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Identification and Therapeutic Targeting of Paracrine Senescence Factors in the Prostate Tumor Microenvironment

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14. ABSTRACT
The Purpose of this proposal is to examine how senescence in the prostate may be caused by medical treatments for prostate cancer, and to identify senescence-associated factors which may mediate resistance of neoplastic epithelium. To date, our major findings present a mixed picture of chemotherapy-induced senescence. Senescence-associated β-galactosidase staining has not identified significant chemotherapy-induced senescence, but quantitation of gene expression changes reveal a pervasive pattern of senescence changes. Correlation of chronological aging and senescence is seen. Detailed investigations into a putative secreted marker of senescence, STC1, find significant decreases in cancer compared to benign prostate glands, establish STC1 secretion as a response to numerous microenvironmenal stressors including chemotherapy, and find increased STC1 expression in human prostate tissue after neoadjuvant chemotherapy. Finally, abrogation of the senescence-associated IGF pathway is being tested in a clinical trial of neoadjuvant anti-IGF-1R antibody therapy with combined androgen deprivation prior to prostatectomy.

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Introduction

Deaths due to prostate cancer- the second leading cause of cancer death in men in the United States - could be prevented with more effective treatments. Overcoming tumor cell resistance to the effects of androgen deprivation and chemotherapies would significantly improve the morbidity and mortality of prostate cancer. We ***hypothesize*** that the induction of cellular senescence in the tumor microenvironment by androgen deprivation and cytotoxic chemotherapy promotes the resistance and survival of carcinoma cells. We further ***hypothesize*** that targeting senescence-associated pro-survival paracrine factors will enhance the effects of these therapies and enhance response rates.

To address these hypotheses, we have three aims: First, to identify senescence changes in prostate tissue induced by androgen deprivation and chemotherapy, specifically focusing on identifying factors with the potential to influence the survival/resistance of neoplastic epithelium via paracrine mechanisms. Second, to evaluate the effects of inhibiting specific senescence-associated pro-survival factors using *in vivo* models. Third, to develop and execute clinical trials designed to inhibit senescence-associated paracrine survival mechanisms and determine if enhanced tumor responses can be achieved.

Body

The following summarizes the research accomplishments of the third year of this proposal, as associated with each task in the Statement of Work.

Technical Objective 1: To determine the effects of chemotherapy and androgen ablation therapy on the frequency, type, and location of senescent cells in the prostate, and to identify changes in levels of senescenc-associated signaling molecules found in those tissues.

Objective 1a. *Identify senescent cells in pre- and post-chemotherapy prostate tissues from 58 patients.*

Task 1. Perform histochemical staining of a defined set of senescence biomarkers. (Months 1-6) As previously described, histochemical staining for the "gold standard" marker of senescence, the Senescence-Associated -Galactosidase (SA- -Gal) on post-chemotherapy prostate tissues from a random sample of 25 treated patients yielded highly variable results in an optimized assay with positive staining only noted in 6 of the patients. Given these results, it was our judgement that analyses of pre-treatment biopsy tissues were not likely to yield useful data with this method. The p16(INK4a) and DcR2 markers of senescence were also evaluated using Tissue Microarray of Aging and Prostate Cancer.

To compare staining pre- and post-treatment samples, it is necessary to be able to stain frozen tissues, as the pre-treatment biopsy cores obtained for research purposes in this study of neoadjuvant chemotherapy were preserved in this fashion to allow gene expression analysis, and not fixed and embedded in the standard fashion. Given our interest in modulating possible signaling pathways in senescence and the limited amount of tissue available for staining analyses, further staining to identify senescent cells was deferred in this interim to allow detailed investigations into the expression and function of the secreted signaling molecule STC1. In addition,

deferral of this work will allow simultaneous staining of these tissues with the tissue staining as described in Objective 1b for optimal reproducibility and comparative statistical analyses.

Task 2. Analyze stained tissues, quantitating frequency of positive staining cells in the epithelium versus stroma, in or near benign versus neoplastic prostate glands. (Months 2-8) Prior work demonstrated low levels of staining for p16(INK4A) in the benign epithelium and stroma. In the benign epithelium, approximately 1-3% of the cells had identifiable staining, assuming average staining level of 1+. In the stroma a similar number of cells, approximately 1-2% of cells stained positively for p16. In both compartments, there was an identifiable increase that occurred with aging, but obviously, these cells still represented rare events. By contrast, many more cells in the neoplastic epithelium stained for p16 (about 30%), but there was no change seen with aging.

In comparison, the DcR2 biomarker of aging also stained very few cells in the stroma (0.2-0.7%). With aging, there was a significant increase in these still rarely staining cells. In the neoplastic epithelium, there were very high numbers of cells with positive staining, representing 88-97% of cells. There was no increase with aging. Only in the benign epithelium was there a significant increase in DcR2 staining seen with aging, that occurred in a significant fraction of the cells evaluated. In the young cohort, 16.5% of cells stained positively, compared to 39% of cells in the aged cohort.

Task 3. Perform statistical analyses to determine the significance of staining differences between the various compartments and differences before and after chemotherapy. (Months 2-8) As previously described, and as detailed above, there were statistically significant increases in staining of benign epithelium and stromal compartments in aged men, when compared to young men. However, close evaluation of this staining reveal that only DcR2 staining of the benign epithelium was notable for a reasonable frequency of staining in combination with this statistically significant increase. Based on this, and given the limited number and amount of tissues available for these analyses, DcR2 staining will be further evaluated as a senescence marker in these tissues.

Objective 1b. *Identify senescent cells in pre- and post-androgen ablation prostate tissues from 48 patients.*

Task 4. Perform histochemical staining of a defined set of senescence biomarkers. (Months 12-20) - pending Due to poor accrual rates with this clinical trial, one arm of the clinical trial was discontinued for a revised total enrollment goal of 36 patients. Enrollment has recently been completed and 35 patients have completed treatment on this protocol of neoadjuvant androgen ablation therapy given prior to prostatectomy. In order to ensure optimal results in analyses of these limited tissues, we opted to defer staining of these tissues to synchronize with other analyses. Once the final tissues are acquired, staining will proceed.

Task 5. Analyze stained tissues, quantitating frequency of positive staining cells in the epithelium versus stroma, in or near benign versus neoplastic prostate glands. (Months 13-22)-pending

Task 6. Perform statistical analyses to determine the significance of staining differences between the various compartments and differences before and after chemotherapy. (Months 13-22)-pending

Objective 1c. *Examine senescence-associated signaling molecules in pre- and post-chemotherapy prostate tissues from 58 patients.*

Task 7. Perform histochemical staining of secreted signaling molecules. (Months 3-18)

As described previously, we employed the tissue microarray of aging and prostate cancer to optimize the staining for a set of senescence-associated, secreted signaling molecules, completing staining and analyses for STC1, GDF15, CXCL1 and IL8. Specific stromal localization of STC1 immunoreactivity was investigated using consecutive serial sections of a neoplastic prostate gland, interspersing STC1-stained sections with staining for various other likely and interesting possible constituents of the stroma. STC1 localization was evaluated in post-chemotherapy tissues as per the above Objective 1c. Finally, STC1 staining was examined in a case series of bony metastases within a tissue microarray derived from patients who underwent a rapid autopsy for the purpose of collecting metastatic prostate cancer deposits. These patients had been exposed to a heterogeneous set of medical therapies for metastatic prostate cancer, including hormonal therapies and chemotherapies prior to their death. Thus, these tissues are a valuable resource for examining the combined effect of these therapies in patients with advanced disease.

Task 8. Analyze stained tissues, quantitating staining intensities and locations of positive staining; epithelium versus stroma, in or near benign versus neoplastic prostate glands. (Months 4-20)

As described previously, the staining patterns of STC1 and GDF15 have been analyzed similarly to the p16 and DcR2 analyses as described above in Objective 1a, Task 2 using the Tissue Microarray of Aging. We demonstrated that overall in the TMA, there was definitely a significant amount of variability in STC1 staining of neoplastic glands. This may reflect the inherent variability in the genetic background of different neoplasms and/or variability in senescence changes in different cancers. By contrast, overall there was significantly more staining of STC1 in the benign epithelial glands with an approximately 50% increase in staining score, reflecting the average amount of staining through each sample. In the stroma, there was a heterogeneity in staining, with patterns of diffuse lighter staining, very intense individual cells, and clusters of intensely staining cells. Subsequently, I showed co-localization of scattered individual STC1 immunoreactive stromal cells with the fibroblastic/myofibroblastic markers caldesmon and sm-a-actin. In addition, there was co-localization of the neuronal markers neurofilament and S100 with the intensely staining cell clusters, unequivocally demonstrating that the nerves of the prostate are intensely stained with STC1.

I also previously described work applying the optimized protocol for STC1 immunolocalization to examine prostate tissues for changes in this senescence-associated signaling molecule after chemotherapy treatments. In this work, it was found that excess background staining, particularly compared to the staining achieved with FFPE tissues, limited appreciation of chemotherapy-induced changes in STC1 staining. In addition to staining of these prostate sections, a tissue microarray consisting of various metastatic prostate cancer tissues obtained from men by rapid autopsy shortly after death was stained for STC1. Results were representative of the staining seen in the bone samples of this TMA, and suggested that the staining seen in the primary tumors is retained during the metastatic process.

Exploratory investigations into STC1 in the neoplastic prostate and its response to therapy:

Importance of the hepatocyte growth factor- c-met receptor axis in STC1 secretion

As described in the last annual report, published data demonstrate that hepatocyte growth factor (HGF) is able to stimulate the secretion of STC1 from endothelial cells in vitro and that that HGF secretion is increased by prostate stromal senescence in vitro. Given the importance of HGF for pro-neoplastic processes in the prostate epithelium, this potentially important axis in prostate senescence was hypothesized to contribute the the effects on senescent stroma. In the last interim, I demonstrated that STC1 is increased by in vitro senescence of the prostate stromal cell line PSC27 and that recombinant HGF was able to activate the c-met receptor and increase secreted STC1 levels.

Subsequent to that report, I attempted to replicate these results and found significant variability over time. As part of these experiments, the c-met inhibitor SU11274 was used to treat the prostate stromal cells in hopes of further demonstrating HGF-specific increases in STC1 secretion. Strikingly, STC1 secretion was very consistently and dramatically increased by SU11274 treatment (Figure 1a). Secondly, I constructed a viral overexpression system to allow significantly increased constitutive STC1 secretion. While I was able to successfully demonstrate STC1 overexpression when compared to empty vector control, there was an unexpected effect of growth conditions. Specifically, increasing amounts of serum were found to increase STC1 secretion levels.

STC1 secretion is increased by a broad spectrum of microenvironmental conditions

Together, it was apparent that STC1 secretion in the prostate needed to be fully characterized to allow further experiments. I performed a titration of SU11274 concentrations which demonstrated that STC1 secretion was dose-dependent (Figure 1b). In a similar fashion, a titration of serum concentration demonstrated the dose-dependence of STC1 secretion (Figure 2). To further characterize the serum-dependent STC1 secretion increases, I treated cells with increasing amounts of serum which had been pre-treated by boiling. Compared to native serum, there were slight decreases in STC1 secretion induced by boiled serum, suggesting that although heat labile factors (such as proteins) contribute to STC1 secretion, non-labile factors account for the majority of the serum effect.

In the literature, numerous growth conditions have been previously found to induce STC1 secretion in various tissues, although never tested in prostate tissues or cell lines. Consistent with published literature, STC1 secretion from prostate stromal cells was induced by hypoxia (1% oxygen, compared to normoxia - 20% oxygen) (Figure 3), calcium (but not magnesium) (Figure 4a), phosphate (but not sulfate) (Figure 4b), osmotic stress (sodium chloride) (Figure 4c). As with serum, for each of these factors, there was a concentration-dependence for each of these treatments. I also showed that the calcium, phosphate and osmotic stress treatments increase STC1 secretion under hypoxic, as well as normoxic conditions, but that serum is still the most effective inductive factor (Figure 3, data not shown). Together, these experiments suggested that STC1 secretion may play a central role in responses to diverse alterations in environmental conditions, particularly abnormal or stressful conditions.

Given my identification of STC1 as a secreted factor increased by senescence, I also tested various forms of senescence induction in prostate stromal cells (Figure 5). In addition to oxidative stress (hydrogen peroxide), direct DNA damage (bleomycin exposure), replicative ex-

haustion (telomere exhaustion) (Figure 5a), and oncogene-induced senescence (RAS overexpression), but not p16 overexpression (direct induction of senescence) (Figure 5b) result in increased STC1 expression. Thus, increased STC1 secretion is a largely, although not wholely generalized response to stressful, senescence-inducing conditions.

To identify additional cellular signaling pathways which might be important for increased levels of STC1 secretion, I tested the effects of several small molecule inhibitors of cellular signaling cascades (Figure 6). Particularly in the case of the mitogen-activated protein kinase pathway inhibitor UO126, there was a dose-dependent inhibition of STC1 secretion induced by serum. This was also seen to a lesser extent for the protein kinase A pathway inhibitor H89 and the phosphatidyl inositol-3-kinase pathway inhibitor LY294002. This suggests that all three pathways play a role in the STC1 secretion increases, with the MAPK pathway being most important. However, perhaps even more interestingly, induction - rather than inhibition - of STC1 secretion could be seen for intermediate doses of H89 and LY294002. Thus, the data for these inhibitors in the presence of serum can not be interpreted simply, but must also take into account this induction. Given the possibility that STC1 appears to be secreted in response to cellular stress, the appropriate doses of these drugs may be sufficient to induce the secretion of STC1 in some circumstances as a non-specific stress effect.

Finally, given our interest in optimizing chemotherapy treatments for prostate cancer, I tested whether STC1 secretion from the stromal cells was altered by docetaxel, mitoxantrone and bleomycin (Figure 7). Indeed, I found STC1 to be induced by ongoing docetaxel and bleomycin chemotherapy treatments (as opposed to post-bleomycin senescent cells discussed above), but not mitoxantrone.

As noted, all of the above studies were performed on prostate stromal cell lines, where this factor was originally identified. To more fully understand the interplay of the stroma and epithelium in the prostate tissue, I tested four independent prostate epithelial cell lines representing benign epithelium (Primary epithelial cells- PECs), androgen-dependent epithelium (LNCaP), and castration-resistant epithelium (PC3) cells for STC1 secretion. Interestingly, STC1 could only be detected in the conditioned medium of PC3 cells (Figure 8), even after 10-fold concentration and exposure of the other cell types to the above-defined inducing conditions. However, in the PC3 cells, significantly less STC1 is secreted, compared to secretion levels of stromal cells. There are also similarities and differences noted in the inducing conditions. For example, hypoxia induces PC3 STC1 secretion but there is less effect of osmotic stress induced by sodium chloride. Calcium may have caused cell death as it resulted in no detectable STC1. However, the effect of sodium phosphate was pronounced in both serum-free and normal serum conditions. By contrast with stromal cells, both mitoxantrone and docetaxel both induce STC1 secretion from PC3 cells. However, the SU11274 c-met inhibitor only induced STC1 under serum-free conditions.

STC1 gene expression increases in the prostate after chemotherapy treatment for prostate cancer

Using the tissues from the clinical trial in Objective 1c, laser capture microdissection was used to capture cells from each of three cellular compartments of the prostate- stroma, benign epithelium, and neoplastic epithelium. Isolated and amplified RNA was reverse transcribed and used in a quantitative real-time PCR assay to quantitate levels of STC1 gene expression in these samples (Figure 9). Normalized to the RPL13a housekeeping gene, there were clearly changes in STC1 gene expression after chemotherapy treatment with the combination of docetaxel and mitoxantrone, particularly in the benign epithelium where a nearly 60-fold average increase in

STC1 expression was seen ($p<0.01$). Increased STC1 was also seen in the stroma (6-fold), but it was not statistically significant ($p<0.08$) due to inter-patient variability. Interestingly, when comparing the pre-chemotherapy tissues with one another, the neoplastic epithelium had the highest basal expression of STC1, followed by the stroma, then the benign epithelium. This is in contrast to the immunohistochemistry STC1 protein localization data detailed above and may represent the limitations of the in vitro models currently available or altered correlation between STC1 RNA levels and protein secretion levels in vivo.

Consequences of STC1 in the tissue microenvironment

Given the increases in STC1 secretion in cells exposed to chemotherapy agents, as well as numerous other environmental stressors, we hypothesized that STC1 might play a role in resistance or adaptation of prostate epithelium to these stressors in vivo. As a first step toward testing this hypothesis, I examined the effects of docetaxel and mitoxantrone on cell proliferation in vitro, with and without exogenous recombinant STC1 or a polyclonal antibody against STC1 which was employed to address the possibility of autocrine effects of STC1 secretion. A similar antibody against the HGF protein was used as a control. Again, a series of cell lines were tested in this assay to represent benign (immortalized BPH1), hormone-responsive (LNCaP) and castration-resistant (PC3) (Figure 10). Interestingly, no discernable effect could be seen on cellular resistance or sensitivity to mitoxantrone in any of the tested cell lines (data not shown). By contrast, small but significant effects could be seen on the docetaxel treatment effect. In the BPH1 cell line, addition of anti-STC1 antibodies caused significant increase in sensitivity to docetaxel. In LNCaP cells, addition of exogenous STC1 decreased sensitivity to docetaxel. In the case of PC3 cells, there was a slight trend toward increased resistance with exogenous STC1, but only in the mid-dose level. It is not clear how STC1 affects cellular resistance to docetaxel treatment. Preliminary data would suggest that STC1 potentiates purinergic receptor-dependent growth inhibition and increases the fraction of PC3 cells in the S phase of the cell cycle (data not shown).

Together, these data define a diverse set of environmental, genetic, and treatment conditions which induce STC1 secretion, and begin to define possible functional consequences of STC1 increases.

Task 9. Perform statistical analyses to determine the significance of staining differences between the various compartments and differences before and after chemotherapy. (Months 4-20) As described previously, a statistically significant increase in GDF15 and decrease in STC1 staining of neoplastic epithelium was seen compared to benign epithelium, but there were no significant changes in benign epithelium, neoplastic epithelium, or stroma in young men compared to older men. Further, no age-dependent change in the staining intensities, frequencies, or patterns could be discerned for the GDF15 and STC1 proteins. No significant stromal GDF15 staining was visualized. By contrast, the increased STC1 staining seen in the stroma bordered on the statistically significant ($p=0.09$). In fact, STC1 is the first protein we have examined with intense and widespread stromal staining patterns. Optimized protocols for IL8 and CXCL1 failed to yield staining that could be analyzed in a meaningful fashion.

Objective 1d. *Examine senescence-associated signaling molecules in pre- and post-androgen ablation prostate tissues from 48 patients.*

Task 10. Perform histochemical staining of secreted signaling molecules. (Months 12-36)-pending, see above.

Task 11. Analyze stained tissues, quantitating staining intensities and locations of positive staining; epithelium versus stroma, in or near benign versus neoplastic prostate glands. (Months 13-36)-pending

Task 12. Perform statistical analyses to determine the significance of staining differences between the various compartments and differences before and after chemotherapy. (Months 13-36)-pending

Objective 1e. *Correlate observed senescence staining results from Objectives 1a-1d with clinical outcomes and gene expression data.*

Task 13. Collate senescence staining results and clinical outcomes. (Months 4-40) - pending Attempts to identify a secreted senescence biomarker with altered levels due to therapeutic treatments are ongoing and critical to allow these correlative studies.

Task 14. Collaborate with Nelson laboratory members performing the parallel expression studies to correlate staining patterns with gene expression changes. (Months 4-40) - pending As described previously, we have performed quantitative real-time PCR experiments on RNA isolated from pre- and post-chemotherapy treated tissues. We demonstrated statistically significant increases in the expression of the senescence biomarkers p16 and p21 in all three compartments of the prostate, as a result of chemotherapy treatment.

Technical Objective 2. To examine resistance of senescence-induced carcinogenesis and tumor progression to the effects of docetaxel and/or the anti-IGF-IR antibody IMC-A12 in a nude mouse xenoplant model.

Objective 2a. *Obtain regulatory approval for animal xenoplant trials.*

Task 15. Write animal protocol for therapy trials. (Months 1-2). This has been completed.

Task 16. Obtain necessary animal review board approval. (Months 2-5). Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee approval has been obtained for the proposed studies (IACUC #1743). USAMRMC Animal Care and Use Review Office (ACURO) approval has also been obtained.

Objective 2b. *Determine the resistance of senescent-dependent carcinogenesis and early tumors to docetaxel chemotherapy.*

Task 17. Culture cells and prepare cellular recombinants with BPH1 or PC3 and primary prostate stromal cells, senescent, or not. Perform sub-kidney capsule implantation surgeries. After 2 weeks of recovery, treat mice with weekly docetaxel for 3 weeks. 2 months after completion of therapy, sacrifice mice and retrieve xenoplanted kidneys. (Months 5-12) - pending The experimental work proposed in this Objective, as with Objectives 2c and 2d continued to be largely deferred to allow commitment of time and energies into completion of the clinical trial described below in Objective 3a, Task 26, as well as the experimental studies described above. As described previously, we have completed a series of studies designed to complement the therapy trials and have demonstrated that replicative senescence, as well as senescence induced by hydrogen peroxide, can mediate the growth stimulatory effects we previously described in our proposal. Technical issues with cell culture contamination have also been addressed previously, a vital pre-requisite to prevent infections of the grafted nude mice which will be used in these studies.

Task 18. Measure resulting graft sizes, fix and embed the tissues, then stain with Hematoxylin and Eosin and immunohistochemistry. Evaluate for invasiveness, senescent cell populations and neoplastic morphologies. (Months 8-14 - pending)

Task 19. Perform statistical analyses to determine significance of chemotherapy-induced changes in the senescent-stimulated, compared to the pre-senescent recombinants. (Months 10-16) - pending

Objective 2c. *Determine the effect of IMC-A12 on senescent-dependent carcinogenesis and early tumors.*

Task 20. Culture cells and prepare cellular recombinants with BPH1 or PC3 and primary prostate stromal cells, senescent, or not. Perform sub-kidney capsule implantation surgeries. After 2 weeks of recovery, treat mice with thrice weekly IMC-A12 injections continuously for 2 months, then sacrifice mice and dissect out kidneys. (Months 7-12) - pending. For further details please see explanation under Objective 2b, Task 17.

Task 21. Measure resulting graft sizes, fix and embed the tissues, then stain with Hematoxylin and Eosin and immunohistochemistry. Evaluate for invasiveness, senescent cell populations and neoplastic morphologies. (Months 10-14) - pending

Task 22. Perform statistical analyses to determine significance of chemotherapy-induced changes in the senescent-stimulated, compared to the pre-senescent recombinants. (Months 12-18) - pending

Objective 2d. *Determine the senescence-dependent resistance of advanced tumors to docetaxel chemotherapy and its modulation by IMC-A12.*

Task 23. Culture cells and prepare cellular recombinants with BPH1 or PC3 and primary prostate stromal cells, senescent, or not. Perform sub-kidney capsule implantation surgeries. Two months after implantation, treat mice with weekly docetaxel for 3 weeks, with or without IMC-A12 thrice weekly treatments. 3 weeks after completion of therapy, sacrifice mice and dissect out kidneys. (Months 10-18) - pending. For further details please see explanation under Objective 2b, Task 17.

Task 24. Measure resulting graft sizes, fix and embed the tissues, then stain with Hematoxylin and Eosin and immunohistochemistry. Evaluate for invasiveness, senescent cell populations and neoplastic morphologies. (Months 13-20 - pending)

Task 25. Perform statistical analyses to determine significance of chemotherapy-induced changes in the senescent-stimulated, compared to the pre-senescent recombinants. (Months 15-22) - pending

Technical Objective 3: To develop and execute clinical trials evaluating the effectiveness of inhibiting senescence-associated modulators of cancer cell survival in the treatment of prostate cancer. The lead candidate for such targeting is currently IGF1R in the insulin-like growth factor pathway. Other targets identified in Specific Aims/Technical Objectives 1 and 2 may also present opportunities for treatment of more novel targets. Optimal target selection and clinical trial design will be determined during year 2 of the proposal.

Objective 3a*: Participate in the execution and analysis of a Phase II clinical trial inhibiting senescence-associated modulators of cancer cell survival in combination with androgen ablation*

in the neoadjuvant setting prior to radical prostatectomy. The lead class of modulators are anti-IGF1R antibodies under development by Imclone (IMC-A12) or Pfizer (CP-751,871) which inhibit the insulin-like growth factor pathway. Other candidates/pathways will be considered and optimized during the clinical trial design phase of the project in year 2.
Task 26. Design Clinical Trial (Months 15-18) As described previously, I attended the 2007 ASCO/AACR Methods in Clinical Cancer Research workshop, July 27-August 3, 2007, during which time I had the opportunity to further refine the proposed clinical trial described above. Food and Drug Administration Investigational New Drug application had also been submitted and duly approved (IND 79729), as had University of Washington Human Subjects Review (institutional review board) approval.

As per discussions with Ms. Johanna Kidwell of the Human Subjects Protection Office, Office of Research Protections at U.S. Army Medical Research and Material Command, I am in the process of submitting application for local institutional review board approval. Subsequent to this, HSPO/ORP approval will be obtained prior to my planned laboratory investigations into the senescence changes that may be detected in tissues from these patients.

Task 27. Develop recruitment materials, hold in-service for clinical providers and study coordinator. (Months 18-24) - pending. As described previously, recruitment materials were limited to internet postings on world-wide web sites for the Fred Hutchinson Cancer Research Center/University of Washington Cancer Consortium, University of Washington Health Sciences and the Fred Hutchinson Cancer Research Center web sites. The study was posted on clinicaltrials.gov as required. In-service training sessions were presented to the infusion center nursing staff, familiarizing them with the scientific rationale of the treatment, goals of the study, and the practicalities of administration and monitoring for unexpected or serious adverse events. I also presented the clinical trial to physician providers and co-investigators.

Task 28. Recruit, enroll, and treat patients on study. Monitor and report adverse events. (Months 20-55) – pending

To date, 18 subjects have been enrolled and 14 subjects have completed treatment. There has been one complete response. There have been no serious or unexpected adverse events related to study treatment. No laboratory studies have been initiated to date pending above IRB approvals.

Task 29. Analyze pre- and post-treatment tissues for changes senescent cell content and senescence-associated signaling molecule expression as described above (Objectives 1a. and 1c.). (Months 24-46) - pending

Task 30. Collate and analyze data. (Months 24-50) - pending

Objective 3b*: Design and execute a clinical trial combining docetaxel chemotherapy with an inhibitor of senescence-associated modulation of cancer cell survival in the neoadjuvant setting prior to radical prostatectomy. The anti-IGF1R antibodies such as IMC-A12 (Imclone) or CP-751,871 (Pfizer) are currently the lead candidates for inhibition. Other candidates/pathways will be considered and optimized during the clinical trial design phase of the project in year 3.*

Task 31. Write the protocol and required regulatory documents. (Months 24-30) - pending

Task 32. Obtain regulatory approval from Institutional Review Board. (Months 30-34) - pending

Task 33. Develop recruitment materials, hold in-service for clinical providers and study coordinator. (Months 34-36) - pending

Task 34. Recruit, enroll, and treat patients on study. Monitor and report adverse events. (Months 36-60) - pending

Task 35. Analyze pre- and post-treatment tissues for changes senescent cell content and senescence-associated signaling molecule expression as described above (Objectives 1a. and 1c.). (Months 40-55) - pending

Task 36. Collate and analyze data. (Months 34-60) - pending

Technical Objective 4: To complete data analyses, compile accomplishments and reportable outcomes, and write final project reports and manuscripts.

Objective 4a*: Prepare manuscript 1.*

Task 37. Describe changes in the extent and distribution of senescent cells in the prostate as a function of chemotherapy and androgen ablation therapies. Correlate with alterations in senescence-associated signaling molecules, gene expression studies and clinical outcome measures. (Months 38-44) - pending

Objective 4b: *Prepare manuscript 2.*

Task 38. Describe the effects of docetaxel, mitoxantrone, and IMC-A12 treatment on the nude mouse model of senescence-dependent carcinogenesis and progression. Inter-correlations of presence or absence of tumor, size of grafts, extent and distribution of senescent cells and levels of senescence-associated signaling molecules. (Months 24-30) - pending

Objective 4c*: Prepare manuscript 3.*

Task 39. Describe the clinical efficacy, side effect profile, and laboratory correlates data resulting from the combination of IMC-A12 with androgen ablation. (Months 50-60) - pending

Objective 4d*: Prepare manuscript 4.*

Task 40. Describe the clinical efficacy, side effect profile and laboratory correlate study results from the clinical trail combining IMC-A12 with chemotherapy. (Months 54-60) - pending

Key Research Accomplishments

The exploratory investigations of the senescence-associated, secreted factor STC1 as described in the last annual report were significantly expanded in this interim.

• STC1 secretion from prostate stromal cells was found to be significantly increased by the c-met inhibitor SU11274 in a dose-dependent fashion, as well as by increasing serum concentrations. In the serum, there is a heat-labile factor which accounts for a small portion of the STC1 secretion effect, but most of the effect is heat-insensitive.

• Calcium, phosphate, osmotic stress, and hypoxic conditions all increase STC1 secretion from prostate stromal cells in a concentration-dependent fashion.

• Most, but not all, forms of senescence induction result in senescent prostate stromal cells which have increased levels of STC1 secretion. These include oxidative stress (hydrogen peroxide), DNA damage (bleomycin), replicative exhaustion (telomere shortening), and Ras oncogene-induced (Ras overexpression), but not p16 overexpression.

• The MAPK signaling pathway appears to play the largest role in mediating increased STC1 secretion in response to environmental conditions, but the PKA and PI3K pathways also contribute.

• Prior to establishment of the senescent cellular state, STC1 secretion is increased by ongoing chemotherapy treatments with docetaxel and bleomycin, but not mitoxantrone in the prostate stromal cell lines.

• In addition to the prostate stromal cell lines, PC3 cells, but not primary epithelial cell lines or LNCaP cells are found to secrete dectectable (albeit significantly lower than the prostate stromal lines) levels of STC1. STC1 secretion from PC3 cells is induced by similar, although not identical conditions as with the prostate stroma.

• In the human prostate, quantitation of STC1 expression finds significantly increased STC1 expression levels, particularly in the benign epithelial compartment. STC1 expression levels are also increased in the stroma, but of borderline significance. STC1 expression levels change minimally in the neoplastic epithelium.

• In the various prostate epithelial cell lines, increased docetaxel sensitivity was seen in BPH1 cell lines with anti-STC1 antibody treatment, while increased doctaxel resistance was seen by addition of exogenous STC1 to the LNCaP cell line.

• STC1 may function in concert with ATP through purinergic receptors to potentiate growth inhibition and increase the fraction of PC3 cells in S-phase.

• Work on Objective 2 has been again been deferred to allow further work on Objectives 1 and 3.

• I have written the IRB application for the laboratory analyses for Objective 3a as per my discussions with the HSPO/ORP/USAMMRMC which will be imminently submitted to the FHCRC IRB. To date, 18 patients have been enrolled on this trial of a planned 28 patients.

Reportable Outcomes

Publication:
Dean JP, Higano CS. Does Chemotherapy Have a Role Before Hormone-Resistant Disease Develops? Current Urology Reports, 10(3)226-35. 2009.

Abstract:
Dean JP, Plymate SR, Dalkin BL, Ellis WJ, Lin DW, Wright, JW, Corman, JM, Lange PH, True, LD, Montgomery, B. Neoadjuvant IMC-A12 and combined androgen deprivation with

prostatectomy for high risk prostate cancer, a phase II trial, Clinical Trials in Progress, ASCO Annual Meeting, 2010.

Manuscript in Preparation:
Dean JP, Coleman I, Sun Y, Martin DB, Nelson PS. Senescence-associated alterations in the secreted proteome of the prostate stroma: Identification of STC1 as a stress-responsive factor.

Conclusion

The research accomplished during this interim has more fully investigated STC1 as a novel biomarker of senescence and explored its role as a response to microenvironmental stressors, both in the stromal and in the epithelial compartment. Changes in STC1 secretion were seen in response to increased serum, inhibition of c-met signaling, increased calcium and phosphate, osmotic stress, hypoxia, induction of senescence, and ongoing treatment with chemotherapy. The MAPK signaling pathway appears to be of primary importance in the STC1 secretion increases with stress, but the PKA and PI3K pathways also contribute. Together, these data suggest a model whereby STC1 secretion is a generalized response to stressful microenvironmental conditions.

An in vivo role for STC1 is strongly implied by changes in STC1 gene expression after neoadjuvant chemotherapy treatment. The consequences of this are suggested by changes in chemotherapy resistance upon modulation of STC1 activity in cell culture models.

Accrual to the clinical trial of androgen deprivation is finally complete. Accrual to the IMC-A12 clinical trial is progressing nicely. Further institutional review board application to review proposed laboratory analyses of senescence changes is in process and will be duly submitted to HSPO/ORP/USAMMRMRC subsequent to approval.

Although delayed in order to allow the extensive investigations into STC1 alterations and functions in the prostate microenvironment (now under preparation for publication), it is anticipated the work of the second specific aim will be addressed in the upcoming months and that the results will be useful in interpretation of IHC results.

As we move forward, continuation of the work detailed for the first Technical Objective will more fully define the relationships of androgen deprivation therapy and chemotherapy with the induction of senescence in the prostate gland. Use of the in vivo mouse model of the effects of stromal senescence on prostate epithelial cells to examine how senescence changes may interfere with therapeutic efficacy. These data may provide the impetus and the insight necessary to allow us to ameliorate a potentially important source of resistance to medical therapies, optimizing outcomes for patients. Finally, the Phase II investigation of the insulin-like growth factor receptor antagonist IMC-A12 together with combined androgen deprivation therapy will start to test whether pathways of importance in senescence changes may also be important in cellular responses to standard medical therapies.

References

none

Appendices

none

Supporting Data

FIGURE 1. STC1 secretion in response to HGF and the c-met inhibitor SU11274.

Equal numbers of prostate stromal cells were treated with the indicated agents for 3 days, then conditioned medium was collected and equal quantities probed for STC1 by Western Blot.

FIGURE 2. STC1 secretion in response to varied concentrations of native and boiled fetal bovine serum.

Equal numbers of prostate stromal cells were treated with the indicated serum concentration for 3 days, then conditioned medium collected and equal quantities probed for STC1 by Western Blot.

FIGURE 3. STC1 secretion in response to hypoxia- additive effects of serum and osmotic stress.

Equal numbers of prostate stromal cells were treated with the indicated additives for 3 days under hypoxic or normoxic conditions, then conditioned medium was collected and equal quantities probed for STC1 by Western Blot.

FIGURE 4. STC1 secretion in response to calcium, phosphate and osmotic stress.

Equal numbers of prostate stromal cells were treated with the indicated additives for 3 days, then conditioned medium collected and equal quantities probed for STC1 by Western Blot.

FIGURE 5. STC1 secretion from senescent prostate stromal cells.

Equal quantities of medium conditioned by prostate stromal cells made senescent by each of the displayed methods (ASB- Bleomycin, ASH- Hydrogen Peroxide, RS- replicative exhaustion, pRAS- Ras oncogene overexpression, p16- p16 overexpression, pDEST- control vector) were collected and equal quantities probed for STC1 by Western Blot.

FIGURE 6. STC1 secretion after treatment with cell signaling inhibitors.

Equal numbers of prostate stromal cells were treated with the indicated agents for 3 days, then conditioned medium collected and equal quantities probed for STC1 by Western Blot.

FIGURE 7. STC1 secretion from prostate stromal cells undergoing chemotherapy treatments.

Equal numbers of prostate stromal cells were treated with the indicated agents for 3 days, then conditioned medium collected and equal quantities probed for STC1 by Western Blot.

FIGURE 8. STC1 secretion from PC3 cells, response to environmental conditions.

Equal numbers of prostate PC3 cells were treated with the indicated agents under the indicated conditions for 3 days, then conditioned medium collected and equal quantities probed for STC1 by Western Blot.

FIGURE 9. STC1 gene expression in the human prostate before and after neoadjuvant chemotherapy treatment

STC1 gene expression was quantitated using quantitative real-time PCR, normalized to RPL13a, after laser capture microscopy, RNA isolation, amplification, and reverse transcription. 2-sample, 2-tailed T-test was used to calculate p-values.

FIGURE 10. Effects of exogenous STC1 or anti-STC1 antibodies on prostate epithelial resistance to docetaxel.

Each prostate epithelial cell line was plated and treated with a range of docetaxel concentrations, in the presence or absence of exogenous STC1, anti-STC1 antibody, or the control anti-HGF antibody. Error bars represent one standard deviation.

Figure 1.

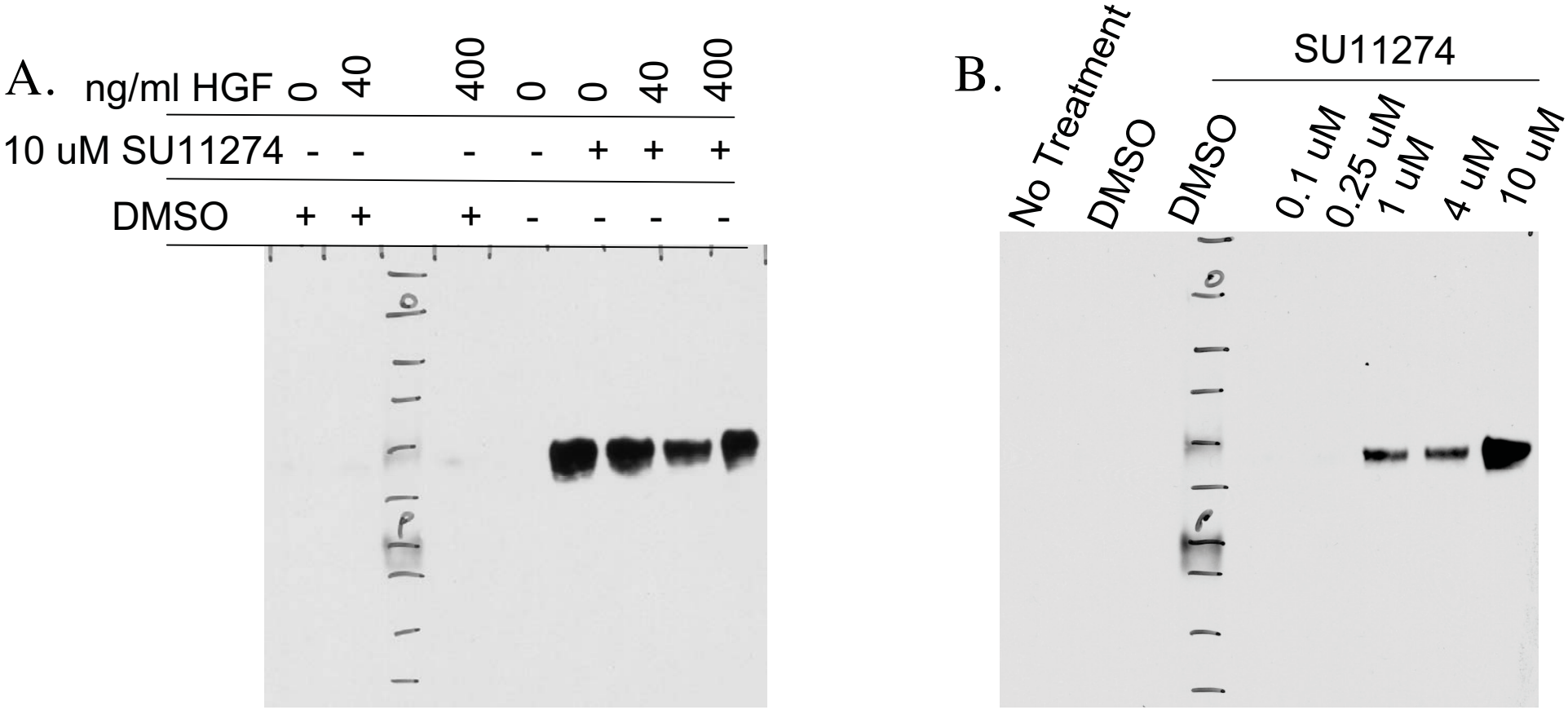

A.
ng/ml HGF 0 40 400 0 0 40 400
10 uM SU11274 - - - - + + +
DMSO + + + - - - -
B.
SU11274
No Treatment
DMSO
DMSO
0.1 uM
0.25 uM
1 uM
4 uM
10 uM

Figure 2

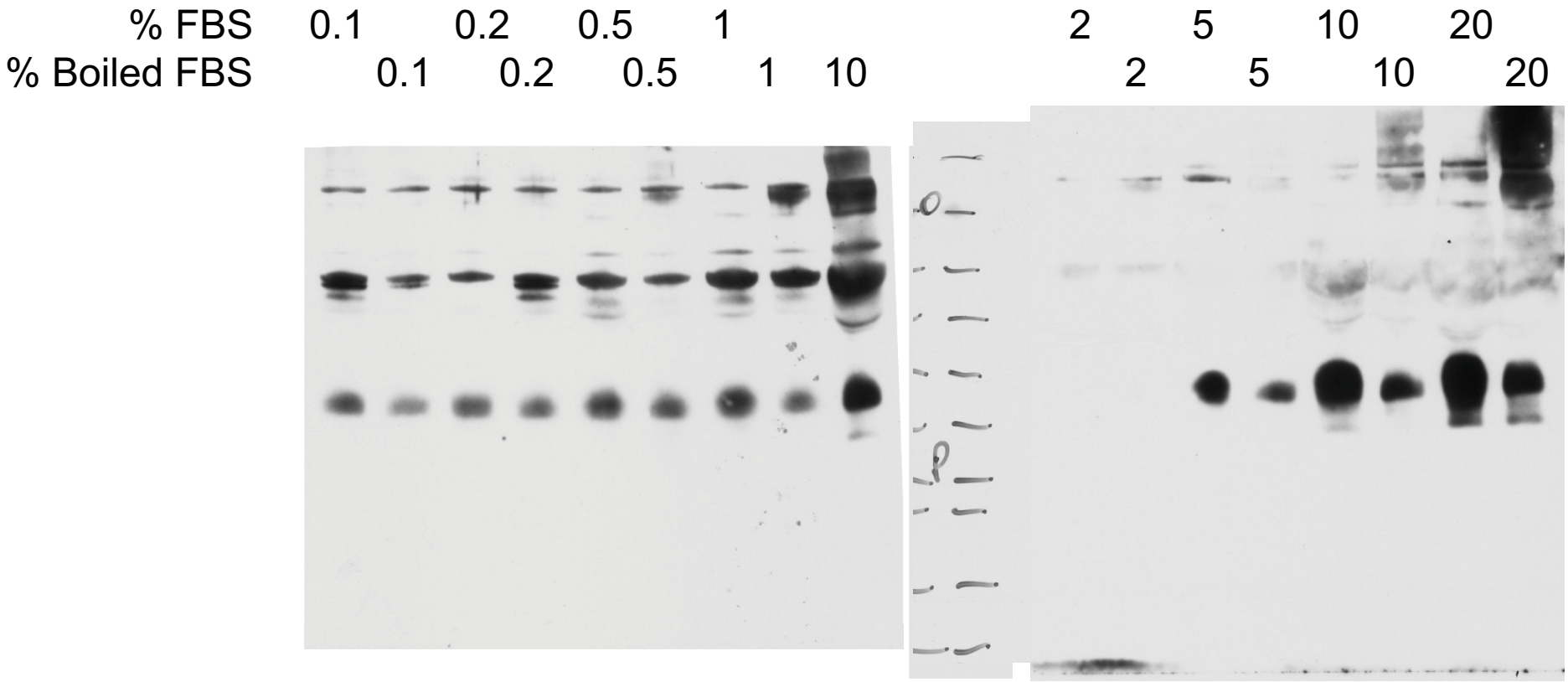
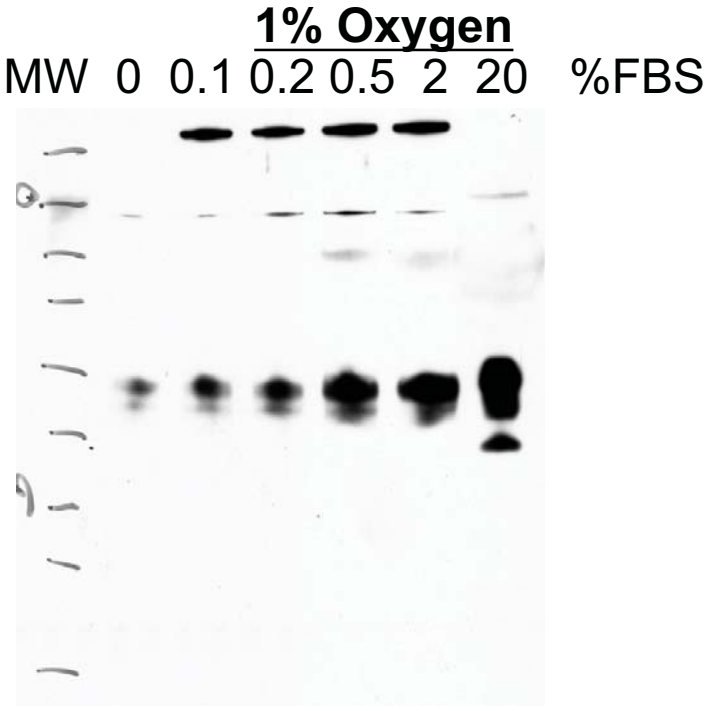


Figure 3

A.


1% Oxygen
MW 0 0.1 0.2 0.5 2 20 %FBS

B.

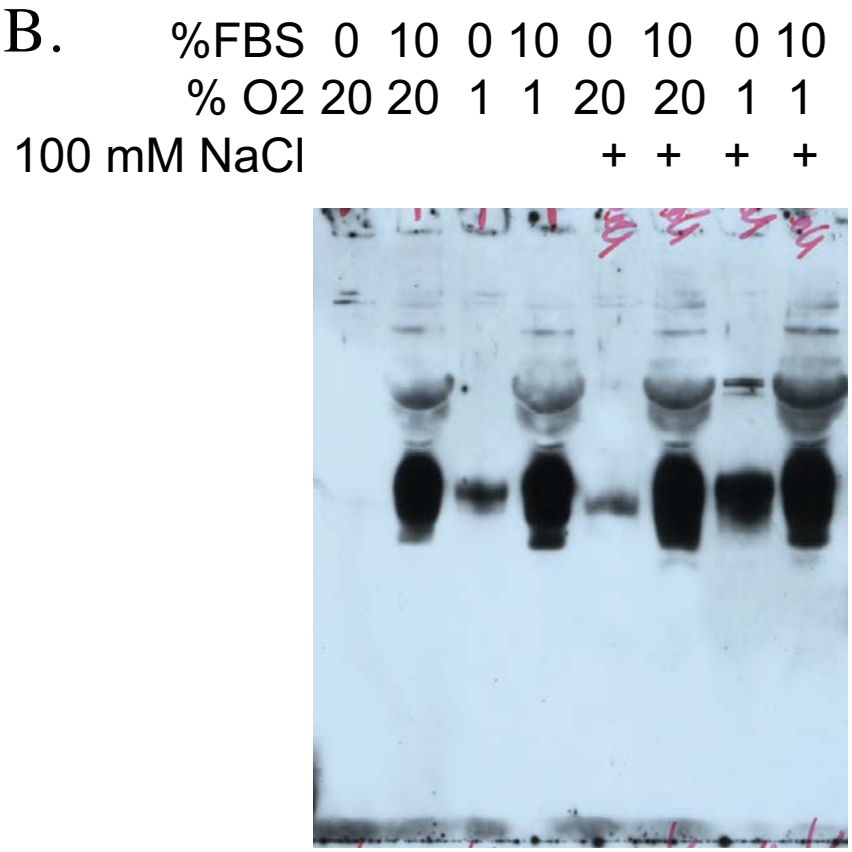
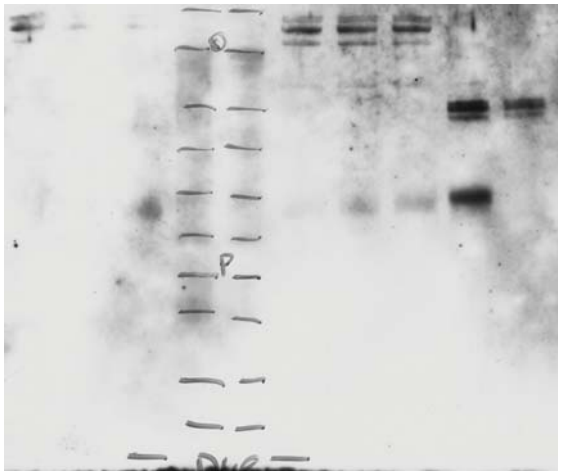

%FBS 0 10 0 10 0 10 0 10
% O2 20 20 1 1 20 20 1 1
100 mM NaCl + + + +

Figure 4

A.

mM CaCl2 0 1 5 20 100

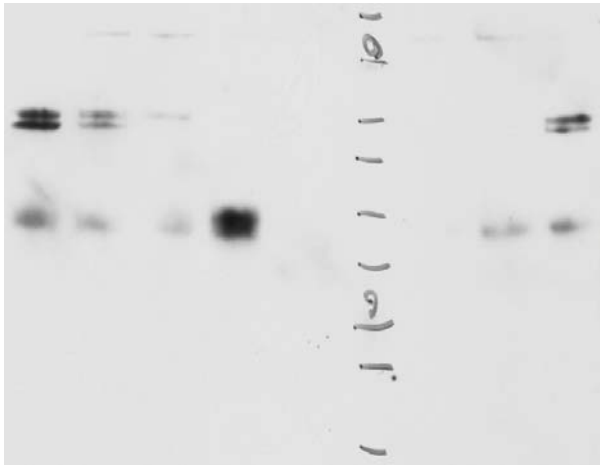
mM MgCl2 1 5 20 0



B.

mM NaPhosphate 0 1 5 10 50

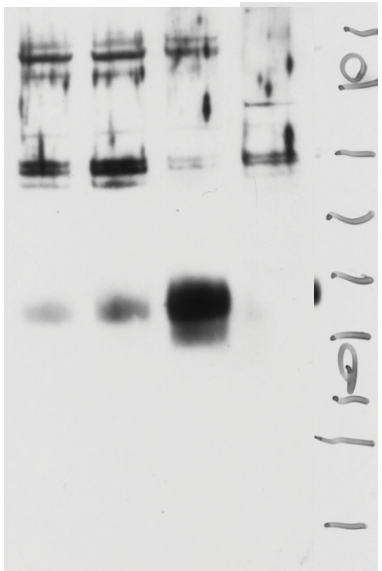
mM NaSulfate 0 1 5 10



C.

Serum-Free

mM NaCl 0 50 100 200



Serum-Containing

mM NaCl 0 50 100 200

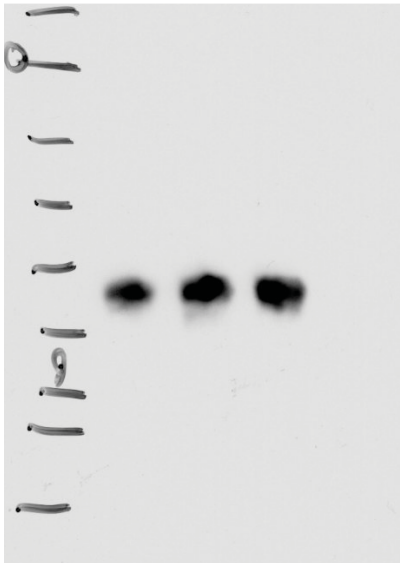
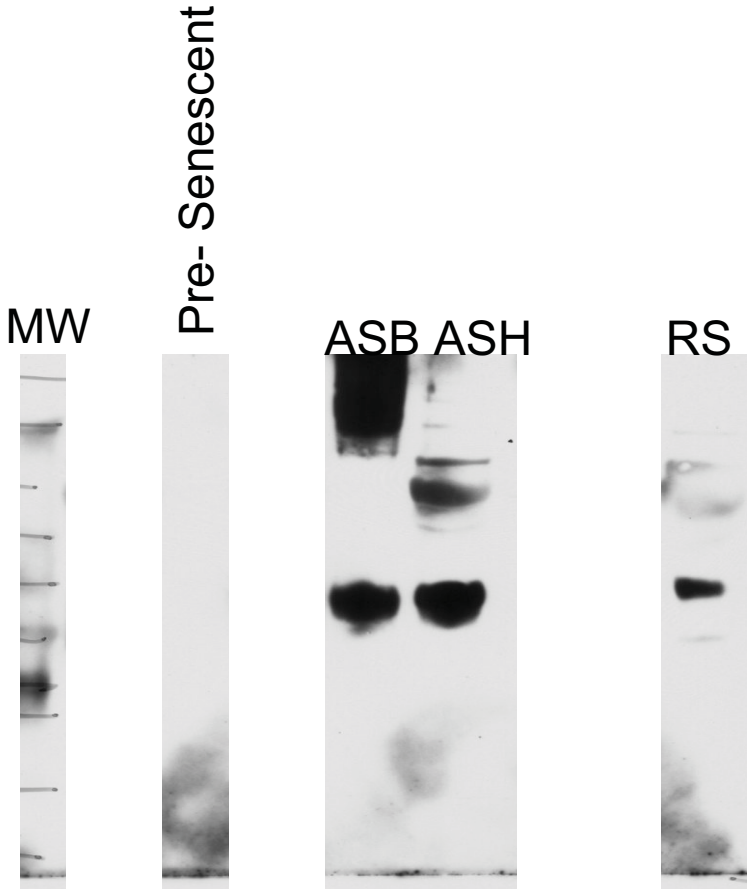


Figure 5.

A.



B.

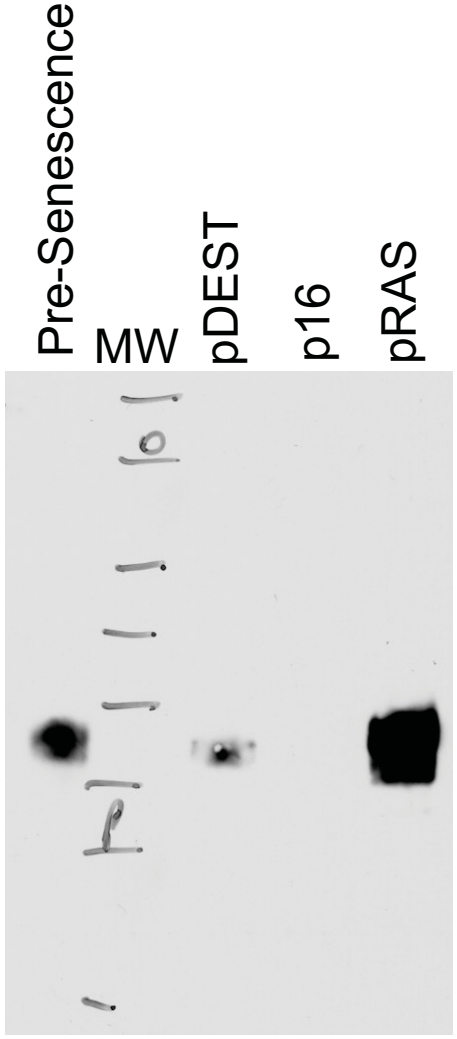
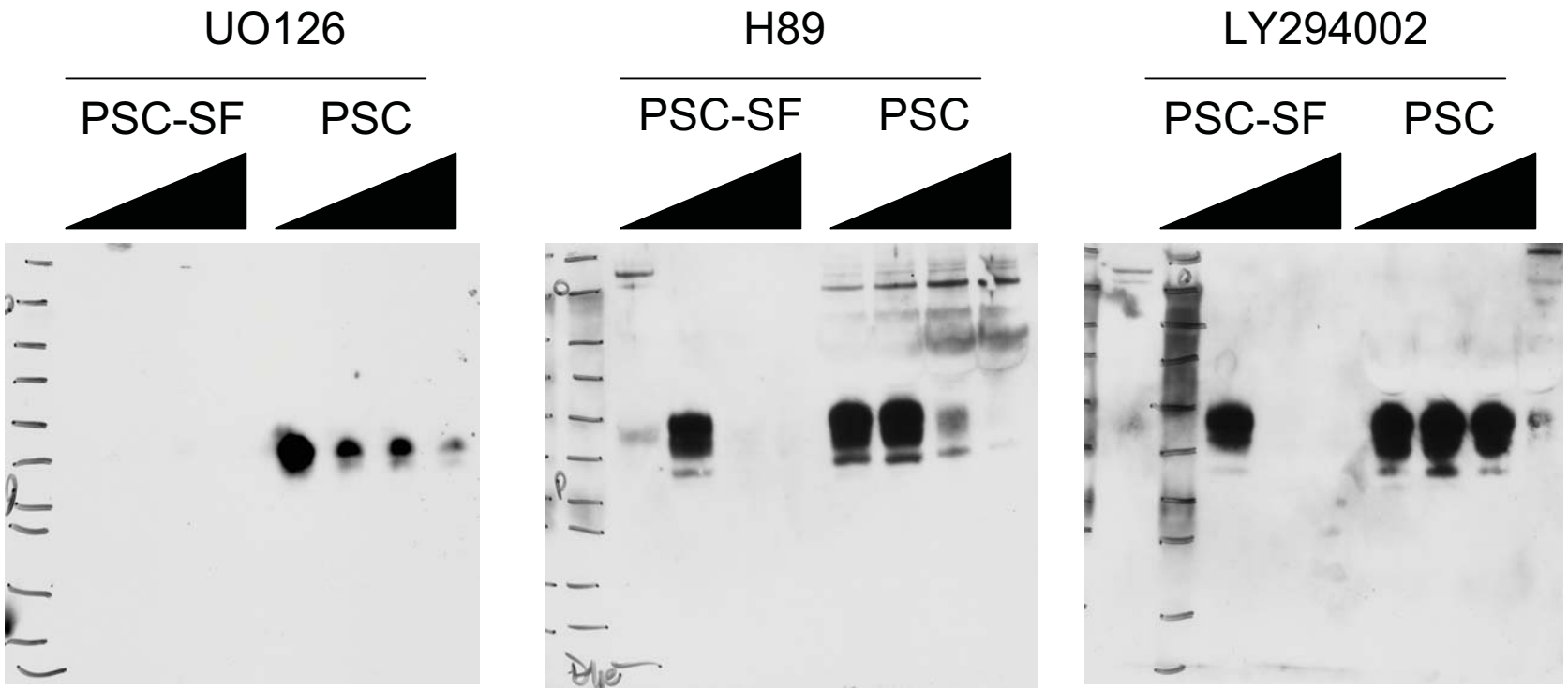


Figure 6



MAPK inhibitor

PKA inhibitor

PI-3K inhibitor

Figure 7

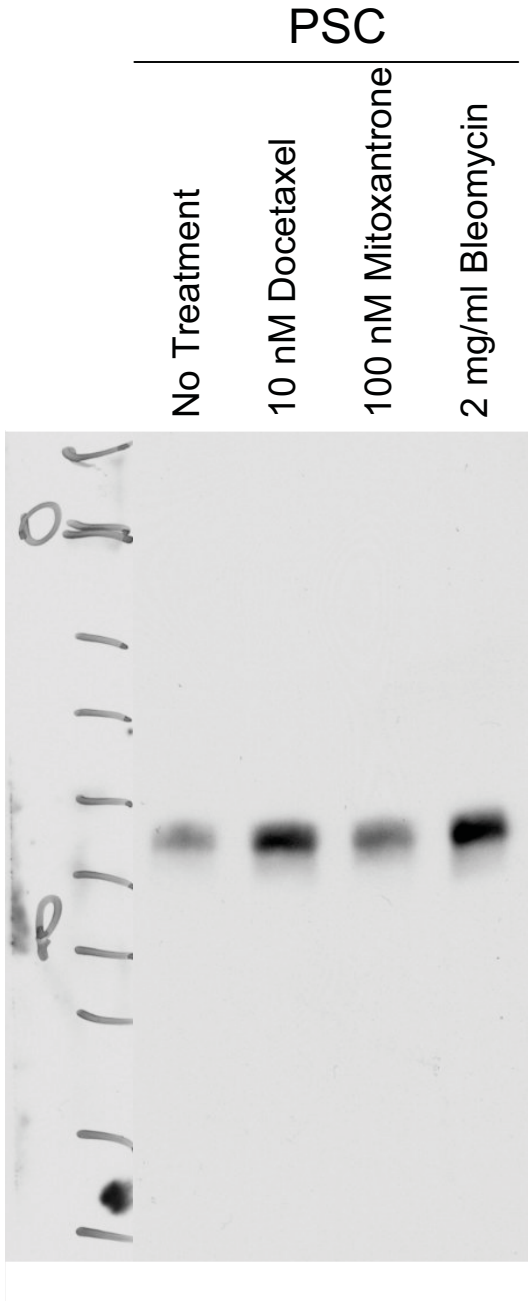
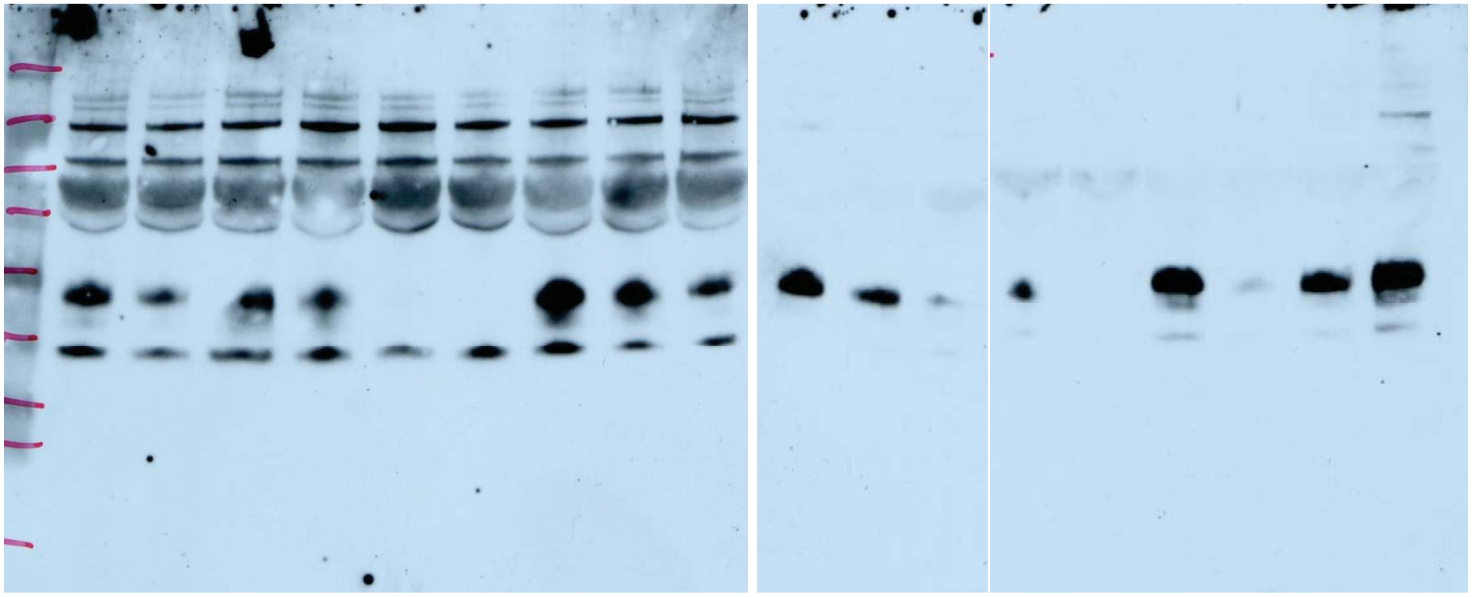

PSC
No Treatment
10 nM Docetaxel
100 nM Mitoxantrone
2 mg/ml Bleomycin

Figure 8



Oxygen	1%	20%	1%	20%	1%	20%	1%	20%	1%	20%	1%	20%	1%	20%	1%	20%	1%	20%
Treatment	none	none	100 mM NaCl	100 mM NaCl	20 mM CaCl2	20 mM CaCl2	10 mM NaPhos	10 mM NaPhos	Mitoxantrone	Mitoxantrone	20 mM MgCl2	20 mM MgCl2	2 mM EGTA	2 mM EGTA	SU11274	SU11274	Docetaxel	Docetaxel

Figure 9

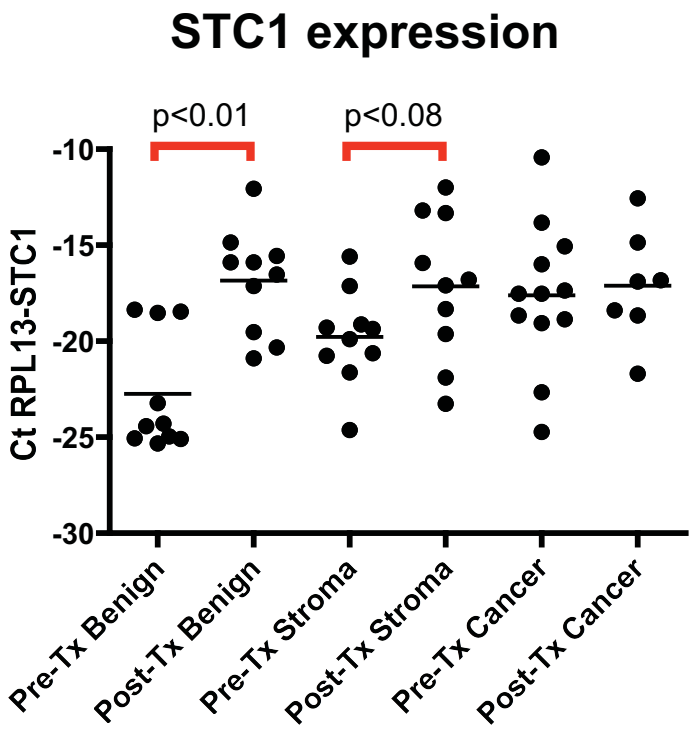
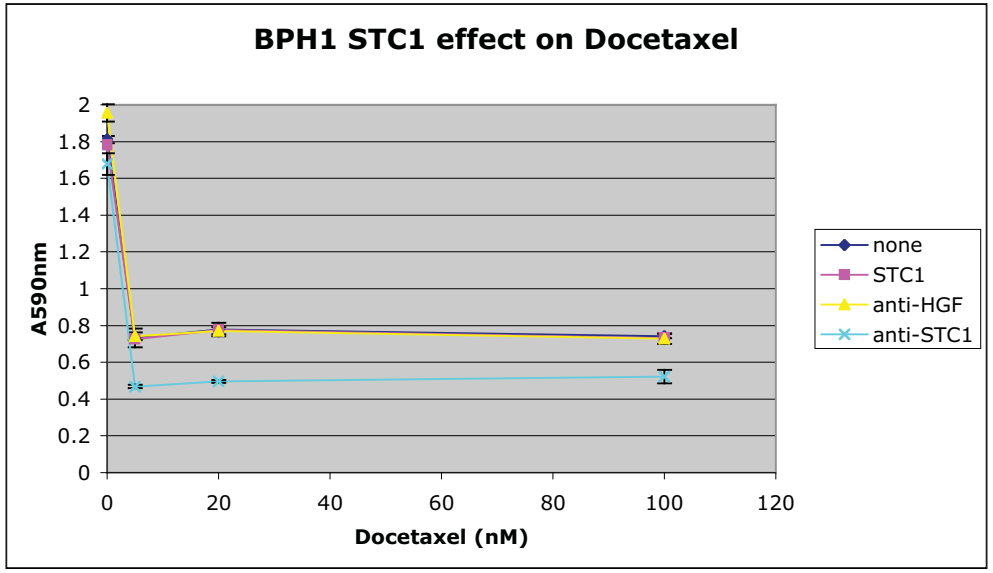
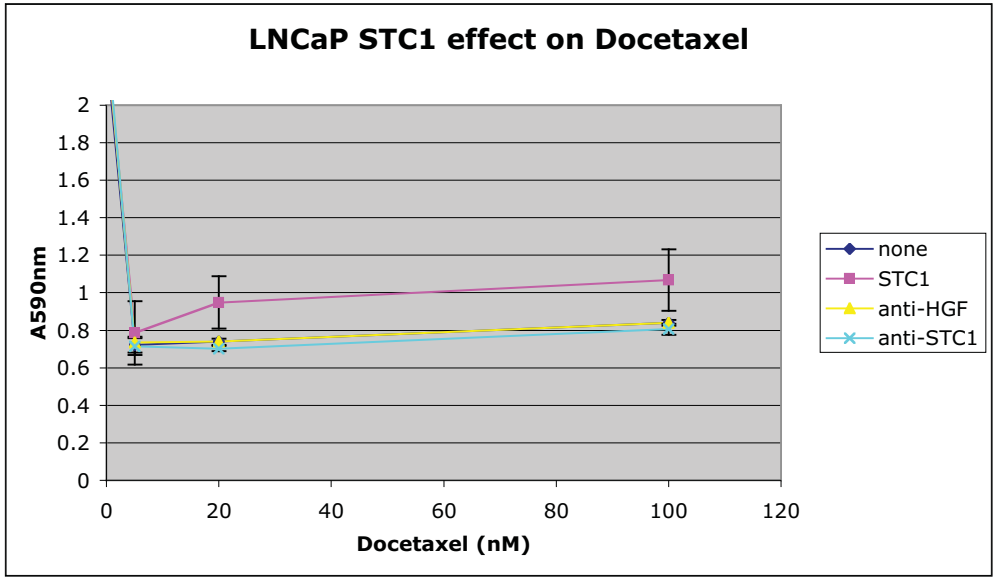
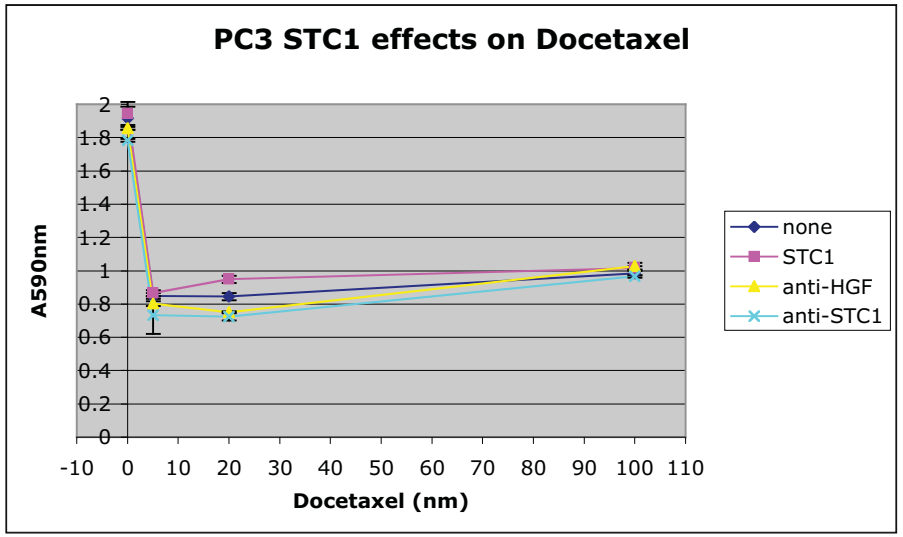

STC1 expression
p<0.01
p<0.08
Ct RPL13-STC1
-10
-15
-20
-25
-30
Pre-Tx Benign
Post-Tx Benign
Pre-Tx Stroma
Post-Tx Stroma
Pre-Tx Cancer
Post-Tx Cancer

Figure 10


BPH1 STC1 effect on Docetaxel
A590nm
Docetaxel (nM)
none
STC1
anti-HGF
anti-STC1


LNCaP STC1 effect on Docetaxel
A590nm
Docetaxel (nM)
none
STC1
anti-HGF
anti-STC1


PC3 STC1 effects on Docetaxel
A590nm
Docetaxel (nm)
none
STC1
anti-HGF
anti-STC1