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TITLE: Targeting Tumor Oct4 to Deplete Prostate Tumor- and Metastasis-Initiating Cells

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Targeting Tumor Oct4 to Deplete Prostate Tumor- and Metastasis-Initiating Cells

Identification of genes driving prostate carcinogenesis will lead to new cancer treatment. The human chromosome 8q24.21 region has been linked with increased risk for prostatic carcinoma but the how this region contributes to prostate carcinogenesis is unknown. We cloned a candidate gene, POU5F1B (also called POU5F1P1), in this gene desert of 1.2Mb between FAM84B and the c-MYC oncogene. POU5F1B is a pseudogene of embryonic Oct4 (POU5F1). A recent study found that tumor Oct4 found in prostate cancer cells is due to the gene expression of POU5F1B, not embryonic Oct4 (POU5F1). In a dataset of 171 patients, it was found that tumor Oct4 was significantly increased in primary tumors and markedly increased in metastatic tumors, when compared to normal prostate or adjacent normal tissues. Based on the analyses and our preliminary data, we think, tumor Oct4, expressed from POU5F1B in the prostate cancer susceptibility loci 8q24, is a driver of prostate tumor formation and progression, and therefore, this driver is a novel target of intervention to eliminate prostate cancer. We propose to further determine the roles of tumor Oct4 in prostate tumor formation and metastasis. We hope we can validate whether tumor Oct4 can be targeted to inhibit prostate cancer progression and metastasis. In addition, we will map out the regions critical for Oct4 to promote prostate carcinogenesis so that we can target this region to develop therapeutics for cancer treatment in the future.

OCT4, cancer stem cells, prostate cancer, metastasis, tumor formation
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Introduction

Background: Genome-wide association studies (GWAS) have linked human chromosome 8q24.21 region with increased risk for prostatic carcinoma but the how this region contributes to prostate carcinogenesis is unknown. In this gene desert of 1.2Mb between \textit{FAM84B} and the \textit{c-MYC} oncogene, \textit{POU5F1B} (also called \textit{POU5F1P1}) is a candidate gene with coding capacity. It is a pseudogene of embryonic Oct4 (POU5F1). A recent study found that tumor Oct4 found in prostate cancer cells is due to the gene expression of POU5F1P1 (Hugo name: POU5F1B), not embryonic Oct4 (POU5F1). Our \textit{in silico} analysis found a significant increase in Oct4 (\textit{POU5F1B}) in primary tumors and a marked increase in metastatic tumors, when compared to normal prostate or adjacent normal tissues. Tumor Oct4 expression was higher in tumorigenic prostate cancer cells than in non-tumorigenic RWPE-1 cells. Depletion of tumor Oct4 in prostate cancer cells reduced their tumorigenic potential. We cloned tumor Oct4 and found that increased expression of tumor Oct4 in prostate cancer cells stimulated tumor cell motility. Further a significant divergence was found between tumor Oct4 and embryonic Oct4 in regulating \textit{Wnt/β-catenin} signaling. It is our hypothesis that tumor Oct4, expressed from \textit{POU5F1B} in the prostate cancer susceptibility loci 8q24, is a driver of prostate tumor formation and progression, and therefore, this driver is a novel target of intervention to eliminate prostate cancer.

Objective: The objective is to determine whether tumor Oct4 promotes tumor formation and metastasis, to determine whether tumor Oct4 can be targeted to treat prostate cancer progression, and to elucidate the mechanism involved for tumor Oct4 to promote prostate carcinogenesis.

Specific Aims: 1) Investigate whether tumor Oct4 promotes prostate tumor initiation and metastasis.

2) Determine whether tumor Oct4 can be targeted to reduce prostate tumor formation, progression, and metastasis.

3) Elucidate the mechanism involved for tumor Oct4 in promoting prostate carcinogenesis.
BODY OF REPORT

Scientific portion:

Task 1. Investigate whether tumor Oct4 promotes prostate tumor initiation and metastasis.

The overexpression of tumor POU5F1B in prostate cancer LNCaP cell lines and subsequent effects on tumor cell growth in vitro and tumor formation and growth in vivo were described in the 2014 report.

The expression of POU5F1B in prostate tissue, and its cloning, sequencing and characterization have been reported in the 2015 report.

Here we describe some findings previously not reported.

1.1 POU5F1B gene is frequently amplified in prostate cancer especially in metastatic, castration resistant prostate cancer.

With cancer genome data increasingly available, we first determined whether POU5F1B is clinically relevant in different cohorts.

Figure 1. Amplification of POU5F1B gene loci in various cohorts of prostate cancer. Red bar, amplifications. Green bar, mutation. Blue bar, deletion. The data were obtained from cBioportal TCGA public databases.
As shown in Figure 1, POU5F1B gene is frequently amplified in various cohorts of prostate cancer. In the Trenton/Cornell/Broad cohort (Beltran et al., 2016), POU5F1B is amplified in about 44% of castration resistant prostate cancer specimens. Similar frequency amplification of POU5F1B was found in another cohort of metastatic prostate cancer (FHCRC) (Kumar et al., 2016). In other cohorts, the amplifications of POU5F1B are in the range between none to up to 15%, dependent upon the stages of specimens. The data suggest that POU5F1B is frequently amplified in metastatic, castration resistance prostate cancer including neuroendocrine prostate cancer.

1.2. POU5F1B and MYC were co-amplified in same subset of prostate cancers

Next analyzed the patterns of POU5F1B amplifications in association with clinical parameters such as cancer type, tumor sites, and ploidy. As shown in Figure 2A, POU5F1B amplifications can be found both in castration resistance prostate cancer (CRPC) adenocarcinoma and CRPC neuroendocrine (NE) cancer in the Trenton/Cornell/Broad cohort (Beltran et al., 2016). The amplifications seem associated with those specimens with increased ploidy, suggesting that the gain of chromosome or chromosome segments likely lead to POU5F1B amplifications. Since MYC gene, like POU5F1B, is also located in the chromosome 8q24 region, we determined the pattern of MYC amplifications in the same cohort. As shown in the figure 2A, MYC is also frequently amplified in the similar subsets of tumor specimens from castration resistance prostate cancer.

Similar co-amplifications of POU5F1B and MYC were also found in the FHCRC cohort of metastatic prostate cancer (Kumar et al., 2016) (Figure 2B). The data suggest that the POU5F1B is frequently amplified with MYC gene loci in more than 40% metastatic or castration resistant prostate cancer.
1.3 POU5F1B expression in prostate carcinoma tissue is associated with androgen receptor (AR).

In the previous reports, we found that we detected Oct4 protein expression in prostate cancer cells as well as in tumor tissue specimens. With the availability of RNAseq data from the Trenton/Cornell/Broad cohort (Beltran et al., 2016), we first checked whether amplifications of POU5F1B can lead to increased...
POU5F1B expression. As shown in Figure 3A, amplifications of POU5F1B gene loci did not necessarily lead to its increased expression, as observed in metastatic prostate cancer. The data suggest that other factors are required for increased POU5F1B expression in metastatic prostate cancer.

(A)

(B)

Figure 3. POU5F1B expression in prostate carcinoma tissue (A) and its association with AR (B).
Next we determined whether POU5F1B is associated with androgen receptor (AR) since it plays an important role in castration resistance and metastatic progression of prostate cancer. As shown in Figure 3B, there is correlation between these two in terms of the expression at RNA levels, with Pearson and Spearman coefficients as 0.30 and 0.32, respectively. The functional significance of the correlation is not immediately known.

1.4 Alignment of PC3 POU5F1B ORF sequence with NCBI POU5F1B sequence (NM_001159542.1)

The enzyme digestion confirmed constructs were sent for sequencing. When we aligned the sequence of insert with POU5F1B (NM_001159542.1), two mismatches was noted, CAG$^{684}$ $\rightarrow$ CAA$^{684}$ (Glutamine/Q $\rightarrow$ Glutamine/Q) and G$^{712}$AG $\rightarrow$ C$^{712}$AG (Glutamic acid/E $\rightarrow$ Glutamine/Q). The first mismatch is a silent mutation, which will not cause amino acid change. The second mismatch will cause glutamic acid to glutamine change (Figure 4A). These two mismatches have been reported as SNP rs6998254 and SNP rs7002225 respectively (Figure 4B).

When compare PC3 POU5F1B amino acid sequence with NCBI POU5F1B amino acid sequence, only one amino acid changed, E$^{238}$ in NCBI POU5F1B but Q$^{238}$ in PC3 POU5F1B. When compare POU5F1B amino acid with POU5F1, we found fifteen amino acids are different, eight of them located at N domain, two located at POU specific domain, one in linker region, one in POU homeodomain and three in C domain (Figure 4C). Some amino acids change may contribute to protein structure, such as R33L, from basic, positive, polar to neutral hydrophobic; G97S, hydrophobic to polar; D108N, acid, negative to neutral; T118P, polar to hydrophobic; E135K, acidic, negative to basic, positive; T170I, polar to hydrophobic; T182K, neutral to basic, positive; Q259 polar was deleted; T351I, polar to hydrophobic (Table 1). Hans R. Schöler showed the linker between two POU domains of mouse OCT4 is exposed to the surface of the protein and it is very important for reprogramming activity of OCT4 and protein-protein interaction(Esch et al., 2013). To map the sequences or residues which are critical for the different function between POU5F1B and OCT4 would provide more clues for better understanding why it is POU5F1B not OCT4 is expressed in prostatic carcinoma and surrounding prostatic tissue.
(A)

Insert 661  TGCAAAACGAGAAAACCTCTCAGAGCCGCCAAGAAAGAAAGCGAACGATTAGCGACAAGCCGA  720
POU5F1B  661  TGCAAAACGAGAAAACCTCTCAGAGCCGCCAAGAAAGAAAGCGAACGATTAGCGACAAGCCGA  720

Insert 721  GTGAGAGGCAACCTGGAGAAAATGTTTTCTCCTGAGTGCCGGAAACCACATCTCGAGACGC  780
POU5F1B  721  GTGAGAGGCAACCTGGAGAAAATGTTTTCTCCTGAGTGCCGGAAACCACATCTCGAGACGC  780

Insert 781  CACATCGCCAGAGCTTGGGCTGAGAAAGATGTGGTCCGAGTGGTTCTCGTAACCGG  840
POU5F1B  781  CACATCGCCAGAGCTTGGGCTGAGAAAGATGTGGTCCGAGTGGTTCTCGTAACCGG  840

(B)

rs6988254 [Homo sapiens]
21.
TATGCAAAACGAGAAAACCTCTCAGAGCCGCCAAGAAAGAAAGCGAACGATTAGCGACAAGCCGA
Chromosome: 8:127416578
Gene: LOC101930333 (GeneView) POU5F1B (GeneView)
Functional Consequence: intron variant, synonymous codon
Validated: by 1000G, by 2hit2alleles, by cluster, by frequency
Global MAF: G=0.4793/1044

rs7002225 [Homo sapiens]
22.
CCAGAAACGAGAAAACCTCTCAGAGCCGCCAAGAAAGAAAGCGAACGATTAGCGACAAGCCGA
Chromosome: 8:127416578
Gene: LOC101930333 (GeneView) POU5F1B (GeneView)
Functional Consequence: intron variant, missense
Validated: by 1000G, by 2hit2alleles, by cluster, by frequency, by hapmap
Global MAF: C=0.4969/1080

(C)

POU5F1  1  MAGHLASDFASPPPPGGGDPGGPEPGWDRPTWLSFQPQPQPGPVGPGSEVWG  60
POU5F1B  1  MAGHLASDFASPPPPGGGDPGGPEPGWDRPTWLSFQPQPQPGPVGPGSEVWG  60
PC3 POU5F1B  1  MAGHLASDFASPPPPGGGDPGGPEPGWDRPTWLSFQPQPQPGPVGPGSEVWG  60

POU5F1  61  PCCPPFFYPECAGMAYCGPQGVGLVPQGGLETSQPEAGVGEVSNAGASPEPCTVPG  120
POU5F1B  61  PCCPPFFYPECAGMAYCGPQGVGLVPQGGLETSQPEAGVGEVSNAGASPEPCTVPG  120
PC3 POU5F1B  61  PCCPPFFYPECAGMAYCGPQGVGLVPQGGLETSQPEAGVGEVSNAGASPEPCTVPG  120

POU5F1  121  AVKLEKEQNLQNEKSDIKALQKELEQFAKLLQKRTLGYTQADVGLTLVLFKVF  180
POU5F1B  121  AVKLEKEQNLQNEKSDIKALQKELEQFAKLLQKRTLGYTQADVGLTLVLFKVF  180
PC3 POU5F1B  121  AVKLEKEQNLQNEKSDIKALQKELEQFAKLLQKRTLGYTQADVGLTLVLFKVF  180

POU5F1  181  QTITCRFALQLSFKNMCMLRLPQKVVEADVENLENLQCICKAETLQARKRKT2I  240
POU5F1B  181  QTITCRFALQLSFKNMCMLRLPQKVVEADVENLENLQCICKAETLQARKRKT2I  240
PC3 POU5F1B  181  QTITCRFALQLSFKNMCMLRLPQKVVEADVENLENLQCICKAETLQARKRKT2I  240

POU5F1  241  VRGNLEFLFCFKPTLQISHAQQQLGKEKDVVRWFCNRQQKQKRSDDQAQRDEFA  300
POU5F1B  241  VRGNLEFLFCFKPTLQISHAQQQLGKEKDVVRWFCNRQQKQKRSDDQAQRDEFA  300
PC3 POU5F1B  241  VRGNLEFLFCFKPTLQISHAQQQLGKEKDVVRWFCNRQQKQKRSDDQAQRDEFA  300

POU5F1  301  AGSSHFSGGGVPSSPLAGPHHFTPQYPSHPHTALYSVVFPEEFAFPFSVS1TTSGLPMHSN  360
POU5F1B  300  AGSSHFSGGGVPSSPLAGPHHFTPQYPSHPHTALYSVVFPEEFAFPFSVS1TTSGLPMHSN  359
PC3 POU5F1B  300  AGSSHFSGGGVPSSPLAGPHHFTPQYPSHPHTALYSVVFPEEFAFPFSVS1TTSGLPMHSN  359
Figure 4. Alignment of PC3 POU5F1B with NCBI POU5F1B (NM_001159542.1) and NCBI POU5F1 (NM_002701.5).

(A) Alignment of insert sequence cloned from PC3 cDNA with NCBI POU5F1B (NM_001159542.1) sequence. 2 mismatches were found as showed in red boxes.

(B) Two reported SNPs in POU5F1B gene. SNP rs6998254 showed CAG^{684}\rightarrow{CAA}^{684} variant allele, and SNP rs7002225 showed G^{712}\rightarrow{C}^{712} variant allele.

(C) Alignment of PC3 POU5F1B putative amino acid sequence with NCBI POU5F1B putative amino acid sequence and NCBI POU5F1 amino acid sequence. Yellow highlight indicate POU specific domain, green highlight indicate POU homeodomain, and blue highlight indicate linker between these two domains. Amino acids in red mean differences between them.

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<td>G</td>
<td>W</td>
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<tr>
<td>24</td>
<td>P</td>
<td>A</td>
<td>N domain</td>
</tr>
<tr>
<td>33</td>
<td>R (Basic, Positive, Polar)</td>
<td>L (Neutral, Hydrophobic)</td>
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<td>K (Basic, Positive)</td>
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<td>V</td>
<td>M</td>
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<tr>
<td>351</td>
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Table 1 Different amino acid between POU5F
1.5 POU5F1B changes cell morphology in DU145 cells

We noticed that DU145 cells with POU5F1B overexpression have different cell morphology when compared with DU145-pCDH myc vector cells. DU145 pCDH-myc vector showed tightly packed and formed clustered structures, typical of epithelial cells and suggestive of strong cell-cell adhesion (Figure 5 left panel). DU145 cells overexpressed POU5F1B showed reduced cell-cell adherens junctions and suggestive of increased cell motility (Figure 5 right panel). This observation are more significant under 200 magnification. This morphology change is consistent with previous data which showed POU5F1B can promote cell migration.

Figure 5. Comparison of cell morphology between DU145 pCDH myc vector and DU145 POU5F1B cells. The tight cell-cell adherent junctions were found in DU145 pCDH myc vector cells, but were reduced in DU145 POU5F1B cells.
1.6. POU5F1B can induce EMT in DU145 cells, but not in nontumorigenic RWPE1 cells

We observed the cells lost tight cell-cell adhesion when POU5F1B is overexpressed, which suggests the cells undergo EMT process. Down-regulation of E-Cadherin is one of the hallmarks of EMT. First, we assessed the cellular localization and expression of E-Cadherin in DU145 pCDH myc vector and DU145 POU5F1B cells. Immunocytochemistry staining showed typically E-Cadherin localization at cell-cell junction in pCDH myc vector cells, but such staining pattern was disapperaed in POU5F1B overexpressed cells (Figure 6 A). Furthermore, immunoblot confirmed the down-regulation of E-Cadherin after POU5F1B overexpression (Figure 6B). The reductidion of E-Cadherin was not found in immortalized normal prostate epithelial cell line RWPE-1 cells with POU5F1B overxpression (Figure 6B). This suggest that POU5F1B decrease E-Cadherin is cell-dependent. E-Cadherin is the main component of the cell-cell adhesion junctions, loss of its expression will increase cell mitility.

To investigate other EMT-related gene expression, we did EMT-related gene microarray. As showed in Figure 7, the epithelila marker, CDH1 gene, which encoding E-Cadherin decreased almost 10 fold. The mesenchymal marker, CDH2, N-Cadherin encoding gene and CLDN1, claudin-1 encoding gene are increased. Twist, SIP-1 and Vimentin are the mesenchymal marker that are decreased in DU145 POU5F1B cells compared with DU145 pCDH cells.
Figure 6. Effect of POU5F1B on E-Cadherin cellular localization and expression.

(A) Immunocytochemistry of E-Cadherin in DU145 pCDH and DU145 POU5F1B cells. POU5F1B causes loss of E-Cadherin localization at cell-cell junctions. DU145 pCDH myc vector and DU145 POU5F1B cells grown on coverslips were fixed, subjected to double staining with anti-E-Cadherin (red) and DAPI (blue) and examined by fluorescence microscopy.

(B) Immunoblot of E-Cadherin in RWPE-1 pCDH and POU5F1B, DU145 pCDH and POU5F1B cells. It was noticed that POU5F1B could decrease E-Cadherin expression in DU145 cells, but not in RWPE-1 cells.
**Figure 7. POU5F1B overexpression in DU145 regulates EMT-related genes expression.** Scatter plot comparing EMT-related gene expression profiles between DU145 pCDH and DU145 POU5F1B cells. Red lines indicate fourfold increase in DU145 POU5F1B compared with DU145 pCDH cells. Green lines indicate fourfold decrease in DU145 POU5F1B compared with DU145 pCDH cells. CT value was normalized to beta-actin CT.
1.8 Suppression of DU145 tumor growth, but not the formation of tumors, in vivo by POU5F1B

In previous reports, we found POU5F1B, when overexpressed in LNCaP-T (FGC) cells, stimulated tumor growth. Due to prostate cancer heterogeneity, we wished to extend the findings to other prostate cancer cell lines. Surprisingly we found POU5F1B actually suppressed the growth of tumors from PC3 or DU145 cells in the last report.

We repeated the studies. As shown in Figure 8, POU5F1B indeed suppressed the growth of DU145 tumors, but not the formation of tumors.

![Graph showing suppression of DU145 tumor growth by POU5F1B.](image)

**Figure 8.** Suppression of DU145 tumor growth by POU5F1B.

1.9 The effect of POU5F1B on cell invasion and anchorage independent growth
Next, we assessed the effect of POU5F1B on cell invasion. LNCaP, DU145, and PC3 cells expressing PCDH-vector or PCDH-POU5F1B were seeded into invasion chambers (BD, #353097). 16 hours later, cells were stained with crystal violet and the cells that did not migrate were removed using a cotton swab. The chambers were then imaged. POU5F1B expression significantly increases prostate cancer cell invasion (Figure 9 A). We then tested the effect of POU5F1B on anchorage independent growth. LNCaP cells expressing pCDH or pCDH-POU5F1B were seeded into soft agar and grown for 12 days. Cells were then stained with crystal violet and imaged. POU5F1B dramatically increased LNCaP anchorage independent growth (Figure 9 B).

Figure 9. The function of POU5F1B gene. (A) Prostate cancer cells were seeded into invasion chambers. Cells that express POU5F1B showed higher invasion than pCDH cells. (B) LNCaP cells were grown in soft agar. Cells that express POU5F1B formed more colonies.
**Task 2: Determine whether tumor Oct4 can be targeted to reduce prostate tumor formation, progression, and metastasis.**

We have introduced shRNA constructs into PC3MM and DU145 cells and screened many clones for the knockdown of POU5F1B. Interestingly we found that in addition to POU5F1B, POU5F1, the putative gene for Oct4, is also expressed in the prostate cancer while we were characterizing the clones. This raises two questions: First, PCa cells may express POU5F1, in addition to its pseudogene. Second, knockdown of POU5F1B, may have stimulated the expression of POU5F1. Since the shRNAs cannot differentiate POU5F1B from POU5F1, this complexity confound the interpretation of the data.

Currently we are using CRISPR approaches to delete POU5F1B, to determine whether its deletion can lead to reduced tumor formation, and metastasis.

**Task 3: Elucidate the mechanism involved for tumor Oct4 in promoting prostate carcinogenesis.**

In the last report, we found that POU5F1B induced many stemness gene signatures in DU145 and PC-3 cells. The changes suggest that POU5F1B may have reprogrammed DU145 and PC-3 stem-like phenotypes: The ability to form tumors, but not necessarily to stimulate tumor growth.

One of characteristics for cancer stem cells is the resistance toward therapeutics. The drug resistance is partially mediated by the slow cycling cells and/or ATP-binding cassette (ABC) efflux transporters. We next studied whether POU5F1B expression is related to ABC transporters. As shown in Figure 10, POU5F1B is associated with ABCC1 and ABCC4. ABCC4 is found upregulated in prostate cancer. Currently we are investigating the significance of the association in terms of drug resistance.
Figure 10. Association of POUSF1B with ABC efflux transporters ABCC1 (A) and ABCC4 (B).
KEY RESEARCH ACCOMPLISHMENT and REPORTABLE OUTCOMES

Presentations and abstracts:


Research articles published:

The manuscript requires further revisions.
Conclusions and significance (So what?):

Our studies found that:

1. POU5F1B, the pseudogen of POU5F1 localized in prostate cancer susceptibility loci 8q24, is frequently amplified in castration resistant metastatic prostate cancer.
2. POU5F1B expression is associated with AR expression level expressed in prostate cancer.
3. The expression of POU5F1B was found increased in prostate tumors when compared to adjacent normal tissues or normal prostate tissues and it was markedly increased in metastatic prostate carcinoma, compared to primary tumors or normal prostate tissues.
4. POU5F1B was cloned from prostate cancer cells. Sequencing found two SNPs when compared to the reference sequence, but the SNPs did not cause amino acid sequence changes.
5. Prostate cancer cells with increased POU5F1B expression had increased migratory phenotype and decreased E-cadherin expression at protein or mRNA level.
6. POU5F1B effect on tumor growth can be context dependent. In some cells (LNCaP), POU5F1B promoted tumor growth but in other cells (PC-3 and DU145), POU5F1B suppressed tumor growth, but not the formation of tumors. The divergence may be due to their differential dependence on AR signaling.
7. POU5F1B expression led to increased “stemness” signature and associated expression of ABC transporters ABCC1 and ABCC4.

Our studies suggest complex roles of POU5F1B in prostate carcinogenesis. More studies are needed to determine when and how POU5F1B can be targeted to reduce prostate cancer metastasis.
APPENDICES

N/A

SUPPORTING DATA

Embedded in the reporting body

REFERENCES

