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

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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.
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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. **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\(^8\). 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 occurs in the majority of patients \((8)\), and often progresses to metastatic disease. 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 steroidogenesis 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. Paracrine Hedgehog Induced Prostate Stromal Cell Steroidogenesis Contributes Significantly to Tumoral Steroid Milieu (Manuscript in progress)

This related project investigates the contribution of the benign prostate stromal cells to steroidogenesis in the PCa microenvironment when stimulated with Hh pathway Smo agonist \(Ag1.5\) \(1\text{nM}\) or co-cultured with Shh overexpressing LNCaP cells (LNSHH). We have demonstrated by QRT PCR and LC/MS that induction of the steroidogenic enzymes in PrSC by Hh signaling is ligand dependant, and can be inhibited once Smo agonist Ag1.5 withdrawal (Figure 1a and b). This is an important finding in consideration that, in the developing leydig cells, once steroidogenesis is initiated, it no longer requires ligand \((17)\). Therefore, the Hh pathway is a feasible target for decreasing steroidogenesis in PCa.
Effect of 1nM Ag1.5 on steroidogenesis in PrSC. (A) Treatment with Ag1.5 increases mRNA expression of key steroidogenesis enzymes after 48hrs; down-regulation follows 48hr withdrawal of Ag1.5. Expression was measured by ΔΔCT method QRT PCR and shown + SE. (B) Steroid production, with 10μM OH-pregnenolone precursor, by PrSC is increased by treatment with Ag1.5, with Ag1.5 necessary for continued upregulation. Steroid levels were measured by LC/MS, normalized to cell number, and expressed as fold change + SE.

Furthermore, we have discovered that, on a per cell basis, PrSC in the presence and absence of Hh agonist are generally equal to some of the most common PCa cell lines (LNCaP and 22RV1) in steroid output when fed 22-OH cholesterol, a precursor for de novo steroidogenesis; however, especially under the influence of Ag1.5, PrSC cells produce much more pregnenolone, the first steroid in the steroidogenesis pathway (Figure 2a). This leads us to hypothesize that pregnenolone from the Hh activated stroma may be used as a precursor for steroidogenesis in tumor cells, or that secreted steroids may be “traded” between stromal and epithelial cells. Likewise, PrSC cells produce a much greater amount of the steroids in the backdoor pathway, such as androsterone, especially with the addition of Ag1.5 (Figure 2b). We have also demonstrated much higher levels of T are produced by coculturing LNSHH cells than with parental LNCaPs or by either cell line alone when fed 30μM OH-cholesterol (Figure 3). As LC/MS experiments are economically prohibitive, these are experiments optimized in the singular, then repeated for triplicates.

Figure 2: Comparative steroid outputs of commercially available PCa cell lines to PrSC cells with and without 1nM Ag1.5 with 10μM OH-cholesterol as a precursor. (A) Much higher output of
pregnenolone and (B) androsterone, a key metabolite in the backdoor pathway in PrSC cells treated with Ag1.5 compared to PCa cell lines, as measured by LC/MS, normalized to cell number + SE.

Finally, we have demonstrated, by LC/MS, that by blocking Hh signalling in castrate LNCaP tumours with Tak441 Smo antagonist, we can dramatically reduce the levels of T and DHT in the tumors (Figure 4). A recent publication from these same tumours showed that Tak441 only effects signalling in the surrounding stromal, and not tumour cell signaling, and inhibits tumour growth (18).

**Taken together, these results demonstrate that Hh signalling between PCa and stromal cells is crucial to tumour steroidogenesis and may be an attractive therapeutic target in both local and metastatic PCa.**

Figure 3: Coculture of PrSC cells with LNCaP cells or Shh overexpressing cells (LNSHH) in either mock coculture (MC)- conditioned PrSC medium transferred to LNCaP cells, or direct coculture (CC) compared to PrSC or LNCaP alone (72hr).

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<th>LNSHH</th>
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Figure 4: Effect of Tak441 Hh inhibitor on steroid levels in castrate LNCaP xenografts. Previous publications on these tumours showed that Tak441 has effects only on the Hh pathway in stromal cell. Tumors treated with 25mg/mg Tak441 had much lower levels of (A) T and (B) DHT. Steroid levels are normalized to tumor weight (n=3, +SE).

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<tr>
<th>Steroid</th>
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**3b2. Simvastatin Blocks Paracrine Hedgehog Induced Prostate Stromal Cell Steroidogenesis via Multiple Avenues**

Because the Shh ligand is highly lipid modified in order to be functional, requiring a cholesterol moiety for auto-catalytic cleavage to its active form, as well as a palmitoyl addition (19, 20), we investigated whether simvastatin, which blocks the 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) and therefore the rate limiting step for cholesterol synthesis, would inhibit steroidogenesis by interfering with the autocatalysis of Shh necessary for activation and secretion. We discovered, by ELISA, that simvastatin appears to increase output of Shh; however, simvastatin still prevents an increase in PrSC steroidogenesis when used in mock-co-culture (serum free medium is added to LNSHH or parental cells and let incubate in the presence or absence of 5µM
simvastatin before the conditioned medium is transferred to PrSC cells with the addition of OH-cholesterol as a precursor), as demonstrated by QRT PCR and LC/MS (Figure 5a and b). This may indicate that simvastatin interferes with Shh in alternative manner than anticipated. Additionally, we demonstrate that treating PrSC cells with simvastatin also blocks steroidogenesis induced by Ag1.5 agonist; however, this does not affect the expression levels of most of the steroidogenesis enzymes at the mRNA level.

As literature suggests that statins are associated with lower risk of PCa and metastatic PCa(21-23), part of the rationale may be that statins inhibit Hh induced steroidogenesis.

Figure 5: Effects of Simvastatin on PrSC paracrine steroidogenesis. (A) When LNCaP or LNSHH cells are incubated for 72hrs in the presence or absence of 5µM simvastatin before incubation of PrSC with conditioned medium from those cells there is a suppression of Hh induction of steroidogenesis enzyme mRNA, with the exception of HMGCR and AKR1C3, which may increase as a compensatory effect (48hr, fold change +SE). (B) Incubation of LNCaP or LNSHH cells with simvastatin before 72hr incubation of PrSC with conditioned medium blocks Hh induction of increase in steroid production (fold-change, +SE, normalized to cell number).

3b3. The Stromal Contribution to the Development of Resistance to New Generation Drugs by Castration Resistant Prostate Cancers- Data Obtained so Far

We began our experiments by purchasing commercially available bone marrow stromal cells (BMS) (Stemcell Technologies, cat# MSC-001F), which are most often used to propagate and investigate mesenchymal stem cells. For our purposes, we attempted to maintain them as BMS cells. Under recommended conditions of 10% FBS in DMEM, these cells grew very slowly, hindering our experimental abilities. We were able to complete two key experiments with these cells.

Firstly, we investigated the effect of increasing dosages (50, 100, 200nM) of SAG Hh agonist on steroidogenesis enzyme mRNA after 48hrs (Figure 6a). At 48hrs, all 3 concentrations significantly upregulated Gli1 signaling, which is the transcriptional regulator most often associated with active Hh signaling(24); however, only at 200nM SAG did we see significant upregulation of Gli2, the transcriptional regulator on which we have demonstrated prostate stromal cell (PrSC) steroidogenesis to depend(6). We also demonstrated, by QRT PCR, significant upregulation of key steroidogenesis transcription factors SF-1 (2.6-fold, p<0.05), SREBP (1.6-fold, p<0.05), and AKR1C3 (1.35-fold, p<0.05), which converts androstenedione into T. Most importantly, we have demonstrated, by LC/MS, that exposure of commercially available BMS cells to either 100nM
SAG or 1nM Ag1.5 increases steroidogenic output when fed an 10µM OH-cholesterol intermediate for 72hrs (Figure 6b); SAG and Ag1.5 induced 72 and 20-fold increases in 4-pregnen-17-ol-3,20-dione (backdoor pathway), respectively, 20 and 2-fold increase in DHEA, and only SAG produced a 5.5-fold increase in T output. As treatment with 100nM SAG unexpectedly gave much higher increases in steroid output, we are in the process of determining the stability of our Ag1.5 stock.

Figure 6: Effect of Hh agonists on 1nM Ag1.5 on steroidogenesis in commercially available BMS cells. (A) Treatment with increasing concentrations of SAG showed increased mRNA expression of key steroidogenesis enzymes after 48hrs with 200nM SAG. Expression was measured by ΔΔCT method QRT PCR and shown +SE. (B) Steroid production, after 72hrs, with 10µM OH-cholesterol precursor, was increased by treatment with both 1nM Ag1.5 and 200nM SAG. Steroid levels were measured by LC/MS, normalized to cell number, and expressed as fold change + SE.

Following the cessation of experiments with commercially available BMS cells, we undertook the lengthy process of obtaining primary bone marrow stromal cells established by our collaborator and co-mentor, Dr. Eva Corey (25) (University of Washington), dubbed BMS-EC. We allowed cells to come to 70% confluence before starving in serum free medium for 48hrs. We then treated the cells with 1nM Ag1.5 for 48hrs, and observed mRNA increases in SF-1 (1.65-fold), SREBP (1.9-fold), CYP17A1 (2.6-fold), HSD3B2 (6.8-fold), AKR1C3 (3.55-fold), and HSD17B3 (3.9-fold), with increases of Gli1 and Gli2, 2.9 and 2.5-fold, respectively. Additionally, treatment with either 1nM Ag1.5 or 100nM SAG resulted in increased steroid output after 48hrs treatment, with OH-pregnenolone precursor. The highest outputs with Ag1.5 treatment were DHEA and androstenedione, 6.5 and 3.2-fold, respectively. Treatment with SAG increased outputs of pregnenolone (2.2-fold), progesterone (2.5-fold), 17-OH progesterone (2-fold), androstenedione (3.9-fold), T (2-fold), and androsterone (4.7-fold). Taken together, these results suggest that both commercially available BMS cells and BMS-EC cells are capable of de novo steroidogenesis when stimulated with Hh pathway agonists, and that this paracrine interaction with PCa cells may contribute to the favorable conditions for bone metastasis growth, making the interaction a promising target for therapeutic intervention.
Figure 6: Effect of Hh agonists on 1nM Ag1.5 on steroidogenesis in BMS-EC cells. (A) Treatment with 1nM Ag1.5 showed increased mRNA expression of key steroidogenesis enzymes after 48hrs. Expression was measured by ΔΔCT method QRT PCR and shown +SE. (B) Steroid production, after 48hrs, with 10µM OH-pregnenolone precursor, was increased by treatment with both 1nM Ag1.5 and 100nM SAG. Steroid levels were measured by LC/MS, normalized to cell number, and expressed as fold change + SE.

We expect to do the gene Chip analysis on BMS-EC cells within the next 2 month, and submit an open-access manuscript on Hh induced BMS steroidogenesis within 6 month.

3b4. Other Training Project: A Spontaneous Developmental Lineage Plasticity that Underlies the Response of Prostate Cancer Cells to Androgen Deprivation (Manuscript in progress; poster presented at AACR April 2014; presentation given at Lorne Sullivan Research Day, Vancouver, June 2014; presentation scheduled for Koc University, Istanbul, October 2014)

Androgen-dependent prostate cancer (PCa) cells can acquire features of neuroendocrine- (NE-) cells under acute androgen deprivation (AD); other neuroectodermal/mesenchymal (NE/M) cell phenotypes develop under chronic AD. We propose that a transient PCa stem-like cell mediates these transdifferentiations. We sought to reveal/maintain this stem-like state using a special “stem-transition medium” that could give rise to these complex lineages from LNCaP, CWR22r1, LAPC4, or VCaP cells after at least 14days. The medium converted these cells, en masse, to small, rounded proliferating cells that grew as 3D rosettes and spheroids. QRT and CD antigen profiling of converted LNCaP cells were most consistent with a neural/neural crest stem-like (N/NCSL) identity. N/NCSL cells fail to express androgen receptor (AR) or PSA and are significantly more resistant to hypoxia and enzalutamide than parental LNCaPs. The similarities and differences between the afore mentioned parental and stem-like cell lines were compared using microarray analysis and were found to be enriched for many of the same neural, stem-like, and developmental pathways.
Androgen-free differentiation medium allowed LNCaP N/NCSL cells to express biomarkers of neuron-, glia-, oligodendrocyte or osteocyte-like cells. Following redifferentiation in androgen-containing medium, they reverted to epithelial cells resembling parental LNCaPs but they overexpressed AR and retained expression of nestin, a neural stem marker. LNCaP-N/NCSL cells have significantly increased tumor-initiating capability, forming tumors in nude mice from only 100 xenografted cells vs \(10^6\) parental cells, and are metastatic when injected intra-prostatically. They are invasive in zebrafish where we observed cells metastasizing to the brain and spine, whereas parental LNCaPs remain in the yolk sac. Likewise, when injected in the neural crest area of developing chicken embryos, only LNCaP-NCSLs migrate throughout the neural tube. Lastly, the neural crest marker BRN3A is upregulated in our stem model and immunostaining shows that increased staining is strongly correlated with patient recurrence and inversely correlated with survival.

In summary, we show 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.

4. KEY RESEARCH ACCOMPLISHMENTS:
   - 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.

5. 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. 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.

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.

Future experiments will seek further elucidate the regulation of steroidogenesis related enzymes in BMS-EC cells by Hh using gene CHIP analysis (Aim1, Major task 1, subtask 3) and to determine the Gli2 dependence of BMS-EC cells by siRNA knockdown and by transduction with constitutently active Gli2 lentiviral vector (Aim1, Major task 3, subtask 1 and 2). These findings will be compiled into an open-access research article. Furthermore, we will use our co-culture system to illuminate the paracrine signaling between BMS cells and LNCaP cells, parental and Shh over-expressing, and the growth inducing effects of BMS cell steroidogenesis on LNCaP cells (Aim 1, Major task 5-6).

We will also test whether acute or chronic exposure of Hh-induced PrSCs or BMS cells to abiraterone or MDV3100 will further upregulate the expression of steroidogenesis enzymes (by QRT PCR) and/or output of T/ DHT by LC/MS (Specific Aim 2).

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

6a. Publications: Nothing to Report

6b. Abstracts:


6c. Presentations:

1) **Lubik, AA**: Targeting Sonic Hedgehog Signalling with Simvastatin Blocks Prostate Stromal Cell Paracrine Steroidogenesis by Multiple Avenues. Presentation given for Vancouver Prostate Centre, Vancouver, BC, Canada, March 2014.

2) **Lubik, AA**: A New Model of Prostate Cancer Stem Cells and its Relevance to Castrate Resistant Prostate Cancer. Presentation given at Lorne Sullivan Research Day, Vancouver, Vancouver, BC, Canada, June 2014

3) **Lubik, AA**: Developmental Plasticity of Prostate Cancer and Hormone Resistance. Presentation scheduled for Koc University, Istanbul, Turkey, November 2014.

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report

8. REPORTABLE OUTCOMES: 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.

9. OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT

- 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-Dimas, 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).

10. OTHER ACHIEVEMENTS: Nothing to report.

11. REFERENCES