Prostate cancer is a highly heterogeneous disease and the second most common type of cancer around the world, accounting for 15% of all cancer diagnoses. The standard treatment for prostate cancer is hormone deprivation therapies that are usually effective initially. However, 30% of patients eventually develop castration resistant prostate cancer (CRPC) that becomes metastatic and fatal. Although there are intensive efforts to search for the causes of CRPC using bulk studies, the mechanisms for CRPC are still largely undetermined. Because of limited treatment options currently available to CRPC patients, understanding the genetic mechanisms that lead to CRPC is critical, and any discoveries leading to novel therapies have the potential to improve prostate cancer patients’ life quality.

**Introduction**

In order to understand the progression of castration resistance systems in prostate cancer, we utilized single cell RNA-seq (scRNA-seq) to profile the heterogeneous global expression patterns in LNCaP and PC3 representing castration sensitive and resistant cell lines as well as a transitional cell line (ABL) with androgen-independency. SCRNA-seq (1) of the three PCA cell lines revealed 335 and 2393 differential expression (DE) genes in ABL and PC3 respectively when compared to LNCaP. Most of them were upregulated. About 43 and 163 pathways were enriched in ABL and PC3 respectively. Those pathways included focal adhesion, adherens junction, leucocyte transendothelial migration, ECM-receptor interaction, regulation of actins, gap junction, TGFβ, WNT, Hippo, Ephrin and etc. The gene expression in the Hippo and Ephrin pathways were further validated using qRT-PCR and western blot. This data consistently confirmed that scRNA-seq data is reliable and robust. Nine pathways associated with castration resistance were further verified using small molecule inhibitors and siRNA knockdown as shown to be involved in cell proliferation, invasion and migration in CRPC. We found that the Hippo and Ephrin pathways were significantly represented in the list of upregulated genes in PC3, suggesting that they may play a role in aggressive PCa. We knocked down the expression of Ephrin B2 and SRC, which are major players in Ephrin pathways affecting PC3 and Du145 migration and invasion. Together, our results indicate that the Hippo and Ephrin pathways play an important role in PCA aggressiveness, and may serve as useful biomarkers for prognostic tests and targets for novel CRPC treatments.

**Results**

1. **Figure 1.** The schematic illustration of single cell RNA-seq using SMART-seq2 approach.
2. **Figure 2.** The differential gene expression in ABL and PC3 as compared with LNCaP cells. A. Volcano plot showed differentially expressed genes identified using single cell differential expression algorithm as shown in pink and red. B. Hierarchical clustering heatmap of DE genes. C. Venn diagram showed the gene intersection of ABL and PC3 specific DE genes.
3. **Figure 3.** Heatmap of DE genes in Ephrin pathway and western blot of Hippo pathway in CRPC. The majority of DE genes were upregulated in castration resistant PC3 suggesting their role in CRPC.
4. **Figure 4.** Enriched pathways in CRPC and functional validation using small molecules. A. Pathway enrichment analysis identified 43 and 163 pathways using DAVID algorithm. Small molecule inhibitors were used to verify the function of 9 pathways in CRPC in four cell lines for B. proliferation, C. migration and D. invasion.
5. **Figure 5.** Knock down of Ephrin B2 and SRC of the Ephrin pathway in PC3 and Du145. Figure 4 shows that Dasatinib (Ephrin inhibitor) and verteporfin (Hippo inhibitor) reduced PCa proliferation, invasion, and migration significantly in PC3 and Du145, but did not affect ABL very much. The inhibition of Ephrin not only slowed the spread and proliferation of PCa in PC3 and Du145, but was specific to only Du145 and PC3 and did not affect androgen dependent cells.
6. **Figure 6.** The inhibitory phenotypes of PC3 and DU145 with siRNA knock down of Ephrin A1, Ephrin B2 and SRC were consistent with small molecule inhibition experiment data indicating that the Ephrin pathway is important in castration resistant systems of PCa.

**Conclusion**

1. We identified 335 and 2393 differentially expressed (DE) genes respectively in ABL and PC3 prostate cancer cell lines.
2. About 43 and 163 pathways were enriched in ABL and PC3 respectively. Those included focal adhesion, adherens junction, leucocyte transendothelial migration, ECM-receptor interaction, regulation of actins, gap junction, TGFβ, WNT, Hippo, Ephrin pathways and etc. These indicated that the castration resistance systems in CRPC may be composed of multiple pathways that are increased along with castration resistance progression.
3. Nine CRPC-associated pathways were further verified using small molecule inhibitors and the data showed that they are involved in cell proliferation, migration and invasion in the CRPC cell lines, PC3 and Du145.
4. The perturbation of Ephrin B2 and SRC reduced the migration and invasion of the CRPC cells, PC3 and DU145. The small molecule and siRNA inhibition experiments strongly suggested that the Hippo and Ephrin pathways are important in CRPC.

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**References**