us that he used Lipofectamine 2000 successfully to transfet the cells. We tested different combinations of DNA-lipofectamine ratios and found that a ratio of 4μg DNA to 10μl lipofectamine gave the highest transfection rate. PC3 cells were transfected using pVgRXR at this ratio and selected using 200mg/ml zeocin. Thirteen colonies grew and were isolated using glass cloning rings.

These colonies were worked up and months 18-19 were spent testing these clones to see if they were inducible for FGFR4 by transient transfection of the pIND-constructs. **Figure 2** is a single clone, representative of how all the other clones were tested for incorporation of pVgRXR. We transfected in pcDNA3-FGFR4-WT (lane 1) as a positive control for FGFR4 expression. In lanes 2-3, we transfected only the pIND-FGFR4-WT plasmid and lane 3 was induced with Ponasterone A. If the RXR clone stably incorporated pVgRXR, lane 3 should have induction of FGFR4 WT, but none of the 13 clones showed any induction of FGFR4 WT. As an additional control, we transiently transfected both the pIND-FGFR4-WT as well as the pVgRXR DNA into lanes 4-5 and lane 5 was induced with ponasterone A. As shown in **Figure 2 lane 5**, when we transiently transfected pVgRXR with pIND-FGFR4-WT, induction of the FGFR4 product was achieved, indicating that the clone does not stably express the pVgRXR on its own and only expresses FGFR4 when both plasmids were transiently transfected (compare lane 3 to lane 5).

During month 20-21, we next tried to linearize the pVgRXR plasmid as before, but this time we purified the cut DNA using a phenol extraction. This linearized DNA was transfected using lipofectamine 2000 and selected using zeocin. At the same time, we attempted to co-transfect both the linearized pVgRXR, as well as linearized pIND-FGFR4-WT. The co-transfected cells were selected with 200μg/ml zeocin plus 200μg/ml G418. Six clones from each transfection were grown up and tested for inducibility as before, rendering none of the 12 clones tested inducible for FGFR4.

While transiently transfecting PC3 cells with pcDNA3 FL-FGFR4 or PTD-FGFR4 constructs, we discovered that the expression of FL-FGFR4, and especially PTD-FGFR4, dramatically decreased over time. During a time
course experiment we found that 48 hours after transfection the PTD-FGFR4 protein was not detectable (see Figure 3, lanes 1-4).

![Figure 3. PTD-FGFR4 expression in PC3 cells is unstable.](image)

The results of the decrease in PTD-FGFR4 expression led us to believe that a potential problem in creating the RXR cell lines might be the length of time between transfection and selection of the cells. We had previously waited 48 hours before adding antibiotics. When it appeared that the RXR was not being expressed after 48 hours, we tried one last attempt to create the RXR cell line. Months 22-24 were spent trying to stably introduce the pVgRXR plasmid into PC3 cells, but this time we selected cells after only 8 hours of transfection. Disappointingly, after testing 6 clones by transient transfection of pIND-FGFR4, once again no RXR-positive cells were found.

The second task of this proposal was to examine the effects of FGFR4 expression on downstream signaling components, comparing full-length and truncated forms of FGFR4 and G388R. As we found ourselves unsuccessful in generating PC3 cell lines inducible for expression of FGFR4, we proceeded to examine downstream effects of the various constructs by transient transfection of PC3 cells. During month 25, we looked at activation of the MAPK pathway as well as altered NCAM expression and found no significant differences when comparing WT and G388R FGFR4 in either the full-length or truncated mutants, as seen in Figure 4.

![Figure 4. FGFR4 effect on downstream signaling components.](image)

Due to the low transfection efficiency of PC3 cells, detecting any differences in endogenous downstream signaling components in total cell lysates was not possible. One way to avoid this problem was to look at signaling components by immunofluorescence. In this way, we would be able to examine only the cells expressing our FGFR4 constructs to view the effect on downstream signaling components. During month 26, we transiently co-transfected the FGFR4 constructs with GFP-STAT1 and GFP-STAT5b expression plasmids into PC3 cells and examined STAT1 and STAT5 localization after FGF2 ligand stimulation. Our preliminary results indicated that there was no difference in STAT1 or STAT5 localization when comparing FL-FGFR4-WT to FL-FGFR4-G388R, or when comparing PTD-FGFR4-WT to PTD-FGFR4-G388R (see Figure 5).
PC3 cells were seeded onto glass coverslips and transfected with the appropriate STAT and FGFR4 constructs. Cells were starved for 24 hours. They were either left untreated, or treated for 15 minutes with 100ng/ml FGF2 in the presence of 1μg/ml heparin. Coverslips were fixed with 3% paraformaldehyde and processed for indirect immunofluorescence. FGFR4 expression was detected with rabbit polyclonal antibody and rhodamine-conjugated antirabbit secondary antibody. Nuclei were visualized by Hoechst dye added to the Rh secondary antibody mix.

We have found other interesting avenues of research relating to the G388R polymorphism and its role in prostate cancer (albeit unexpected and circuitous to our original statement of work), which we are continuing to pursue. We completed a yeast two-hybrid screen to identify novel proteins that interact with FGFR4. Using the intracellular domain of FGFR4 as bait, we identified a variety of interacting proteins, including IKKγ. Since then during months 26-28, we have confirmed the validity of this interaction using a filter-lift β-galactosidase assay, and have also demonstrated that IKKβ coimmunoprecipitates with FGFR4 in HEK293 cells. These experiments revealed that IKKβ not only associates with FGFR4 but also, unexpectedly, undergoes tyrosine phosphorylation in response to FGFR4 activation. Typically, IKKβ is activated by Ser phosphorylation within the activation loop (6). Upon activation, IKKβ phosphorylates IκBα on serine residues, leading to its degradation by the 26s proteasome. This releases NFκB from its inhibited, bound state to translocate to the nucleus, which eventually leads to transcription of a variety of genes (7).

Our preliminary results generated during months 29-30 are presented in Figure 6 and Figure 7. The demonstration that a member of the FGFR family, in this case FGFR4, stimulates tyrosine phosphorylation of IKKβ provides an entirely novel link from FGFR signaling pathways to NF-κB signaling pathways, and may explain how FGFR signaling plays an important role in cell survival. There is precedent in the literature (8,9) for IKKβ activation by Tyr phosphorylation by the src protein kinase in gastic cancer, although this has not been extensively examined by other researchers.

Interestingly, it appears in Figure 7 that the FL-FGFR4-G388R protein has a stronger interaction with IKKβ. To examine the possible altered effects of the G388R mutant on NFκB signaling could be a very exciting avenue of work. It would also be important to study this interaction in prostate cancer cells to illuminate the role of FGFR4 interaction with IKKβ in prostate cancer progression.
Figure 7. FGFR4 interaction with IKKβ. HEK293 cells were cotransfected with FGFR4 derivatives and IKKβ. Cells were lysed in 1% NP-40 lysis buffer, and lysates were immunoprecipitated with IKKβ antisera, separated as before, immunoblotted with (top) FGFR4, and (2nd panel) IKKβ antisera. Cell lysates were separated and analyzed for expression by immunoblotting with (3rd panel) FGFR4, and (bottom) IKKβ antisera.

Key Research Accomplishments (as described more fully above)
- Construction of four different FGFR4 derivatives, as described in this proposal
- Initial characterization of downstream signaling components in prostate cancer cells, as described in this proposal
- Yeast two-hybrid assay with FGFR4, identifying IKKβ as a positive interaction
- Determination that FGFR4 activation leads to tyrosine phosphorylation of IKKβ

Reportable Outcomes
- Using transient transfection, we find no significant differences in MAPK activation or NCAM expression when comparing FL-FGFR4-WT to FL-FGFR4-G388R or PTD-FGFR4-WT to PTD-FGFR4-G388R
- Using immunofluorescence by transiently transfecting PC3 cells, we find no significant differences in GFP-STAT1 or GFP-STAT5 localization when comparing any of the FGFR4 derivatives.
- IKKbeta interacts directly with FGFR4
- Activation of FGFR4 leads to tyrosine phosphorylation of IKKbeta.

Conclusion
Though unsuccessful in generating FGFR4-inducible prostate cancer cells, we have characterized downstream signaling components in response to transient transfection of the various forms of FGFR4 in PC3 prostate cancer cells. No significant differences were found when comparing any of the FGFR4 derivatives; however, the low transfection efficiency may be the reason we are unable to see any distinguishable differences in endogenous downstream signaling components. We have made excellent progress in a new direction that is highly interesting and potentially very important in our understanding of FGFR4’s role in prostate cancer progression. Understanding the role of the IKKβ interaction with FGFR4 and what significance the FGFR4-induced tyrosine phosphorylation on IKKβ has to the cell will be of great interest as we continue our research. It will also be of interest to discern whether the G388R polymorphism of FGFR4 has any altered binding or increased kinase activity toward IKKβ.
References


Appendices

N/A