Award Number: W81XWH-13-1-0379

TITLE: The Stromal Contribution to the Development of Resistance to New Generation Drugs by Castrate Resistant Prostate Cancer

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REPORT DATE: May 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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The project will test the hypothesis that benign tumor support (stromal) cells have a significant role in acquired resistance of a prostate tumor to hormone therapies. Based upon our preliminary data showing that primary prostate stromal cells (PrSCs) acquire a steroidogenic phenotype under the influence of a paracrine Hedgehog (Hh) signaling microenvironment, we will test whether human bone marrow stromal cells (BMSCs) likewise convert to an androgen secreting phenotype under paracrine Hh stimulation. Aim 2 will test whether Hh-primed steroidogenic BMSCs or PrSCs can adapt to acute or chronic abiraterone treatment by further increasing their expressions of steroidogenic genes and by further increasing their outputs of T and DHT. Finally, in Aim 3, I will attempt to demonstrate in cell co-culture models that Sonic Hh produced from PCa cells activates steroidogenesis in BMCs and PrSCs and test whether the addition of abiraterone to this model co-culture system further boosts production of androgen from the co-cultured stromal cells. Collectively, this work will significantly impact on our understanding of the mechanistic process leading to CRPC by showing that benign tumor support (stromal) cells within a bone metastasis have a supportive role in the development of CRPC through their ability to synthesize T and DHT upon Hh stimulation from metastatic PCa cells, and whether this adaptation can increase resistance to new generation therapies, which may lead to more productive combined therapies not only targeting steroidogenesis, but the supporting hedgehog pathway.

15. SUBJECT TERMS
Prostate Cancer, Hedgehog signaling, Hormone Therapy, Intratumoral, Steroidogenesis, Androgens, Smoothened Agonists, Benign Prostate Stromal Cells, Bone Marrow Stromal Cells, Cell Co-culture.

16. SECURITY CLASSIFICATION OF:
   a. REPORT Unclassified
   b. ABSTRACT Unclassified
   c. THIS PAGE Unclassified

17. LIMITATION OF ABSTRACT Unclassified
18. NUMBER OF PAGES 15

19a. NAME OF RESPONSIBLE PERSON USAMRMC
19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION:
Prostate cancer (PCa) is the most prevalent solid tissue malignancy in men in Western countries(1). Local disease is often curable by radiation or surgery; however, patients with advanced (metastatic) disease are treated by androgen deprivation therapy (ADT) that ablates testicular androgen synthesis that is needed for continued tumor growth(2). Unfortunately, ADT is usually only palliative since the tumor often recurs in a state that is refractory to the low systemic androgen levels in ADT-treated patients. This phenotype, termed castrate resistant PCa (CRPC), is the main cause of mortality from the disease. Recently, we learned that progression to CRPC can be linked to acquired intratumoral steroidogenesis (IS) that supports renewed activity of the androgen receptor (AR) in tumor cells thus enabling tumor re-growth(3). While new generation of drugs (abiraterone [abi] or Enzalutamide) that target IS or renewed AR activity have shown the ability to increase survival of CRPC patients(4), most patients treated with these drugs also soon develop resistance that limits the drug’s effectiveness. Resistance to the new generation drugs is thought to be due to adaptions of the tumor cells themselves that further increase IS(5). This hypothesis, though, neglects any potential contributions to IS of cancer-adjacent stromal cells that might also acquire a steroidogenic phenotype upon exposure to the Hedgehog (Hh) signaling microenvironment of an androgen deprived prostate tumor(6). Our work is innovative in that it seeks to illuminate the steroidogenic interaction between PCa cells that produce Hh ligands (hhs) in response to androgen deprivation and the stromal cells in the tumor microenvironment at both primary and metastatic sites. Furthermore, we seek to show that tumor stromal cells, like the cancer cells, can adapt to the new prostate cancer therapeutics and contribute to resistance to these drugs.

2. KEYWORDS: Prostate Cancer, Hedgehog signaling, Hormone Therapy, Intratumoral, Steroidogenesis, Androgens, Smoothened Agonists, Benign Prostate Stromal Cells, Bone Marrow Stromal Cells, Coculture.

3. ACCOMPLISHMENTS/ OVERALL PROJECT SUMMARY:
3a. Brief Overview of the Clinical Problem Addressed by the Project:
In adult males, the normal functioning of the prostate is dependent on interactions with the surrounding stromal environment, much as the development of the prostate gland is dependent on the stromal mesenchyme for proper form and function. In cancers, these interactions are interfered with, disrupting homeostasis, differentiation, and growth; sometimes evoking tumor inhibition, at other times enhancing tumor progression(7). Recent studies from our laboratory have shown that, under androgen deprived conditions, PCa cells upregulate and secrete large amounts of Sonic Hedgehog ligand (Shh), which interacts with its receptor Patched-1 (Ptch-1) on the stromal cells to initiate a signaling chain through de-repression of Smoothened (Smo) and activation of Gli2 transcription factor signaling which culminates in the activation and secretion of steroidogenic enzymes and steroids, respectively(6). These steroids can feed back to the tumor cells, which, after ADT, have hypersensitive AR(8). Stromal steroidogenesis may play a significant part in the steroid milieu of the tumor microenvironment in addition to ADT driven upregulation of the steroidogenic potential of tumor cells, which are able to synthesize steroids from adrenal precursors, such as Dehydroepiandrosterone (DHEA), or de novo from precursors such as cholesterol or acetate through either the classical steroidogenesis pathway, or the backdoor pathway, which bypasses testosterone (T) as an intermediate for dihydrotestosterone (DHT) (3, 9-11).
After ADT, recurrence as castration-resistant disease is common (8). For PCa, the most common site for metastasis is bone, and these metastases are a major cause of morbidity due to replacement of haematopoietic tissue with tumor and decreased bone stability(12). Bone overgrowth at metastatic sites causes pain, fracture and spinal cord compression in patients. It has already been demonstrated that bone mesenchymal cells can be differentiated into steroidogenic cells (13) by overexpression of steroidogenic factor-1 (SF), and that SF-1 is a Hh target gene in Leydig cell differentiation (14). Furthermore, enzymes for androgen production are highly expressed in bone core metastases samples (15). LNCaP cells co-cultured with BMS cells increase their output of PSA (16). Collectively, these data suggest a potential role for Hh-induced steroiodogenesis in the interactions between PCa cells and the stromal cells in the bone metastatic microenvironment.

It is the focus of my project to investigate and comprehend the interactions between PCa cells which secrete Hh and the stromal cells of both the primary and bone microenvironment which become steroidogenic under the influence of Hh signaling. We are also interested in whether this interaction could facilitate resistance to new generation drugs against PCa, such as Enzalutamide or Abiraterone (abi), which target the AR or cytochrome p450 (CYP17)A1, respectively (4). By the way of the “seed and soil” paradigm for metastasis, it maybe that Hh signaling from PCa cells causes a differentiation to a steroidogenic phenotype in the BMS cells, which then provides androgens or precursors for tumor growth and resistance to next generation drugs, such as abi.

3b. Summary of Results

3b1. Brief Summary of First Year Results as Outlined in First Annual Report

We have shown that (a) 1nM Ag.15 Smo agonist treatment increases expression of steroidogenic enzyme mRNA in benign prostate stromal cells (PrSC) as well as (b) steroid output, including T, from those cells; furthermore, (c), this effect is attenuated if the agonist is removed from the treated cells, showing that the Hh pathway is a feasible target for decreasing steroidogenesis in PCa. We have also demonstrated that (d) blocking the stromal Shh pathway in castrate LNCaP tumours greatly quells the DHT and T levels in the tumours. We further demonstrated that (e) simvastatin 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) inhibitor effectively blocked Shh induced steroidogenesis in PrSC cells.

For the project of “The Stromal Contribution to the Development of Resistance to New Generation Drugs by Castration Resistant Prostate Cancers,” we demonstrated that treatment of either commercially available BMS cells, (Stemcell Technologies, cat# MSC-001F), or BMS cells obtained from our collaborator Dr. Eva Corey (University of Washington) (25) with 1nM Ag1.5 or 100nM SAG agonist resulted in (f) upregulation of steroidogenesis enzyme mRNA, as well as (f) steroid output, including T (repeated only once). Due to the very slow growth of the commercially purchased cells, our subsequent experiments were carried out solely with BMS cells from Dr. Corey. We allowed cells to come to 70% confluence before starving in serum free medium for 48hrs before treatments.

3b2. The Stromal Contribution to the Development of Resistance to New Generation Drugs by Castration Resistant Prostate Cancers- Data Obtained until December 2015.
Since the last reporting period, we have demonstrated that Gli1 induction in BMS cells can be quelled with 100nM KAAD-cyclopamine (figure 1), which is accompanied by an inhibition of SF-1 induction, and apparent inhibition of HMGCR and CYP17A1 mRNA induction. The induction of SREBP by KAAD cyclopamine is not readily explainable; however, it is possible that inhibition of this pathway is triggering a feedback of mRNA transcription.

In order to show that T output is statistically and repeatably increased by BMS cells with the addition of Shh pathway agonist, we incubated BMS cells with Ag1.5 (1nM) or SAG (100nM) for 72hrs. Incubation of BMS cells, in triplicate, with 22-OH cholesterol precursor (10µM) resulted in approximately double the T output compared to control as measured by ELISA, figure 2. ELISA was used because LC/MS has become economically prohibitive.

Much of our research during this period has been to establish a coculture system to look at the interaction and mutual steroidogenesis stimulation between PrSC or BMS cells and LNCaPs, either parental (LNp) or over expressing Shh (LN-Shh). During the previous reporting period we established a system where we incubated either LNp or LN-Shh for 72hrs in serum free medium to build Shh in the conditioned medium. That medium was then harvested and applied to PrSC for 48 or 72hrs, in the presence of a 22-OH cholesterol precursor, to measure changes in steroidogenesis mRNA or steroids in the medium, respectively. In following the same procedure using BMS cells, we have shown that incubation with conditioned media from LN-Shh cells significantly upregulates expression of StAR, HSD3B2, and AKR1C3, close to significant upregulation of CP17A1 and Gli1, figure 3a; furthermore, analysis of steroidogenesis by LC/MS demonstrates significant increase over 2-fold in T and androsterone output in LN-Shh conditioned medium treated cells compared to LNp, figure 3b, with a trend to increases in output of most steroids. We also compared the steroid production levels of BMS cells on a per-cell basis compared to LNCaP cells and demonstrated that they are at least as steroidogenic, with significantly higher levels of...
pregnenolone, as well as higher levels of steroids in the backdoor pathway, particularly when activated by conditioned medium from LN-Shh cells (figure 3c).

We also attempted to demonstrate in direct co-culture that incubation of PrSc or BMS cells with LN-Shh would contribute paracrine steroidogenesis, resulting in increased PSA expression due to
high paracrine steroid levels when a 10µM OH-cholesterol precursor was applied. We did not see co-culture in transwell dishes resulting in greater PSA mRNA when stromal cells were incubated with LN-Shh compared to LNp; however, we demonstrated that even without increased contribution of paracrine Hh signalling from LN-Shh, incubation with stromal cells resulted in increased PSA expression, as measured by PSA output for co-culture with PrSC (figure 4a) and mRNA for co-culture with BMS cells (figure 4b), likely due to a more steroid-rich microenvironment.

Figure 4: Effect of transwell co-culture on LNCaP PSA expression. (A) Co-culture with PrSC resulted in approximately double the PSA output of LCaP cells after 72hrs. (72hr fold-change, +SE) PSA output in medium was measured by COBAS. (B) Co-culture with BMS cells also resulted in approximately double the PSA expression as measured by mRNA levels. Expression was measured by ΔΔCT method QRT PCR and shown +SE.

To further investigate the effect of co-culture, or conditioned medium “mock” co-culture on LNCaPs, conditioned mediums were collected from Ag1.5 treated or control cultures and were applied to 48 hr androgen-starved LNCaP cells to determine subsequent growth of LNCaP cells using metabolic/ DNA quantifying (cyquant) assay. We found that incubation with conditioned medium from BMS cells incubated with Ag1.5 resulted in more cell growth than with conditioned medium from control cell, figure 5. This effect can be quelled with 10µM MDV, which indicated the increased growth may be due to AR stimulation by androgen from conditioned medium.

Figure 5: Effect of BMS conditioned medium on LNCaP cells as measured by Cyquant assay. Treatment of LNCaP cells with BMS medium from cells incubated with Ag1.5 showed increase in growth compared to controls. MDV treatment abrogated this effect. Experiments were done in 6 replicates, shown +/-SE.
One of the goals of our project was to test whether acute or chronic exposure of Hh-induced PrSCs or BMS cells to abiraterone (abi) or MDV3100 will further upregulate the expression of steroidogenic genes and further increase steroid output. Current literature suggests that prolonged exposure to abi may force upregulation of steroidogenesis enzymes, particularly CYP17A1, resulting in resistance to abi treatment (5). We hypothesized that this may also occur in stromal cells, further compounding the problem. We tested our hypothesis at various time points, and found abi (100nM) increased levels of mRNA expression in BMS cells at both 2 and 6 days of exposure. At 6 days, co-treatment with Ag1.5 agonist appeared to increase expression of key steroidogenic enzymes even further (figure 6a). We also measured the steroid output of the cells at 6days, demonstrating increased output of steroids, particularly DHEA, T, and androsterone, as measured by LC/MS (figure 6b). We are in the process of repeating this experiment in triplicate. We also tested the effects of long term, 10day, abi treatment on BMS steroidogenesis mRNA expression, combined with 48hr Ag1.5 treatment, and found increased expression of key enzymes, including, HMGCS, HSD3B2, AKR1C3, and Gli2, though the effect did not appear additive (figure 6c).

Finally, we tested the effect of conditioned medium from our 10day abi treated cells in the presence and absence of Shh pathway agonist on LNCaP cell growth and showed medium from abi treated cells increased cell proliferation as measured by cyquant assay (figure 6d).

Figure 6: Effect of abiraterone on stromal cell steroidogenesis. (A) Treatment of BMS cells with 100nM abi +/- 1nM Ag1.5 resulted in increased expression of key steroidogenic enzyme mRNA (shown as fold change using ΔΔCt method, +SE). (B) Treatment with abi also resulted in increased steroid outputs as measured by LC/MS and normalized to cell number. (C) Treatment of BMS cells for 10days also resulted in upregulation of steroidogenesis enzyme mRNA (shown as fold change using ΔΔCt method, +SE). (D) Treatment of LNCaP cells with conditioned medium from 10day abi treated BMS cells or control BMS cells showed increased LNCaP growth with medium from BMS cell treated with Ag1.5, and further increased growth with 10day abi treated cells, though the effects did not appear additive. Growth was analyzed by Cyquant assay.
Taken together, these results suggest that BMS cells are capable of de novo steroidogenesis when stimulated with Hh pathway agonists or conditioned medium from BMS cells, with the same or more steroidogenic potential per cell to LNCaP prostate cancer cells, and that this paracrine interaction with PCa cells may contribute to the favorable conditions for bone metastasis growth. We have also demonstrated that BMS cells may contribute to abiraterone resistance through upregulation of steroidogenesis enzyme mRNA and steroid output. These results suggest the Shh pathway and stromal steroidogenesis may be promising targets for therapeutic intervention.

Future Collaborations:
While presenting in Brisbane for the Prostate Cancer Collaborative Research Symposium, I connected with Dr. Michael Doran from the Australia Prostate Cancer Research Centre of Queensland, who has run a microarray comparing cancer associated bone stromal cells to regular stromal cells. They have seen upregulation of many of our steroidogenic enzymes of interest in the cancer associated stroma and would like to share that information with us, potentially adding to the impact of our forthcoming paper.

3b3. Other Training Project: A Spontaneous Developmental Lineage Plasticity that Underlies the Response of Prostate Cancer Cells to Androgen Deprivation (Manuscript in progress; presentation given at Koc University, Istanbul, October 2014; presentation given at the Prostate Cancer Collaborative Research Symposium, November 2015)
The results reported for the previous reporting period showed that PCa(AR+) cells have a previously unsuspected developmental plasticity allowing them to revert to neural/neural crest stem-like state which may be correlated to PCa progression, therapy resistance and metastasis. LNCaP cells after 14 days in specialized stem conversion medium grow as spheroids, lose their AR and PSA expression, and upregulate the Yamanaka stem cells factors, as well as a panel of neural crest genes. They become more resistant to hypoxia and enzalutamide and are more invasive than parental in intact and castrate xenografts.
Since the last reporting period, we have now repeated the conversion of PCa cells to neural/neural crest stem cells in three other cells types, VCaPs, LAPC4, and 22RV1. We compared these cells and our previously studied LNCaP by microarray and discovered a 62 gene signature. When compared to patient data in a Mayo Clinic database, we discovered that this signature corresponded to more likelihood of prostate cancer specific mortality, biochemical recurrence, and metastasis. We conclude that this neural/neural crest signature may be important to PCa progression and that the signature we have discovered may have high prognostic value.

4. KEY RESEARCH ACCOMPLISHMENTS:
Reporting Period 1:
- Have determined that treatment of BMS cells with Hh agonists upregulates mRNA for key steroidogenesis enzymes in both the classical and backdoor pathways, in 2 different BMS cell lines.
- Have shown that treatment of two BMS cell lines with Hh agonists increases secretion of steroids in both the classical and backdoor pathways.
- Have established a co-culture system with LNCaP and LNSHH cells with PrSC which is likely easily adapted to BMS-EC.

Reporting Period 2:
• Have determined that treatment of BMS cells with Hh agonists and conditioned medium from LN-Shh upregulates mRNA for steroidogenesis enzymes in BMS cells.
• Have shown that treatment of two BMS cell lines with Hh agonists increases T production from BMS cells and incubation with conditioned medium from LN-Shh increase secretion of steroids in both the classical and backdoor pathways.
• Have shown incubation of LNCaP cells with either PrSC or BMS cells in the presence of OH-cholesterol increases PSA expression.
• Have shown medium from BMS cells stimulated with Ag1.5 increase grown of LNCaP cells, which can be attenuated by MDV.
• Have shown incubation of BMS cells with abiraterone (abi) increases there steroid output and mRNA expression.
• Have demonstrated that incubation of LNCaP cells with conditioned medium from BMS cells incubated with abi for 10days causes increased cell growth as measured by DNA content.

CONCLUSION:
Our work has confirmed the importance of paracrine Hh signaling in PCa, as it actives/upregulates steroidogenesis in PrSC and BMS cells. We have demonstrated that exposure to Hh agonists upregulates key steroidogenesis enzyme mRNAs present in both the classical and backdoor pathways and also increase production and secretion of steroids and androgens. We have demonstrated that these benign stromal cells, when activated by Hh, readily convert very upstream precursors OH-cholesterol and OH-pregnenolone to active steroids and androgens, signifying the capacity to contribute to de novo steroidogenesis. In PrSC cells, upregulation of pregnenolone synthesis was most notable; however, in commercially available BMS cells, the steroid with the highest increase in output was 4-pregnen-17-ol-3,20-dione, which is a key metabolite of the backdoor pathway. Furthermore, we have shown that incubation of BMS cells with conditioned medium from LN-Shh shows the same or greater steroidogenic potential for BMS cells compared to LNCaPs on a per cell basis, with much more production of pregnenolone and 4-pregnan-3,17-ol-20-one, and much more androsterone in the presence of LN-Shh conditioned medium. In all of the stromal cell lines, we have seen upregulation of GLi2, which we had previously demonstrated to be necessary for Hh mediated steroidogenesis(6). Importantly, we have demonstrated that blocking the stromal steroidogenesis pathways in murine tumors can inhibit T and DHT production and slow tumor growth rate (18). We have also demonstrated that Simvastatin may be a useful inhibitor of these pathways, in the context of PrSC.

Further, we have shown that conditioned medium from BMS cells activated by Ag1.5 can stimulate growth in LNCaP cells, which can be quelled by MDV, and have demonstrated a rise in PSA expression when LNCaP cells are cocultured with stromal cells from the bone or prostate.

We have also demonstrated that exposure of BMS cells to abi leads to an upregulation of steroidogenesis enzymes and steroid output. Additionally, conditioned medium from BMS cells stimulated with abi for 10days results in increased cell growth, which our previous results suggest is likely due to increased steroids in the medium.
Collectively, our work strongly supports that Hh signaling is involved in the progression of PCa to CRPC, at which time cells become refractory to androgen ablation by synthesizing their own steroids(3), and that targeting of this pathway would be beneficial for tumors that involved steroidogenesis evoked by paracrine Hh signaling, as compliment to traditional targeting of the AR and steroidogenesis pathways. We have also demonstrated that stromal steroidogenesis may contribute to abiraterone resistance through increases in steroidogenesis.

Future experiments will complete our present investigations with abiraterone resistance and steroidogenesis. We will also endeavor to establish a coculture system in which we can see the effects of Shh pathway induction; to that end, we are experimenting with a new matrix system called Alvetex scaffolding, which is postulated to be very realistic in mimicking the microenvironment.

5. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

5a. Publications:


5b. Abstracts:

Reporting Peroid 1:


Reporting period 2:

1) **Amy A Lubik**, Mannan Nouri, Parvin Yanki, Mazyar Ghaffari, Hans Adomat, Eva Corey, Emma Guns, Michael Cox, and Ralph Buttyan. Paracrine hedgehog signalling


5c. Presentations:
Reporting Period 1:

Reporting period 2:
1) Lubik, AA. Spontaneous developmental lineage plasticity that underlies the response of prostate cancer cells to androgen deprivation. Presented at the Prostate Cancer Collaborative Research Symposium, Brisbane, Australia, November 2015.

6. REPORTABLE OUTCOMES:
Reporting Period 1: Have shown that OH-cholesterol and OH-pregnenolone are readily converted to T and DHT are useful and applicable precursors for steroid conversion/ synthesis. Most laboratories use DHEA to mimic an adrenal precursor; whereas, we are more closely mimicking de novo steroidogenesis.
Reporting Period 2: Have shown that conditioned medium from bone stromal cells treated with Shh agonists can increase growth of LNCaP cells. Have shown that abiraterone exposure increases steroidogenesis in bone stromal cells.

7. OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT
Reporting Period 1:
- Presentation given for Vancouver Prostate Centre, March 2014 “Targeting Sonic Hedgehog Signalling with Simvastatin Blocks Prostate Stromal Cell Paracrine Steroidogenesis by Multiple Avenues”
- Poster presented at AACR April 2014 “A Spontaneous Developmental Lineage Plasticity that Underlies the Response of Prostate Cancer Cells to Androgen Deprivation” Amy Anne Lubik, Josselin Caradec, Mannan Nouri, Manuel Altimarano-Dimis, Na Li, Jennifer Bishop, Mani Moniri, Martin Gleave, Ralph Buttyan
- Presentation given at Lorne Sullivan Research Day, Vancouver, June 2014 “A New Model of Prostate Cancer Stem Cells and its Relevance to Castrate Resistant Prostate Cancer.”
• Presentation scheduled for Koc University, Istanbul, October 2014 “Developmental Plasticity of Prostate Cancer and Hormone Resistance.”
• Project Management Workshop through University of British Columbia Postdoctoral Association (Registered, November 2014).

Reporting Period 2:

• Presentation for Koc University, Istanbul, October 2014 “Developmental Plasticity of Prostate Cancer and Hormone Resistance.”
• Co-authored on the Human Health Effects of the Kinder Morgan Trans Mountain Pipeline Expansion for submission to the National Energy Board May 2015.
• Poster presented at Lorne Sullivan Research Day, Vancouver, June 2015 “Paracrine hedgehog signalling upregulates steroidogenesis in primary prostate and bone stromal cells.”
• Poster presented Prostate Cancer Collaborative Research Symposium November 2015 “Ablation of Intratumoural Androgens in Prostate Tumour Cell Xenografts by a Hedgehog Signalling Inhibitors.”
• Presented at Prostate Cancer Collaborative Research Symposium November 2015 “A Spontaneous Developmental Lineage Plasticity that Underlies the Response of Prostate Cancer Cells to Androgen Deprivation” Amy Anne Lubik.
• Volunteer experience: Head volunteer and organizer for non-partisan social justice organization Leadnow, TriCities Chapter. Port Moody, BC, May 2013-ongoing.

8. OTHER ACHIEVEMENTS: Nothing to report.

9. REFERENCES

16. A. Mizokami et al., Prostate cancer stromal cells and LNCaP cells coordinately activate the androgen receptor through synthesis of testosterone and dihydrotestosterone from dehydroepiandrosterone. *Endocrine-Related Cancer* 16, 1139 (2009).