Identification of Prostate Cancer Prognostic Markers

The objective of this project is to develop prognostic markers for prostate cancer (PCA) and identify potential therapeutic targets. Specific aims are: 1) To profile bone metastasis samples to identify genomic alterations of PCA metastases that can be retrieved in their corresponding primary tumors, 2) To evaluate the prognostic value of specific genomic alterations in localized primary tumors with clinical follow-up database, 3) To ascertain the relevance to disease progression of genes residing in genomic alterations of prostate cancer metastases. Standard procedure for PCA bone metastases resection material collection was established and DNA, RNA and protein analysis is currently ongoing (AIM1). Chromosome 10q23 (PTEN) deletion is currently being assessed and its prognostic value is being confirmed on the full McGill TMA (328 cases), that will be combined to chromosome 16p13 gain status, which is already available (AIM2). Under AIM3, we generated stable clones for reduced GABARAPL2 expression in PC-3 cells and will assess the consequences on cell growth and migration. Stable knockdown of PDK1, which gene is located at 16p13 (gained in PCA metastases), confirmed previous results from transient knockdown on reduction in motility. Another target located at 16p13, ECI1, is also being assessed for its role in cellular energetics and growth. So far, our results suggest that genomic alterations may serve as prognostic markers that would improve the clinical management of PCa.

prostate cancer, genomic alteration, chromosome gain and deletion, fluorescence in situ hybridization (FISH), prognostic markers, biomarkers, tissue microarrays, autophagy
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1. INTRODUCTION:

Prostate cancer (PCa) is a very heterogeneous disease ranging from indolent to metastatic deadly disease. A major challenge in clinical management of PCa is to predict, at the time of diagnostic, the outcome of the tumor and consequently choose the best treatment option for each patient. Current markers - preoperative serum prostate specific antigen (PSA) levels, tumor stage and biopsy Gleason score (GS) - cannot accurately predict individual patient outcome. For advanced and metastatic disease there is no curative treatment. Genomic profiling studies have identified specific genomic alterations such as chromosome gains and deletions associated with aggressive PCa [1] [2] [3]. This research project aims to develop new prognostic markers and identify the relevant tumor genomic alterations associated with disease progression.

2. KEYWORDS:

Prostate cancer, castrate resistant prostate cancer (CRPC), DNA copy number alteration, chromosome gains and deletions, fluorescence in situ hybridization (FISH), biomarkers, tissue microarray (TMA), colony formation assay, cell growth, bone metastasis.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The hypothesis of the project is that specific genomic alterations, determinant of tumor behaviour, could be detected in primary tumor and predict clinical outcome. The objective is to develop prognostic markers for PCa and identify potential therapeutic targets. To that goal we proposed the following specific aims:

- Specific AIM 1: To profile bone metastasis samples to identify genomic alterations of PCa metastases that can be retrieved in their corresponding primary tumors;
- Specific AIM 2: To survey selected cohorts of clinical prostate samples for genomic changes and evaluate their prognostic value;
- Specific AIM 3: To ascertain the relevance to disease progression of genes residing in genomic alterations of PCa metastases.

Our progress is summarized below, with figures supporting the data shown in the appendices (section 9). Please note that all data presented in this report are unpublished.

What was accomplished under these goals?

Specific AIM 1: To profile bone metastasis samples to identify genomic alterations of PCa metastases that can be retrieved in their corresponding primary tumors.
To obtain PCa bone metastasis (PCaBM) samples (Task 2 in Statement Of Work (SOW)), we established a collaboration with McGill orthopaedic surgeon Dr. Michael H. Weber, as mentioned in the 2015 Progress Report. We also established a standard procedure to process samples after their collection.

Upon receiving the tissues from the operating room, each fresh sample is cut into pieces and:

i) embedded within optimal cutting temperature (OCT) compound and snap frozen in liquid nitrogen and stored at -80°C.

ii) formalin-fixed, decalcified and paraffin-embedded (FFPE).

iii) put in cell culture after collagenase dissociation.

iv) blood is collected for isolation of peripheral blood mononuclear cells (PBMC), serum, RNA in PaxGene tube and DNA on dry card.

Those processes are being performed to:

i) visually assess tumor content by H&E on frozen sections and extract RNA and DNA for genomic profiling;

ii) provide high quality tissue for fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC);

iii) maintain primary PCaBM cells, perform cytogenetic analysis (FISH, chromosome painting), immunocytochemistry (ICC), RNA-DNA-proteins isolation, in vitro experiments;

iv) extract germline genomic DNA from PBMC as control and save circulating tumor cells (CTCs), circulating DNA and RNA, and exosomes.

Our first bone metastasis sample, PCaBM-1, was isolated from a vertebrae of a 62 years old patient and processed according to our protocol (i to iv). H&E staining of one frozen section was reported last year and showed that our sample contains tumor cells. After decalcification in 14% EDTA pH 7.1 (adjusted with NH₄OH) at room temperature with gentle shaking, the formalin-fixed piece was paraffin-embedded and sectioned. H&E staining showed the presence of malignant cells (Figure 1 A-B). This sample stained positive in IHC for Vimentin, a marker of epithelial to mesenchymal transition (EMT) (Figure 1 C-D), and their epithelial origin was confirmed by their positive staining with pan-cytokeratin cocktails CK8/18 (Figure 1 E-F) and CKAE1/3 (Figure 1 G-H). Cell cultures derived from this sample do not grow very well and we are not sure whether they are malignant cells. They are being kept in culture for expansion and characterization.

We obtained a second sample, PCaBM-2, from a voluminous lumbar mass (spinal segment L4-L5) of a 56 years old patient. As the mass was resected, 3 areas were collected, based on their anatomical location; lumbar soft tissue, lamina and epidural regions. Each of them was processed in parallel as per our protocol. Cells in culture from all 3 areas (summary of procedures in Figure 2) grew at a surprisingly fast rate, and clearly showed 2 mixed but distinct cell populations, one being more epithelioid and the second more fibroblastoid (Figure 3A, yellow and white arrows,
respectively). To enrich the epithelioid fraction, we incubated cells with trypsin for a short time, collecting the cells detaching easily (a characteristic of fibroblasts) and washing and keeping the cells that didn’t detach (a characteristic of epithelial cells). With 2 rounds of such differential trypsinization, we derived 7 different cell cultures with this process.

The pathology report revealed that the patient had an extremely rare case of PCa with sarcomatoid differentiation, as assessed by 2 independent pathologists of the McGill University Health Centre. Sarcomatoid differentiation in PCa is extremely rare [4] and very aggressive [5]. With that knowledge, we trypsinized and seeded at low density an epithelioid-rich cell subculture, and isolated 12 single colonies of epithelioid and 1 of sarcomatoid appearances (previously referred to as fibroblastoid). Figure 3B and C respectively show examples of isolated epithelioid and sarcomatoid colonies. Figure 3D shows sarcomatoid cells as in C, when more confluent. A total of 36 different cell cultures were derived from the 3 different areas of resected material, including some after differential trypsinization, as previously mentioned, and colony isolation (Table 1). They are currently all being harvested for cryopreservation, protein (lysis in RIPA buffer), RNA (TRIzol) and DNA (QIAGEN gDNA kit) isolation.

In parallel, pieces of formalin-fixed tissue from the 3 anatomical areas (lumbar soft tissue, lamina, epidural) were decalcified, as described above, before being paraffin-embedded. Four microns (4 μm) consecutive sections from lumbar soft tissue were H&E stained and subjected to IHC for Vimentin (Vim), a marker positive in a majority of sarcomatoid differentiated cells [6], CK8/18 and CKAE1/3, markers of epithelial cells. In Figure 4, 1X and 8X magnifications of H&E staining (A-B) revealed infiltration of mixed sarcomatoid and epithelioid cell populations, IHC showed that Vimentin expression (C-D) was strongly positive where sarcomatoid cells were found in the H&E staining. IHC for CK8/18 (E-F) and CKAE1/3 (G-H) revealed strong staining in epithelial-rich regions, that were unstained for Vimentin. This leads us to hypothesize that in this PCaBM case, epithelial cells are Vimentin-, CK8/18+ and CKAE1/3+, while sarcomatoid cells are Vimentin+, CK8/18- and CKAE1/3-.

Figure 5 shows 1X and 8X magnifications of H&E and IHC for Vimentin, CK8/18 and CKAE1/3 on lamina area of the PCaBM-2 tumor. This area of tumor showed few epithelial cells, both in H&E (A-B) and demonstrated by many cells positive for Vimentin (C-D) and few positive for CK8/18 (E-F), while almost no cell were positive for CKAE1/3 (G-H). Epithelial cells positive for CK8/18 are surrounded by cells positive for Vimentin, with the same pattern displayed as in Figure 4 for lumbar soft area.

Epidural area (Figure 6) displays overall very few epithelial cells in H&E staining (A-B), and IHC shows cells that are strongly positive for Vimentin (C-D) and weakly positive for CK8/18 (E-F). IHC for CKAE1/3 will be performed on a slide consecutive to those mentioned above.
To evaluate copy number alterations (CNA) in those cultured cells derived from PCaBM-2 tumor, and eventually on FFPE tissue, we are currently setting up a five (5)-color multiplex FISH assay, with specific probes for chromosomal regions 10q23 (PTEN), control centromere 10, 8q24 (MYC), control centromere 8, counterstained with DAPI (gold, green, red, aqua and blue, respectively). We successfully tested this multiplex FISH assay on normal lymphocytes metaphase spread. We are optimizing a protocol for colcemid-induced metaphase block on PCaBM-2 cultured cells to perform this multiplex FISH, as well as G-banding and spectral karyotyping (SKY) for chromosome counting and assessment of translocation. This fixation method of cultured cell will also be used to perform ICC for expression markers of epithelial and sarcomatoid cells.

We currently are extracting RNA, DNA and proteins from the 36 cell cultures isolated from PCaBM-2 sample (Table 1), some of which are strongly enriched in epithelioid or sarcomatoid features. Sections from all the frozen material are being cut and stained with H&E to identify areas of tumor, as well as epithelial and sarcomatoid compartments for separate isolation of RNA, DNA and protein. Upon analysis of gene-expression, CNA and protein expression from tissue and cell lines derived from the same tumor, we plan on assessing the molecular landscape of both epithelial and sarcomatoid components. This could lead to molecular evidences of the origin of sarcomatoid differentiation, a very aggressive type of PCa [5], that might share characteristics with other aggressive types of PCa tumours.

Specific AIM 2: To survey selected cohorts of clinical prostate samples for genomic changes and evaluate their prognostic value

In previous Progress Reports, we showed that 16p13.3 genomic gain in primary PCa samples was significantly associated with clinico-pathological features of aggressive PCa. A manuscript comprising these exciting results has been submitted for publication to Clinical Cancer Research, 2017. According to Task 3 of the SOW, we are pursuing the assessment of prognostic value of other genomic alterations (8q24, 10q23) found in metastases and retrieved in primary tumors, which may serve as predictors of progression.

-FISH analysis of 10q23 (PTEN) deletion on primary radical prostatectomy samples on the McGill Urology TMA with complete follow-up.

We had assessed the specificity of the 10q23 FISH probe on normal lymphocyte metaphases and on different PCa samples harboring a hemizygous, homozygous or no 10q23 deletion. This PTEN-specific probe was then used on the McGill Urology TMA representing 328 primary PCa cases. Out of the 269 cases probed so far, PTEN FISH was scorable in 230 cases. We detected hemizygous in 70/230 (31%) and homozygous PTEN deletion in 13/230 (5%) of the primary radical prostatectomy samples (Figure 7). Hybridization and analysis of this 10q23 probe on the remaining cohort is currently being performed. Correlation with the 16p13.3 gain status,
previously performed on the same cohort, will assess the advantage of combining these markers with known clinico-pathological parameters for better recurrence-free survival.

**Specific AIM 3:** To ascertain the relevance to disease progression of genes residing in genomic alterations of PCa metastases.

In our previous Progress Report, under AIM 3 Task 1 of the SOW, we showed that modulation of GABARAPL2 by lack vs. gain (siRNA and overexpression, respectively) could have an impact on cell growth of PC-3 cells. MTT assay (under basic culture conditions, no starvation, and in the context of induced autophagy by Rad001) revealed that downregulation of GABARAPL2 with 2 different siRNAs significantly reduced cell growth, that this effect was rescued by re-expressing GABARAPL2, and that growth was further decreased after induction of autophagy with Rad001. This experiment showed for the first time that downregulation of GABARAPL2 in PC-3, but not in LNCaP nor 22Rv1 cells, reduced cell growth.

-Generation of stable GABARAPL2 knockdown PCa cell lines.

We selected the PC-3 cell line for lentivirus-mediated stable knockdown of GABARAPL2. For this, the optimum multiplicity of infection (MOI) was assessed to be 5 TU/ cell. Cells were transduced with lentiviral particles containing control pLKO.1-puro-tGFP-shCnt or pLKO.1-puro-tGFP-shGABARAPL2 vectors (Sigma “Mission” Lentiviral System) in the presence of polybrene (8 ug/ml), and were subjected to puromycin selection (2-3 weeks) to isolate single isolated colonies (clones), as well as a pooled mix for each condition (shControl and shGABARAPL2). **Figure 8A** shows WB analyses assessing GABARAPL2 expression in 14 knockdown selected PC-3 clones (sh-GABARAPL2-Clone A-F & A2-F2), pool population (sh-GABARAPL2 pool A&B) and controls (sh-Control-Clone A-B& sh-Control-pool). Clones D, E and F showed the least GABARAPL2 expression as compared to control clones. Similar approach was used to generate stable knockdown clones in PC3-M-LN4, a more aggressive variant of PC-3, without significant reduction in GABARAPL2 expression (**Figure 8B**). Another attempt will be performed with this cell line, but the successful stable PC-3 clones will be used to carry out downstream experiments.

In parallel to our work on GABARAPL2, we are studying **ECI1** (enoyl-CoA delta isomerase 1, previously reported as **DCI**), another relevant gene residing in the minimal region of gain of chromosome 16p13.3 [7]. Our previous gene expression analysis of various PCa subtypes revealed that along PDK1, ECI1 was significantly overexpressed in the most aggressive PCa subtype-3, as compared to clinically favourable subtype-1 and normal prostate tissues, as reported in the previous Progress Report. ECI1, which has not been studied in PCa, is a mitochondrial enzyme involved in β-oxidation of unsaturated fatty acids (FA), a metabolic pathway involved in cancer cell survival and growth [8] (**Figure 9**). ECI1 overexpression may thus provide PCa cells a survival advantage. Under AIM3, Task 1, we undertook the
assessment of *in vitro* modulation of ECI1 and its relevance to disease progression. We are currently following up with the findings reported last year that ECI1 stable overexpression in two PCa cell lines (PC-3 and 22Rv1) increased the number of colonies in a colony formation assay.

- FA β-oxidation in stable PC-3 clones overexpressing ECI1

We assessed the effects of ECI1 overexpression on mitochondrial respiration and FA β-oxidation, using a Seahorse Bioanalyzer (Agilent Technologies). For this, the oxygen consumption rate (OCR) in the PC-3 control and ECI1-overexpressing clones was measured following their maintenance in low-nutrient medium for 24h to favor FA β-oxidation. Our preliminary analyses demonstrate that stable ECI1-overexpressing PC-3 clones exhibit higher maximal respiration profiles as compared to Mock clones (*Figure 10*). This means that ECI1 overexpression results in an increased amount of ATP that can be generated by oxidative phosphorylation in response to stress or increased energy demand. We are currently repeating our analyses and optimizing protocols to specifically assess FA β-oxidation profiles of these clones when supplied with exogenous FA source (palmitate).

- Generation of stable ECI1 knockdown in 22Rv1 cells

We also are in the process of generating stable ECI1-knockdown in 22Rv1 cells, using the same lentivirus-mediated approach used above for GABARAPL2. Western blotting analysis of selected stable clones and pooled populations revealed minimal knockdown of ECI1 expression in the shECI1 conditions when compared to shCnt (*Figure 11*). We are currently optimizing the shRNA designs to produce new lentiviral shRNA vectors with better knockdown efficiency. Stable ECI1-knockdown cells would be ultimately used to perform rescue experiments for the phenotypes we observe upon ECI1 overexpression.

- Prognostic significance of ECI1 protein expression

Owing to our previous observations regarding overexpression of ECI1 in the aggressive subtype-3 compared to clinically favourable subtype-1 and normal prostate tissues, we optimized a protocol to assess ECI1 protein expression by IHC in FFPE PCa samples (*Figure 12*). ECI1 IHC analyses in 8 PCa tissue specimen revealed that ECI1 expression was negative/weak in 2/8 (25%) samples; intermediate in 2/8 (25%) and strong in 4/8 (50%) of the PCa tissues. More importantly, out of the 4 cases with high ECI1 expression, 3 harbored 16p13.3 gain (as assessed by FISH). The 16p13.3 gain was not found in any of the negative/weak or intermediate ECI1 expression samples. This suggests a possible correlation between ECI1 overexpression and 16p13.3 genomic gain. We are now extending this study on a larger cohort and have successfully performed the ECI1 IHC on the McGill TMA representing 328 radical prostatectomy samples. We are currently measuring its expression in these tissues. We will soon be assessing the association of ECI1 overexpression in this cohort with the clinico-pathologic features of aggressive PCa.
-Generation of stable PDK1 knockdown in PC-3 cells

Our previous publication identified the minimal region of 16p13.3 gain. We demonstrated that, PDPK1 gene, residing in this region and encoding a protein, 3-Phosphoinositide Dependent Protein Kinase 1, was involved in regulating PCa cell motility when assessed by in vitro cell-based wound healing assay [7]. We further want to explore the role of this gene in cell invasion and metastases using an orthotopic PCa mice model.

We used two different shRNA sequences targeting PDK1 (shPDK1-13 and shPDK1-82) cloned into the lentiviral system mentioned above and co-expressing tGFP, for stable knockdown of PDK1 in PC-3 and more aggressive PC3-M-LN4 cell line2. Figure 13 demonstrates PDK1 knockdown in the resulting stable clones and pool populations in those two cell lines. Using the stable PC-3 pool conditions (shCntrl, shPDK1-13, and shPDK1-82), we confirmed and validated our previous results regarding significant modulation of PCa cell motility upon PDK1 knockdown (Figure 14). We also performed a colony formation assay with these stable cells (pooled) and noticed no significant difference between the number of colonies for the control and the knockdown conditions (Figure 15), in accordance with our one of our previous publications [7]. Similar strategy was used in PC3-M-LN4 cell line and successful PDK1 knockdown clones were identified. Clones with optimum PDK1 knockdown for both cell lines will be selected and used for future experiments.

What opportunities for training and professional development has the project provided?

This project is the work of a PhD student (AIM 2-3), a post-doctoral fellow (AIM 3), and my research assistant (AIM 1), all of them in my laboratory under my direct supervision (Yogesh Bramhecha, Shaghayegh Rouzbeh and Karl-Philippe Guérard, respectively). They all using the skills they master and developing new ones by performing the required experiments to complete the specific tasks related to this project. During their graduate studies, both students are actively learning how to meticulously plan and perform experiments to generate scientifically sound results, a notion that will serve them throughout their career. Also, writing and publishing their results in scientific journals will make them develop interpretation capabilities, as well as make them participate in the dissemination of knowledge.

How were the results disseminated to communities of interest?

The results and analyses that stemmed from the experiments described above and in previous progress reports are unpublished, but some of them have been written in the form of manuscripts and submitted to peer-review, public-access scientific journals. Sections 6 of this report will show a submitted article, the list of presentations and
posters presented in regional and national conferences, by the personnel who worked on the project.

**What do you plan to do during the next reporting period to accomplish the goals?**

AIM 1: To pursue the goal of identifying new genomic signatures in bone metastases and in their corresponding primary tumors (Milestone), we will continue collecting bone metastases resection material, with the established protocol (see Section 3). We will perform gene expression analysis (Task 2), IHC and multiplex FISH on the metastases, their corresponding primary tumors (Task 3) and on the cultured cell lines derived from the metastasis samples. In the latter, we also plan on characterizing their aggressive phenotype *in vitro* and its tumor growth potential by injection in nude mice *in vivo*.

AIM 2: We will start FISH analyses of 8q24.21 (*MYC*) probe on the McGill Urology TMA of 400 cases of prostatectomy with clinical follow-up (Task 3). After having completed the analysis of 10q23 (*PTEN*), the deletion will be correlated with 16p13.3 gain status, previously performed on the same cohort. In this manner, we will assess the advantage of combining these markers with known clinico-pathological parameters for better recurrence-free survival.

AIM 3: First, we will assess the effect of GABARPL2 knockdown on cell migration using wound healing assay. Also, after confirming that GABARAPL2-downregulation stable clones affect cell growth *in vitro* (Task 2), *in vivo* metastasis experiments will be performed using BALB/c mice, as described in original proposal (Task 4). The work measuring the effect of ECI1 overexpression (and rescue) on FA β-oxidation and colony formation will continue, in parallel to correlating its expression in the McGill cohort with clinico-pathological data. Finally, selected stable PDK1 knockdown in PC-3 and PC3-M-LN4 cell lines will be selected and used for mouse implantation experiments.

4. **IMPACT:**

   **What was the impact on the development of the principal discipline(s) of the project?**

   Nothing to report.

   **What was the impact on other disciplines?**
Nothing to report.

**What was the impact on technology transfer?**

Nothing to report.

**What was the impact on society beyond science and technology?**

Nothing to report.

5. **CHANGES/PROBLEMS:**

   **Changes in approach and reasons for change**

   Nothing to report.

   **Actual or anticipated problems or delays and actions or plans to resolve them**

   Nothing to report.

   **Changes that had a significant impact on expenditures**

   Nothing to report.

   **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

   Nothing to report.

   **Significant changes in use or care of human subjects**

   Nothing to report.
Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

Publications, conference papers, and presentations

Journal publications.


Books or other non-periodical, one-time publications.

Other publications, conference papers, and presentations.


Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
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<tbody>
<tr>
<td>Jacques Lapointe</td>
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<td>Karl-Philippe Guérard</td>
<td>(no change)</td>
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<td>Yogesh Bramhecha</td>
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</table>
### Project Role: Graduate Student

| Researcher Identifier (e.g. ORCID ID): | - |
| Nearest person month worked: | 12 |
| Contribution to Project: | PhD candidate Yogesh Bramhecha performed experiments related to AIM 2 (FISH) and part of AIM 3 (in vitro functional assays). |

### Name: Shaghayegh Rouzbeh

| Project Role: | Post-Graduate Student |
| Researcher Identifier (e.g. ORCID ID): | - |
| Nearest person month worked: | 5 |
| Contribution to Project: | Post-doc fellow Shaghayegh Rouzbeh worked on GABARAPL2 expression modulation experiments in AIM 3. |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report.

**What other organizations were involved as partners?**

Nothing to report.

### 8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.
9. **APPENDICES:**

**REFERENCES**


Figure 1: H&E stain and IHC for Vimentin, CK8/18 and CKAE1/3 on PCaBM-1 decalcified sample.

After decalcification, formalin-fixed PCaBM-1 sample was put in FFPE block. Four microns (4 µm) consecutive sections were subjected to H&E staining (A-B) for assessment of tissue architecture or IHC stained with Vimentin (C-D), pan-cytokeratin antibody cocktail CK8/18 (E-F) or CKAE1/3 (G-H) showed at 1X and 8X magnifications, respectively. All IHC stained positively. For IHC, briefly, FFPE section on slide was baked for 3 hours at 60°C, deparaffinized in xylene, rehydrated by passing through solutions of decreasing alcohol percentages and then water, microwave-boiled in citrate buffer, washed, blocked for endogenous peroxidase activity, blocked in goat-serum, incubated with primary antibody, HRP-linked secondary antibody, revealed with DAB reagent, counterstained in Hematoxylin, dehydrated in alcohol and xylene, and mounted with Clearmount under a coverslip.
Figure 2: Method of culture and cell lines derived from PCaBM-2 sample. Fresh material from operating room was carried on ice in sterile phosphate-buffered saline to the laboratory. A fraction of the material was directly frozen in FBS+10% DMSO. After overnight incubation with collagenase, dissociated cells were filtered through cell strainers and put in cell culture (RPMI 1640, supplemented with 10% FBS, penicillin-streptomycin, L-glutamine and amphotericin B) in plate “A” and 25 cm² cell culture flask “E”. Cell strainers were then washed with culture medium and wash material put in cell culture plate “B”. Bone/tissue pieces left over from previous step were put in cell culture plate “C”.

Fresh piece of tissue (Lumbar soft, Lamina, Epidural region) → \( \text{LN}_2 \)

Overnight collagenase incubation at 37°C → Dissociated cells + bone/tissue pieces

\( \text{Filtration through Cell Strainers} \)

Centrifugation at 1000 x g, 15 min → Culture in 6-well plate "A"

Wash with culture medium → Culture in 6-well plate "B"

Filtrate + Material in cell strainer → + Culture in 25 cm² flask “E” for Lumbar soft area only
Figure 3: Cells in culture from PCaBM-2 sample show distinct populations. After incubation with collagenase and filtration through cell strainers, cells were cultured in RPMI1640 medium supplemented with 10% FBS, L-glutamine, penicillin/streptomycin and amphotericin B. Cells from lumbar soft tissue area exhibited 2 different populations, of epithelioid and fibroblastoid appearances (A, yellow and white arrows, respectively). After isolation of single colonies based on their morphology from low-density seeding of epithelial-rich culture, pure epithelial (B) and sarcomatoid (C) cultures were isolated. When more confluent, sarcomatoid cells arrange in a distinct monolayer shape (D).
Figure 4: H&E staining and IHC for Vimentin, CK8/18 and CKAE1/3 on lumbar soft tissue area of PCaBM-2 tumor. H&E staining and IHCs were performed as described in previous figures, showed at 1X and 8X on left and right panels, respectively. Inserts at 8X magnification of the same region of the tumor show epithelial cells (H&E (A-B), yellow arrow) surrounded by sarcomatoid cells (black arrows). IHC for Vimentin (C-D) is positive in the sarcomatoid and negative in epithelial compartment, while CK8/18 (E-F) and CKAE1/3 (G-H) are positive strictly in epithelial cells and negative in the sarcomatoid compartment.
Figure 5: H&E staining and IHC for Vimentin, CK8/18 and CK AE1/3 on lamina area of PCaBM-2 tumor. H&E staining and IHCs were performed as described in previous figures, showed at 1X and 8X on left and right panels. In the inserts at 8X magnifications of the lamina region of the tumor, H&E (A-B) shows epithelial cells that are negative in IHC for Vimentin (C-D) but positive for CK8/18 (E-F), while surrounding cells stain negative for CK8/18 and positive for Vimentin. A deeper cut of same block show few epithelial cells positive for CKAE1/3 (G-H).
Figure 6: H&E staining and IHC for Vimentin and CK8/18 on epidural area of PCaBM-2 tumor. H&E staining and IHCs were performed as described in previous figures, showed at 1X and 8X on left and right panels, respectively. In the inserts at 8X magnifications, H&E (A-B) shows overall few epithelial cells, with cells strongly positive in IHC for Vimentin (C-D) and a low number of cells positive for CK8/18 (E-F).
Figure 7: FISH for 10q23 (PTEN) deletion. A) Validation of PTEN FISH probe specificity on normal lymphocyte metaphase, showing 1 green and 1 orange signals (yellow arrow head). White arrows show B) normal interphase nuclei with 2 green and 2 orange signals in a PCa tumor with no PTEN deletion, C) 2 green and 1 orange signals in a tumor harboring hemizygous PTEN deletion and D) 2 green and 0 orange signals in a homozygous PTEN deleted case. E) Tumor sample with nuclei harboring hemizygous (white arrows) or homozygous (yellow arrow) deletions respectively. F) FISH analysis detected hemizygous PTEN deletion in 70/230 (31%) and homozygous PTEN deletion in 13/230 (5%) of the primary radical prostatectomy samples on the McGill Urology tissue microarray analysed so far (n = 230).
Figure 8: Generation of stable GABARAPL2 knockdown clones in PC-3 and PC3-M-LN4 cells. Western blot analysis characterizing A) knockdown of GABARAPL2 expression (Abcam #ab126607) in different shRNA stable PC-3 clones. Clones D, E and F (left panel) have the least GABARAPL2 expression compared to control clones. Single and pooled stable clones for GABARAPL2 knockdown in B) PC3-M-LN4 show negligible knockdown. Percentage shown are GABARAPL2 expression relative to average of sh-Controls and normalized to actin (Millipore #MAB1501R).
Figure 9: Mitochondrial fatty acid β-oxidation overview. ECII, an enoyl-CoA isomerase, plays an important role in the mitochondrial fatty acid (FA) β-oxidation. Etomoxir inhibits FA transport in the mitochondria. Adapted from lipidlibrary.aocs.org/.
Figure 10: Mitochondrial Respiration in ECI1-overexpressing PC-3 Clones. Oxygen Consumption rate (OCR) in PC-3 control (green) and ECI1-overexpressing clones (Orange) measured by Seahorse flux analysis after 24h in low-nutrient conditions to favour fatty acid oxidation. Both basal and maximal (after FCCP injection) respiration are shown. Results are shown as the average of 3 independent experiments ± SEM. ECI1 overexpressing clones had significantly higher maximal respiration compared to control clones as assessed by ANOVA and post-hoc pairwise significance test (n=13-16 samples/group, *p<0.05).

Figure 11: Generation of stable lentivirus-mediated ECI1 knockdown PCa (22Rv1) cell lines. Western blot analysis characterizing different stable 22Rv1 clones transduced by shECI1 or shControl lentiviral particles. All the stable clones and pooled conditions demonstrated negligible ECI1 knockdown. Antibodies used were anti-ECI1 and Actin as control.
Figure 12: ECI1 expression and 16p13.3 gain status. Representative images of IHC for ECI1 expression in the optimization tissue microarray comprising 8 PCa tissues. Left panel represents negative/weak (2/8), middle panel intermediate (2/8), and right panel strong ECI1 staining (4/8). Corresponding 16p13.3 gain status, assessed by FISH, is shown below the images. 16p13.3 gains were only found in strong ECI1 expression cases (3/4).
Figure 13: Generation of lentivirus-mediated stable PDK1 knockdown PCa cell lines. Western blot analysis (anti-PDK1, ECM #PM1461) characterizing different single or pool stable clones, transduced with 2 different shRNAs targeting PDK1 (shPDK1-13 or shPDK1-82), or non-targeting (shControl) lentiviral particles. All stable clones and pooled conditions denoted by * demonstrated optimum PDK1 knockdown. Actin was used as control.
Figure 14: Stable PDK1 knockdown in PC-3 Cells Reduces PCa Cell Motility. A) Wound healing assay for PC-3 cell lines at 0, 9 and 12 hours with shControl (shCtrl) compared to two different shPDK1 (shPDK1-13 and shPDK1-82). Migration results were quantified and are represented in B) in terms of percentage recovery of initial surface area after wounding (hour). C) WB analyses for the stable knockdown cells used for the wound healing assay. * and ** represent statistically significant difference between the shControl vs shPDK1-13 and shPDK1-82 respectively, t-test, p<0.05.
Figure 15: Stable PDK1 knockdown have no effect on colony formation capacity of PC-3 cells. A) Picture representing crystal violet staining for the PC-3 colonies (shControl, shPDK1-13 and shPDK1-82). B) Bar graph representing the mean total number of colonies per well for each condition.
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<th>Isolation method</th>
<th>Culture vessel</th>
<th>Anatomical area of origin</th>
<th>Expansion in flasks/plates</th>
<th>Differential trypsin</th>
<th>2^nd differential trypsin</th>
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Table 1: Cell lines derived from PCaBM-2 sample. Isolation method, culture vessel, anatomical provenance and code names of all cell lines derived from PCaBM-2 sample are shown.