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# Epigenetic regulation of CpG promoter methylation in invasive prostate cancer cells

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## Abstract

**Background:** Recently, much attention has been focused on gaining a better understanding of the different populations of cells within a tumor and their contribution to cancer progression. One of the most commonly used methods to isolate a more aggressive sub-population of cells utilizes cell sorting based on expression of certain cell adhesion molecules. A recently established method we developed is to isolate these more aggressive cells based on their properties of increased invasive ability. These more invasive cells have been previously characterized as tumor initiating cells (TICs) that have a stem-like genomic signature and express a number of stem cell genes including *Oct3/4* and *Nanog* and are more tumorigenic compared to their 'non-invasive' counterpart. They also have a profile reminiscent of cells undergoing a classic pattern of epithelial to mesenchymal transition or EMT. Using this model of invasion, we sought to investigate which genes are under epigenetic control in this rare population of cells. Epigenetic modifications, specifically DNA methylation, are key events regulating the process of normal human development. To determine the specific methylation pattern in these invasive prostate cells, and if any developmental genes were being differentially regulated, we analyzed differences in global CpG promoter methylation.

**Results:** Differentially methylated genes were determined and select genes were chosen for additional analyses. The non-receptor tyrosine kinase BMX and transcription factor SOX1 were found to play a significant role in invasion. Ingenuity pathway analysis revealed the methylated gene list frequently displayed genes from the IL-6/STAT3 pathway. Cells which have decreased levels of the targets BMX and SOX1 also display loss of STAT3 activity. Finally, using OncoPrint, it was determined that more aggressive metastatic prostate cancers in humans also have higher levels of both *Stat3* and *Sox1*.

**Conclusions:** Using this method we can begin to understand which genes are epigenetically regulated in the invasive population compared to the bulk tumor cells. These aggressive sub-populations of cells may be linked to the cancer stem cell hypothesis, making their patterns of epigenetic regulation very attractive for biomarker analysis.

## Background

Cancer is defined as uncontrolled cell growth resulting from genetic mutations or exposure to environmental carcinogens that alter normal regulation. If the cancer is aggressive in nature, invasion of local tissues near the primary tumor site as well as distant metastasis can occur. Current treatment regimens almost always involve a form of surgery to remove the primary tumor and

systemic chemotherapy with localized radiation. However, aggressive cells can remain in the body and evade treatment with these conventional therapies. Additionally, it has been well documented that only a small fraction of epithelial tumor cells have the ability to form colonies *in vitro* or to initiate a new tumor upon injection into a host *in vivo* [1-6]. In order to study the epigenetic regulation of these aggressive cells, we chose to study an invasive population of prostate cancer cells. We and others have developed a novel method for the isolation of these cells from bulk tumor cell populations using Matrigel [7,8]. These cells have a stem-like phenotype [7] and exist within both established cell lines (LNCaP and

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DU145) and in cells isolated from primary prostate cancer tissue (PCSC1-3). The invasive cells have been characterized as undergoing an epithelial to mesenchymal transition (EMT) during the process of invasion, and are also highly tumorigenic when injected into mice [7]. They demonstrate increases in the stem cell regulators *CD44*, *CD133*, *Bmi1*, *Nanog*, and *Sonic hedgehog (Shh)*, as well as increased expression in mesenchymal markers such as *Vimentin* and *Tgfb $\beta$ -1*, and a decrease in the epithelial marker *E-cadherin (CDH1)*. Over the last few years this hypothesis of EMT and cancer progression has been widely supported in models of not only prostate cancer, but also within the breast, colon, lung and pancreas [9-16]. The idea that the same cells which are undergoing the EMT may also be a population of cells called cancer stem cells or CSCs is a relatively new concept.

It is becoming more evident that CSCs are not governed by the same type of genetic regulation as normal stem cells, and arguably in solid tumors may be an epithelial cell that has up-regulated pathways that have been previously observed in true stem cells. In order to determine the epigenetic profile of these invasive prostate cancer cells, we isolated DNA and performed a very sensitive MeDIP (methylated DNA immunoprecipitation) assay coupled with Agilent's 244 K Human Promoter Tiling Arrays. This allowed for an in-depth analysis of the methylation status within promoter elements, upstream as well as down, in these cells. Differences between the invaded (more stem-like) and non-invaded cells, as well as the bulk tumor cell line (parental cells) were compared. In our analysis, the LNCaP and DU145 cell lines were used, as well as confirmation analysis in two primary prostate cancer cell lines (PCSC1 and PCSC2).

A unique set of genes were found to be expressed in the invasive cells, yet methylated in the non-invasive cells and parental cell lines. This included genes involved in embryonic and tissue/organ development, and specifically in neurogenesis including bone marrow X kinase (*Bmx*), Iroquois homeobox 3 (*Irx3*), *Sine oculis* homeobox homolog 1 (*Six1*) and Sex determining region-Y-box 1 (*Sox1*). Using the available online expression databases in Oncomine, it was determined that *Sox1* plays a significant role in prostate cancer progression and metastasis. Furthermore, Ingenuity pathway analysis determined that the set of differentially methylated genes are involved in cellular functions such as cell-to-cell interaction and cell morphology, as well as development of the hematological system and cancer. The most intriguing data identified many of the methylated targets as members of the IL-6/STAT3 signaling pathway. Further investigation demonstrated that *Stat3* was increased in these invasive cells, and cells infected

with an shRNA against either BMX or SOX1 resulted in decreased levels of activated STAT3. However, only the differentially methylated *Sox1* directly interacts with STAT3. Thus, in our model SOX1 plays a critical role in regulating invasive prostate cancer cells. These aggressive sub-populations of cells may be linked to the cancer stem cell hypothesis, making their patterns of epigenetic regulation very attractive for biomarker analysis.

## Materials and methods

### Cell Lines and Reagents

LNCaP and DU145 human prostate cancer cell lines were obtained from ATCC and cultured accordingly (Manassas, VA). Primary human prostate cancer cells (PCSC1-2) [7] were acquired from Celprogen (San Pedro, CA) and maintained as recommended using specific coated culture plates and defined media. Human bone marrow derived mesenchymal stem cells (hMSCs) were obtained from Lonza (Gaithersburg, MD) and maintained using their recommended conditions. The cultures were maintained in 5% CO<sub>2</sub> air at 37°C. Human serum was obtained from Gemini Bioproducts (West Sacramento, CA). The following inhibitors were also used: Anti-human IL-6 antibody (R&D Systems, Minneapolis, MN), PI3K inhibitor LY294002 (Cell Signaling, Danvers, MA), Tec Kinase inhibitor LFM-A13 (Tocris, Ellisville, MO), MEK inhibitor PD98059 (Gibco, Carlsbad, CA), JAK inhibitor AG490 (Gibco), and STAT3 inhibitor Stattic (Sigma Aldrich, St. Louis, MO).

### Matrigel Invasion Assay

Matrigel-coated 24-well inserts (8  $\mu$ M pore size) and non-coated control inserts purchased from BD Biosciences (Palo Alto, CA) were used according to manufacturer's instructions. A range of 20,000-100,000 cells were seeded for the invasion (higher for less invasive LNCaP cells). Cells were seeded in serum-free RPMI and migrated toward media specific for stem cells (SCM) containing DMEM/F12 with human supplementation of 10 ng/mL bFGF, 20 ng/mL EGF and 5  $\mu$ g/mL insulin along with 0.4% BSA (each from Sigma, St. Louis, MO). Routine invasion assays were performed for 24 hours and then stained with the Diffi-Quick Staining kit (Dade Behring, Deerfield, IL). Three to five microscopic fields (20 $\times$ ) were photographed and counted for each sample. Percent invasion was calculated as average number of cells/field (Matrigel) divided by average number of cells/field (control insert). Values were averaged from 2-5 independent experiments. For the isolation of cells from top 'non-invading' and bottom 'invading' cells, parallel invasion chambers were setup. For non-invading cells, the bottom of the membrane was scrubbed with a cotton swab and cells on top were harvested using 500  $\mu$ L of

Accutase (eBioscience, San Diego, CA) incubated at 37°C for 5 minutes. To obtain the invading cells, the top of the membrane was scrubbed with a cotton swab and the chambers were placed into another 24-well plate containing 500 µL of Accutase incubated at 37°C for 5 minutes.

#### MeDIP Arrays

Matrigel invasion assays were carried out as previously described. For the isolation of DNA from both non-invasive and invasive cells the DNeasy kit from Qiagen (Valencia, CA) was used and parallel invasion chambers were setup. For non-invading cells, the bottom of the membrane was scrubbed with a cotton swab and cells on top were trypsinized and harvested in 200 µL of PBS followed by the direct addition of lysis buffer or stored at -80°C. For bottom 'invading cells' the top of the membrane was scrubbed with a cotton swab and the membrane was removed and placed directly into lysis buffer or stored at -80°C until needed. A modified version of Agilent's (Santa Clara, CA) protocol for Mammalian ChIP on ChIP was used to capture methylated DNA with immunoprecipitation (MeDIP). DNA was quantified and 2 µg (or total yield if less) was digested with MseI overnight at 37°C. Linkers (JW102-5'-GCGGTGACCCGG-GAGATCTGAATTC-3' and JW103-5'-TAGAATTCAGATC-3') were ligated at 16°C using T4 ligase overnight and the next day used as input for the MethylCollector (Active Motif, Carlsbad, CA) assay to isolate methylated and non-methylated fractions of DNA. The kit utilizes histidine-tagged MeBP2 (methyl-binding protein 2) and magnetic bead separation. The isolated methylated and non-methylated DNA from each sample (as little as 5 ng) was then amplified in a series of PCR reactions following the mammalian ChIP on ChIP protocol. The input DNA was labeled with Cy3-dUTP and the methylated DNA with Cy5-dUTP and then immediately applied to Agilent's 2 × 244 K Human Promoter Tiling Arrays for 40 hours at 65°C. The arrays were scanned using a Gene Pix 4000B scanner (Molecular Devices, Sunnyvale, CA) with GenePix Pro software version 6.1 and extracted using Agilent's Feature Extraction software version 9.5.3.1. The data was annotated using Agilent's ChIP Analytics software version 4.0. Normalization was carried out using a blank subtraction model and statistical stringency (p-value) between 0.01-0.05 was applied using a Whitehead Per-Array Neighbourhood Analysis. This analysis allowed for the determination of differentially methylated genes between non-invasive and invasive cells. Ingenuity core analysis was carried out to determine which pathways are of functional significance based on the gene lists identified <http://www.ingenuity.com/>. Genomatix software was used to determine transcription factor binding sites (matrix). A perfect match to the matrix gets a score

of 1.00 (each sequence position corresponds to the highest conserved nucleotide at that position in the matrix), a "good" match to the matrix usually has a similarity of >0.80.

Mismatches in highly conserved positions of the matrix decrease the matrix similarity more than mismatches in less conserved regions.

#### Methylation Specific polymerase chain reaction (MSP-PCR)

A total of 1 µg of DNA extracted from total (parental) DU145 and LNCaP cells was bisulfite modified using the EpiTect Bisulfite kit from Qiagen. PCR was performed using Platinum Taq Polymerase (Invitrogen) and 200 ng of either genomic or bisulfite treated DNA. The PCR method utilized was 94°C for 2 minutes, then 35 cycles (94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute) with a final extension of 10 minutes at 72°C. The unmethylated primers however were run with an annealing temperature of 42°C since their melting temperature values were drastically different from their methylated counter part. A portion of the PCR product was run on a 1% agarose gel containing ethidium bromide.

#### Methylated primers

hBMX-Forward 5'- TGGTGAGACATCATGTGTTC-CATT-3';

hBMX-Reverse 5'- ATGCCCTCAGTTGAGAAC-CACTGT-3';

hSOX1-Forward 5'-ATGATCAGCATGTACTTGC CCGC-3';

hSOX1- Reverse 5'-TCCGCTTCCTCCGTAGGTGA-TAAA-3'

#### Unmethylated primers

hBMX-Forward 5'- TGGTGAGATATTATGTGTTT-TATT-3';

hBMX-Reverse 5'- ATGTTTTTAGTTGAGAAAT-TATTGT-3';

hSOX1-Forward 5'-ATGATTAGTATGTATTTG TTTGT-3';

hSOX1-Reverse 5'-TTTGTTTTTTTTGTAGGTGA-TAAA-3'

#### Quantitative real time polymerase chain reaction (QRT-PCR)

Total RNA was isolated using TRIzol (Invitrogen Corporation, Carlsbad, CA). RNA from 'top' cells was isolated using a cell pellet acquired from trypsinizing cells from one membrane after bottom cells were removed with a cotton swab. Conversely, RNA from the bottom cells was isolated by combining three membranes where the top cells were removed using a cotton swab. The membranes were pooled and placed in TRIzol for 10 minutes at room

temperature, and the conventional procedure for isolation of RNA was then followed. To increase the yield of RNA, 5  $\mu$ g of linear acrylamide (Ambion, Austin, TX) was added prior to precipitation of RNA with isopropanol. Additionally to increase overall yield, 100 ng of RNA was amplified using the MessageAmp aRNA Amplification Kit (Ambion, Austin, TX). cDNA was prepared using the SuperScript<sup>III</sup> First-Strand Synthesis System (Invitrogen Corporation, Carlsbad, CA). Quantitative real time polymerase chain reaction (qRT-PCR) analysis was performed using a StepOne Real-time PCR machine (Applied Biosystems, Foster City, CA) with TaqMan Gene Expression Assay reagents and probes (Applied Biosystems). A total of 4  $\mu$ L of cDNA was used in a 20  $\mu$ L reaction resulting in a 1:5 dilution. The following FAM labeled human probes were used: BMX (Hs00174139\_m1), IRX3 (Hs00273561\_s1), SOX1 (Hs01023894\_m1), MCL-1 (Hs00172036\_m1), MYC (Hs00153408\_m1), STAT3 (Hs01047580\_m1), SURVIVIN (Hs00977611\_g1) and 18S rRNA (Hs99999901\_s1). Relative fold induction of mRNA was compared between non-invasive and invasive cells using the Delta-Delta CT method of quantitation, and 18S rRNA was used as a loading control.

#### shRNA of *Bmx* and *Sox1*

The Trans-Lentiviral pTRIPZ system from Open Biosystems (Huntsville, AL) was used to introduce shRNA against BMX (Clone ID: V2THS\_150067) and SOX1 (V2THS\_197330) along with a non-silencing control vector. The vectors were transfected into HEK293T cells which were seeded in serum-free media at 60% confluency in 10  $\text{cm}^2$  dishes using the Arrest-In reagent provided in the kit. The cells were transfected for 6 hours and then replaced with complete media. After 24 and 48 hours lentiviral supernatants were harvested, spun at 1500 rpms, and filtered using a 0.45  $\mu$ M filter to clear them. The viral titer was mixed 1:1 with DU145 media and placed on sub-confluent DU145 cells for 4-6 hours and changed to complete media. The next day media containing 1  $\mu$ g/mL of doxycycline (Sigma) was added to ensure efficient transfection/infection has occurred. Efficient transfection was observed using a TET inducible TurboRFP (red fluorescent protein) upstream of the shRNA that appears red upon successful infection. The cells were selected for 2 weeks in 1  $\mu$ g/mL of puromycin (Sigma). Single cell clones were then generated and lowered expression was confirmed using Western blotting.

#### Western Blotting and sub-cellular fractions

Total cell lysates were prepared using RIPA buffer (Sigma) and sub-cellular fractions using the NE-PER Nuclear Protein Extraction Kit (Thermo Scientific, Rockford, IL). Samples were loaded onto a 4-20% Tris-

glycine gel and transferred to a PVDF membrane. The membranes were blocked at room temperature for 45 minutes in 5% non-fat milk in TBS-Tween (0.05%). Primary antibodies were as follows: BMX (Abcam-59360), pBMX (Cell Signaling-3211S), STAT3 (Santa Cruz-SC482), pSTAT3/Tyr705 (Cell Signaling-9131S), SOX1 (Cell Signaling 4194S) and Actin (Abcam-8227-50) and incubated overnight at 4°C. The membrane was washed 3 $\times$  for 10 minutes each using TBS-T (0.1%). Secondary antibody was applied for 1 hour at room temperature (infrared goat-anti rabbit or mouse in the 800 channel) and washed. The membrane was developed using the Odyssey from Licor (Lincoln, NE). Protein loading was normalized using actin as a control. Densitometry analysis was performed using ImageJ (NIH, Bethesda, MD).

#### Proliferation Assays

Cells were seeded overnight in a 96 well plate in 100  $\mu$ L of regular media at a density of 2000 cells per well. Cell proliferation was measured using the CellTiter-Glo assay from Promega on Day 1, 3, 5 and 7 using 100  $\mu$ L of reagent and an incubation time of 20 minutes. The relative luciferase units (RLU) were quantified using a Tecan Infinite 200 plate reader.

#### Prostatosphere Formation Assays

LNCaP and DU145 cells were seeded at 1000 cells per mL in replacement media SCM supplemented with KO Serum Replacement (Invitrogen) for LNCaP or B27 (Invitrogen) for DU145 cells in non-adherent 6 well plates coated with Hydrogel (Corning Life Sciences, Chemsford, MA). The prostatospheres were generated for 5-7 days and then quantified or RNA extracted.

#### Immunofluorescence

Staining of invasive or non-invasive cells was performed directly on the Matrigel membrane. Duplicate invasion chambers were used for each antibody; one each for staining invasive cells or non-invasive cells. Cells not being stained were removed from each insert, and cells of interest were fixed to the membrane in 4% para-formaldehyde for 15 minutes at 25°C and permeabilized with 0.5% saponin in PBS for 15 minutes at 25°C followed by a series of washes with PBS. Non-specific antibody binding sites were blocked for 15 minutes with 1% BSA in PBS containing 0.1% Tween-20 (PBS-T). Cells were incubated with either anti-pBMX antibody in PBS-T, SOX1 (Cell Signaling, Danvers, MA), or pSTAT3 (Millipore/Upstate Technologies, Billerica, MA (4°C, overnight). Following 3 $\times$  PBS-T washes, infrared goat anti-rabbit Alexa 488 (Molecular Probes, Carlsbad, CA) was added for 1 hour at 25°C using a 1:500 dilution in PBS-T and again washed, then air-dried. Membranes were mounted on glass slides with

Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Cells were visualized with a Zeiss-510 L5 confocal microscope where separate images were obtained for Alexa-488 and DAPI fluorescence, as well as overlays and 10 slice Z-stacks. Images were analyzed using the Zeiss LSM5 Image Browser (version 3.2.0.115) and further prepared in Adobe Photoshop CS. "Non-invasive" cells were stained on the topside of the membrane, while "invasive cells" were stained on the underside of the membrane. Controls using the secondary antibody and no primary antibody indicated that little, if any, fluorescence was contributed by non-specific binding of this antibody (data not shown).

### Immunoprecipitation

Protein was extracted using RIPA buffer (Sigma) and lysates were incubated with either SOX1, STAT3 or BMX (same antibodies used in Western Blotting) overnight at 4°C with rotation. The next day Protein A sepharose beads were added to the lysate and incubated for 3 hours with rotation at 4°C. The lysate was then spun at 13,000 rpms in a benchtop centrifuge and washed 3× with RIPA buffer. Before loading on a 4-20% Tris-Glycine SDS-Page gel (Invitrogen) 2× loading buffer was added and upon completion the gel was transferred to a PVDF membrane. The membrane was blocked for 45 minutes using 5% non-fat milk in TBS-T (0.1%). The membrane was then incubated overnight at 4°C using either primary antibodies SOX1 or STAT3 diluted in blocking buffer to confirm a direction interaction. The membrane was washed 3× for 10 minutes each using TBS-T (0.1%). Secondary antibody was applied for 1 hour at room temperature (infrared goat-anti-rabbit in the 800 channel) and washed. The membrane was developed using the Odyssey from Licor. Protein loading was normalized using actin from previous Westerns.

### EMSA

The Licor EMSA buffer kit was used according to the manufacturer's instructions. Infrared (IR) and unlabeled STAT3 oligos were ordered from IDT and used at 0.625 fmoles/reaction.

Wildtype probes (WT): (800-IR channel and unlabeled)

F- 5'-GATCCTTCTGGGAATTCCTAGATC-3';

R- 5'-GATCTAGGAATCCCAGAAGGATC-3'

Mutant probes (MU): (700-IR channel)

F- 5'-GATCCTTCTGGGCCGTCCTAGATC-3';

R- 5'-GATCTAGGACGGCCCAGAAGGATC-3';

Mutant oligos and unlabeled wildtype oligos were used at 200-fold molar excess. A total of 20 µg of nuclear protein extract was incubated with 1× binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5), Poly (dl-dC) 1 µg/µL (in 10 mM Tris, 1 mM EDTA, pH 7.5), 25 mM DTT/2.5% Tween-20, 1% NP-40, 100 mM

MgCl<sub>2</sub>, and 50% glycerol for 20 minutes at room temperature shielded from light. For supershift experiments, extracts were pre-incubated with 5 µg of STAT3 antibody at 4°C for 30 minutes. DNA/protein complexes were visualized on a native 6% Tris-Borate-EDTA polyacrylamide gel. Gels were immediately removed from cassettes and scanned using the Odyssey in both the 700 and 800 channels.

### Meta-analysis on patient databases

Oncomine and Gene Expression Omnibus (GEO) databases were queried to identify associations between genes. GEO database is available at <http://www.pubmed.org> (GEO profiles) and provides raw expression data from several gene expression arrays. Oncomine 4.2 database analysis tool is available with a subscription at <http://www.oncomine.org>. Selected data was compared for gene expression levels in prostate primary tumor samples as well as their respective metastatic specimens. Data have been selected from [17] because this study was an integrated molecular profiling of gene expression in prostate cancer samples. In this work, a significant concordance between expression of *Sox1* and *Stat3* mRNA was found to correlate with the aggressiveness of the sample.

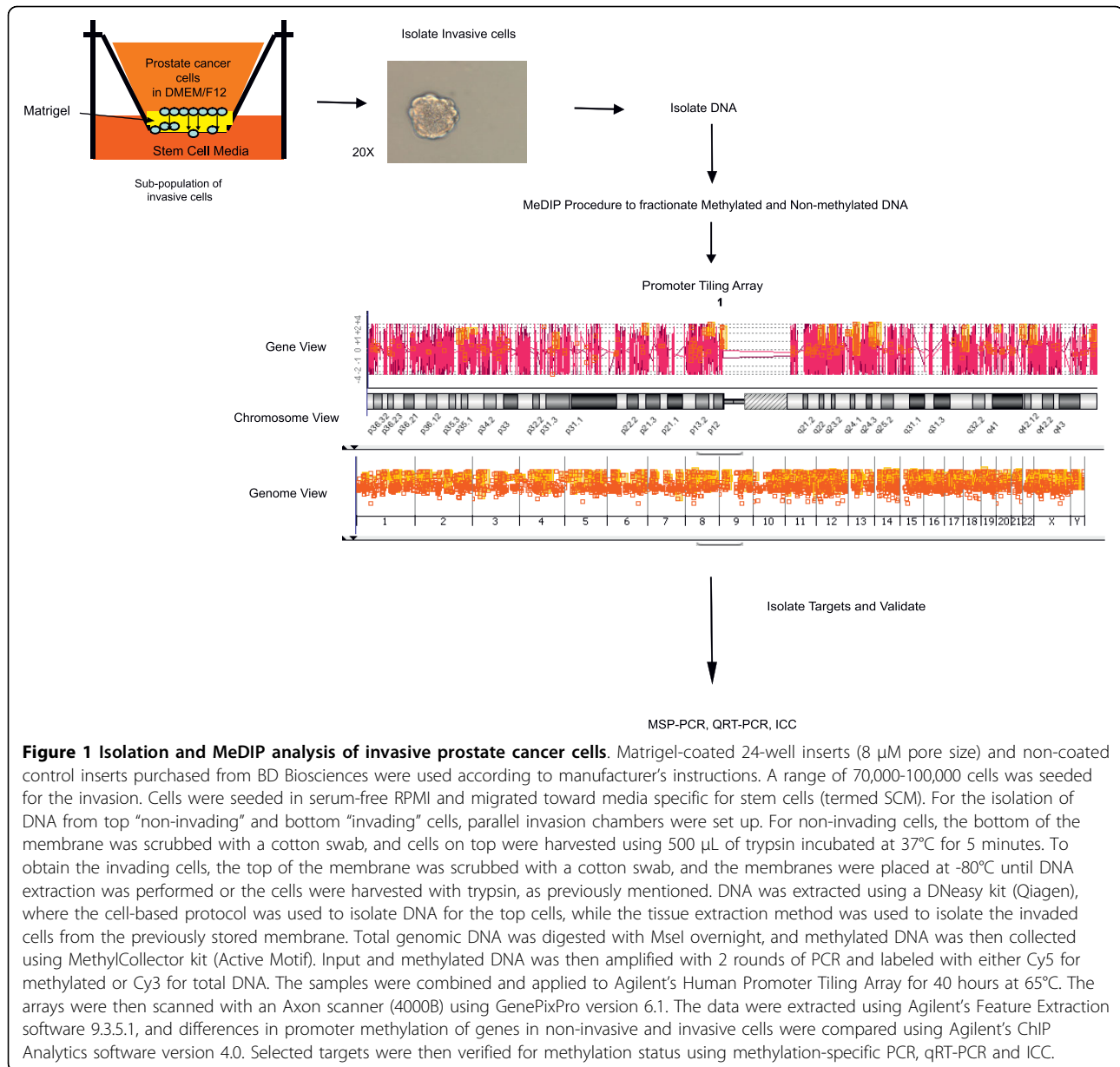
### Statistical Analysis

All statistical calculations were performed using GraphPad Prism Version 5. Comparisons between groups were carried out using either a Student's pair-wise t-test, or a One or Two-way ANOVA with a Bonferroni post-test wherever each test was applicable. Error bars represent the Standard Error of the Mean (SEM) and each experiment has been completed at least twice with samples in triplicate.

### Results

#### Identification of differentially methylated genes in invasive sub-populations of cells

Individual promoter tiling arrays were performed to analyze global CpG promoter methylation for both non-invasive and invasive cell isolates from both LNCaP and DU145 (Figure 1). The cells were allowed to invade the Matrigel toward a highly defined media called stem cell media (SCM) [18]. It was then determined which genes were methylated in the non-invasive cells and not in the invasive fraction of cells. This analysis determined that 869 probes were differentially methylated in the non-invasive LNCaP fraction compared with the invasive and 1015 for DU145 (Additional File 1, Tables S1A and S1B). A very small subset of 44 overlapping genes was methylated in the non-invasive cells and not in the invasive population from both of the prostate cancer lines analyzed. These included genes involved in development



such as *Irx3*, *Six1* and *Sox1*, as well as a type-III 5' deiodinase (*Dio3*), and an embryonic version of myosin (*Myh3*) (Table 1). Using the OncoPrint database we investigated changes in expression patterns for these methylated targets, and we found a significant association between progression of prostate cancer and metastasis with expression of a number of genes including G protein, beta-1 subunit (*Gnb1*), retinoblastoma binding protein 8 (*rbbp8*), secretogranin III (*Scg3*) and *Sox1* (Figure 2). Albeit a number of these proteins have been shown to play a role in cancer, we chose to investigate the role of *Sox1* in our model since it is very homologous to the induced pluripotent stem cell (iPS) regulator *Sox2*, and has been shown to play a role in progression

of lung and nasopharyngeal cancer [19,20]. We also chose to investigate bone marrow tyrosine kinase gene in chromosome X protein (*Bmx*) since it has been shown to regulate hematopoiesis [21] and play a role in the regulation of prostate cancer [22]. However, from our OncoPrint analysis *Bmx* was not shown to significantly affect prostate cancer metastasis (Figure 2).

#### Verification of methylation array data

To verify the results from our methylation specific promoter tiling arrays, we performed methylation specific PCR (MS-PCR) where primers were designed around the probe sequences identified from the arrays. Both *Bmx* and *Sox1* were found to be methylated in the

**Table 1 Summary of genes methylated within the non-invasive LNCaP and DU145 cell lines**

Gene	Agilent ID	Location
ABCF3	A_17_P02824570	chr3:185384633-185384688
ACRC	A_17_P11784482	chrX:70712439-70712498
ARHGEF9	A_17_P11762981	chrX:62889218-62889272
AVPR1A	A_17_P08402446	chr12:61828587-61828646
BCL2A1	A_17_P09781583	chr15:78049283-78049342
BMX	A_17_P11607973	chrX:15428990-15429037
CAPN6	A_17_P11887436	chrX:110391469-110391527
CNIH3	A_17_P00830574	chr1:222866130-222866189
DIO3	A_17_P09520078	chr14:101092190-101092234
DLGAP1	A_17_P10514555	chr18:3832861-3832920
DONSON	A_17_P11359769	chr21:33880442-33880501
EXOC3L2	A_17_P17147769	chr19:50426839-50426891
GNB1	A_17_P00002239	chr1:1744644-1744703
GPR103	A_17_P03395862	chr4:122518949-122518996
GPR81	A_17_P08659974	chr12:121778515-121778572
GUCY2F	A_17_P11879791	chrX:108613896-108613952
HRK	A_17_P08637968	chr12:115801241-115801285
hsa-mir-346	A_17_P07370831	chr10:88012234-88012278
hsa-mir-507	A_17_P12010468	chrX:146120196-146120255
hsa-mir-542	A_17_P11969030	chrX:133503290-133503349
HTR2A	A_17_P08830482	chr13:46365855-46365914
IRX3	A_17_P10024825	chr16:52875157-52875205
LOC339344	A_17_P10976156	chr19:51085104-51085163
MAGEA8	A_17_P12020625	chrX:148768376-148768435
MYH3	A_17_P10227610	chr17:10497672-10497716
NCAM1	A_17_P08036168	chr11:112332051-112332110
NR2E1	A_17_P04970834	chr6:108588654-108588713
NUP85	A_17_P10465401	chr17:70708211-70708270
PARVB	A_17_P11521863	chr22:42723842-42723888
RAMP2	A_17_P10328303	chr17:38161563-38161609
RBBP8	A_17_P10568640	chr18:18762400-18762459
RPS4X	A_17_P11786429	chrX:71410283-71410342
SCG3	A_17_P09654436	chr15:49755368-49755423
SCGB1A1	A_17_P07831562	chr11:61937609-61937653
SIGLEC1	A_17_P11026333	chr20:3615793-3615841
SIX1	A_17_P16707873	chr14:60183180-60183224
SLC5A1	A_17_P11476138	chr22:30766279-30766324
SOX1	A_17_P09141969	chr13:111764575-111764619
SPATA5L1	A_17_P09626282	chr15:43477779-43477838
TCF12	A_17_P09676944	chr15:54992977-54993036
TMEM46	A_17_P08732705	chr13:25520762-25520811
TNRC6A	A_17_P09967235	chr16:24643725-24643784
VSIG1	A_17_P11874787	chrX:107171900-107171959
ZBTB16	A_17_P08041891	chr11:113430193-113430252

Genes methylated within the non-invasive population of LNCaP and DU145 cell lines. Once extracted from the Chip Analytics software, a list of genes which were methylated in the non-invasive cells, yet not in the invasive population was generated. The Agilent ID corresponds to the probe location on the chromosome.

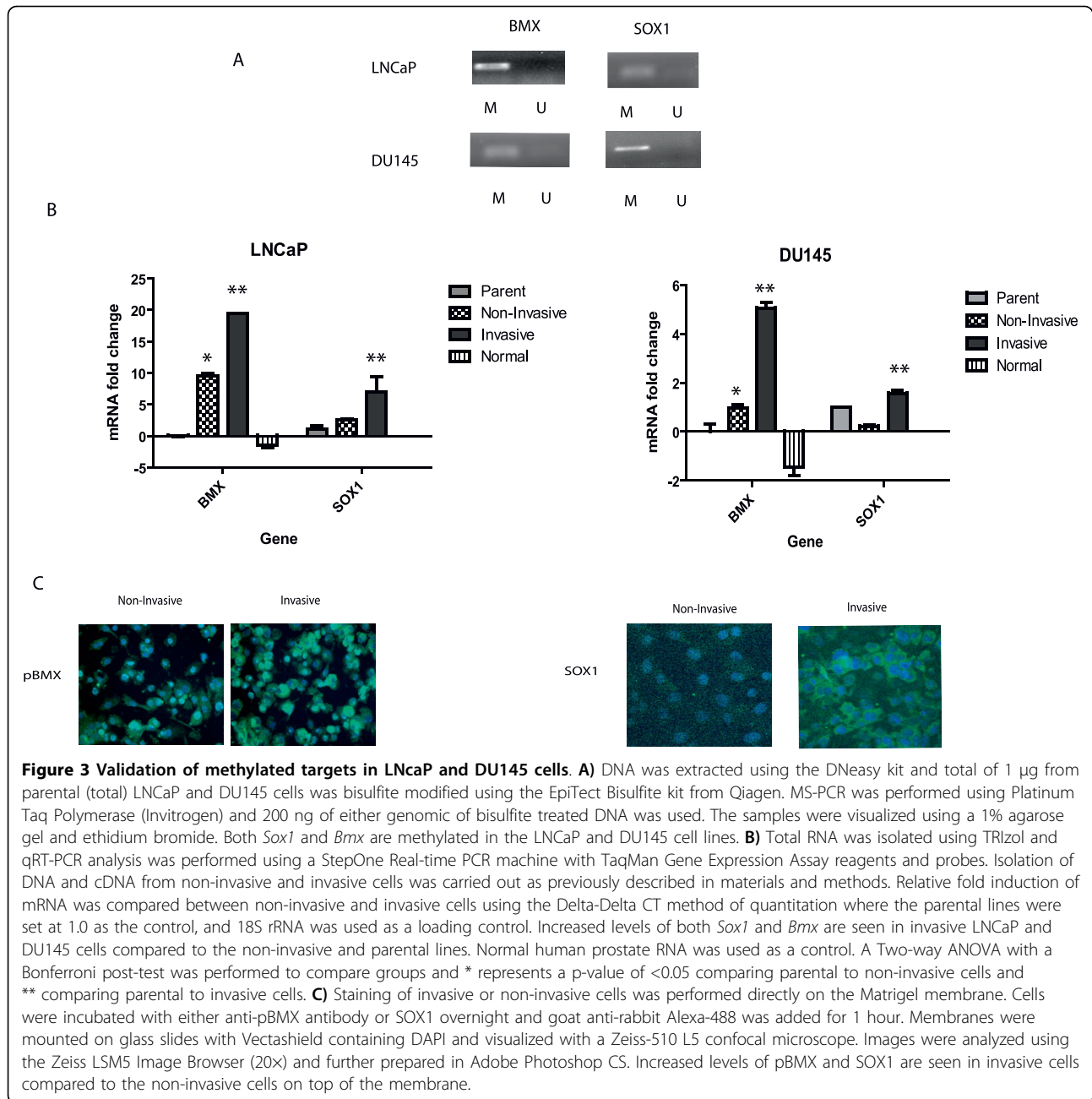
parental (total) LNCaP and DU145 cell lines (Figure 3A), representing the non-invasive phenotype. To determine if this pattern of methylation correlated with the level of gene expression, real time quantitative PCR (qRT-PCR) was performed. Significant differences in the expression of *Bmx* and *Sox1* were seen when comparing the expression in non-invasive and invasive cell populations in both LNCaP and DU145 cell lines (Figure 3B) (Two-way ANOVA; \*compares non-invasive to parental and \*\* compares invasive to parental,  $p < 0.05$ ). To further validate the results, immunocytochemistry (ICC) was performed to analyze differences in protein expression between non-invasive and invasive cells. There is significantly higher expression of activated BMX and SOX1 in the invasive versus non-invasive cells (Figure 3C). Therefore, we validated the methylation and resultant decreased expression of BMX and SOX1 in the non-invasive cells.

#### Functional role of *Bmx* and *Sox1* during invasion

To further determine the role of *Bmx* and *Sox1* during the process of invasion we performed the invasion assay with DU145 cells stably infected with shRNAs directed against *Sox1* or *Bmx* (Figure 4). A significant decrease in expression of SOX1 and BMX following induction with 1  $\mu\text{g}/\text{mL}$  of doxycycline (Dox) for 24 hours was first verified using western blotting. Upon induction with Dox, the shRNA is turned on and a downstream red fluorescent protein (RFP) demonstrates efficiency of this induction (Figure 4A). Densitometry analysis was performed to compare expression of individual clones with the NS cells, and no significant differences in protein expression were seen using the non-silencing (NS) controls (Figure 4B). In addition, SOX1 shRNA cells demonstrated a significant decrease in proliferation compared to either the parental cell line (total cells) or the NS infected line (Figure 4C), as well as a significant decrease in invasion toward SCM (Figure 4D) ( $p$ -value  $< 0.05$ ). However, there was not a significant difference using the shBMX lines, except for a slight reduction in invasion using clone #3. Interestingly, a small increase in proliferation was seen with the shBMX clones (Figure 4C). Further promoter tiling array analysis using two short term cultures primary prostate tumor cell lines, PCSC1 and PCSC2, determined that *Sox1*, and not *Bmx*, was methylated in the invasive population of cells (Additional File 2, Table S2A and B). Overall, we demonstrate that *Sox1* is differentially methylated within the invasive CSC population and the shRNA studies indicate it could be selectively targeted to block invasion.



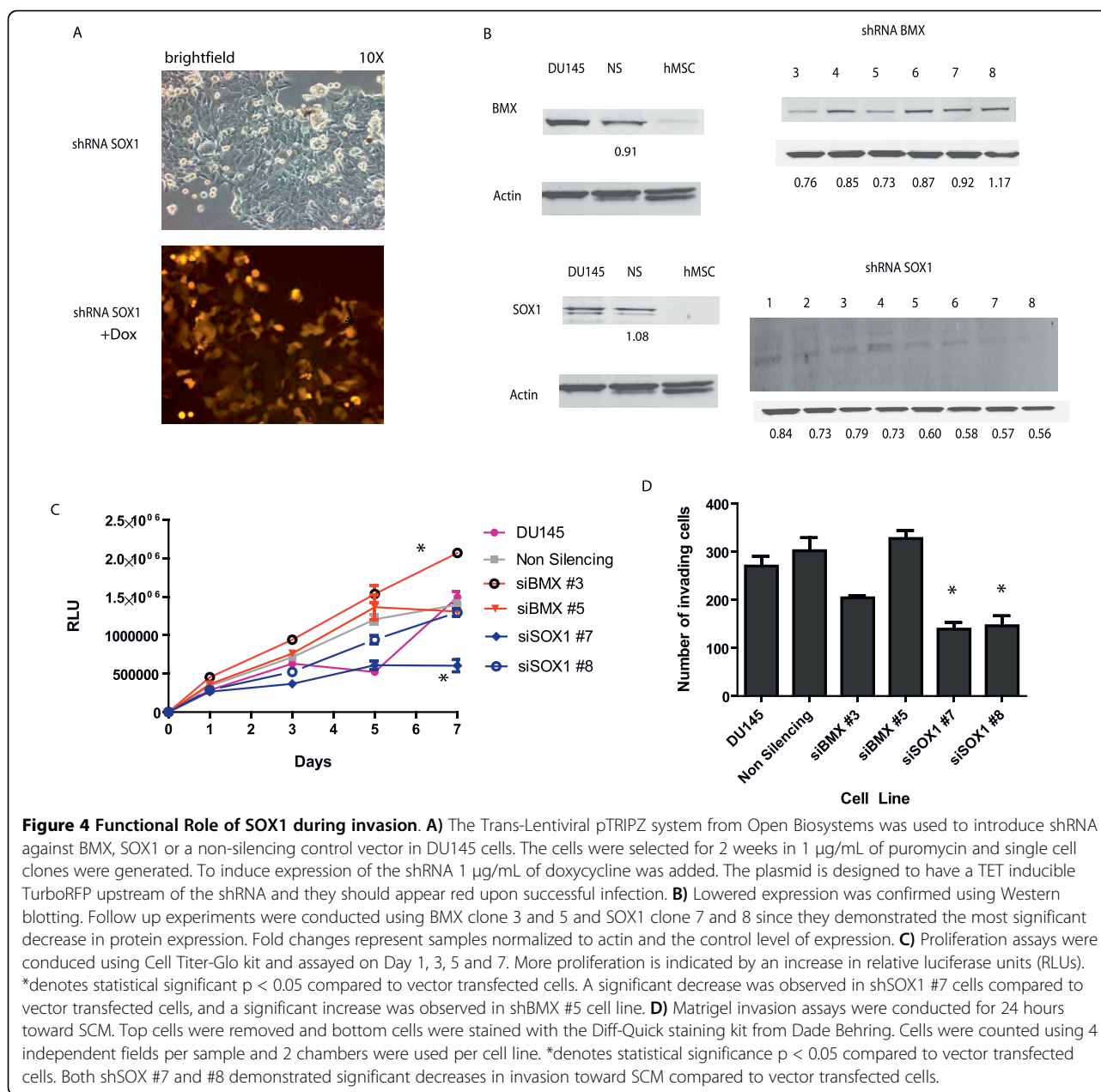




### Role of SOX1 during differentiation

In addition to the method presented here, prostate TICs (tumor initiating cells) can also be isolated by culturing total cells in SCM where structures called prostatespheres are generated [18,23-25]. The prostatespheres are multicellular globes that develop from cells that survive anchorage-independent conditions *in vitro*, and are frequently used when analyzing the ability of TICs to self-renew or differentiate upon the addition of serum. Using this assay as a model, a greater number of prostatespheres were isolated from DU145 NS cells compared

to shSOX1 cells (Additional File 3, Figure S1A and B). When invasive DU145 cells were isolated and cultured in SCM, prostatespheres were maintained for up to 3 passages (Additional File 3, Figure S1C) and if these cells were further cultured in the presence of 1% human serum (Additional File 3, Figure S1C), the vector control (NS) cells rapidly differentiated and proliferated, while the shSOX1 cells did not (Additional File 3, Figure S1D). These observations suggest that not only does *Sox1* play a role in regulating invasion, but it can also regulate the maintenance of 'stem-ness' in culture.



### Ingenuity pathway analysis defines pathways of differentially methylated genes within invasive sub-populations of cells

Each data set of differentially methylated genes was then extracted and uploaded to the Ingenuity server to identify common gene pathways that are regulated during the process of invasion. The most conserved functional pathways between the cell lines are cellular development, cell growth and proliferation, as well as organismal development, nervous system development and function, and tissue development (Additional File 4, Table S3). The full list from the Ingenuity pathway analysis is also included (Additional File 5, Figure S2A and S2B). Additionally, the

IL-6 signaling pathway involving STAT3 had a significant number of contributing methylated genes, a pathway recently found to play a significant role in cancer stem cell regulation [26-34] (Additional File 6, Figure S3A).

### Inhibitor studies further determine the role of IL-6/STAT3 pathway in invasion

Based on the information generated from Ingenuity, we chose to determine how the IL-6 pathway might be regulating this process of invasion. A number of inhibitors of downstream targets of IL-6 regulation were tested for their ability to block invasion toward SCM. We included a neutralizing antibody to interleukin-6 (IL-6) to test

what effect this may have upstream. Downstream of the receptor, the following inhibitors were used; the PI3K inhibitor LY294002, small molecular inhibitor of MEK called U0126 (thus downstream inhibition of extracellular-related kinase (ERK1 and ERK2) mediated responses), a small molecule inhibitor of JAK (Janus Kinase) called AG490 and an inhibitor of its partner signal transducers and activators of transcription-3 (STAT3) called Stattic (Figure 5A). Additionally, we tested the ability of the Tec kinase family inhibitor LFM-A13 based on the potential involvement of BMX during invasion (Figure 5A). The inhibitors which demonstrated the greatest effect at blocking invasion included Stattic, LY294002, and LFM-A13 (Figure 5A). However, a proliferation assay determined that Stattic could be preventing invasion because it was either cytotoxic to the cells or causing them to undergo apoptosis (Additional File 6, Figure S3B). To eliminate this possibility, viable cells were isolated after treating the DU145 cell line with Stattic for 24 hours (data not shown). These cells, although viable as determined by trypan blue staining, were still unable to invade.

#### Direct interaction between the differentially methylated SOX1 and STAT3

Since inhibition of STAT3 demonstrated such a profound effect on invasion toward SCM, we questioned its involvement with the epigenetically regulated targets. Although we did not observe methylation of *Stat3* itself, in both cell lines, the mRNA expression of *Stat3* was increased (p-value < 0.03) when comparing invasive cells to their non-invasive counterpart (Figure 5B). Protein expression of pSTAT3 was also found to be increased in the invasive cells (Figure 5C). Since both SOX1 and STAT3 are known to act as transcriptional activators after forming protein complexes with other proteins [35-40], and BMX is known to activate STAT3 itself [40], we determined whether STAT3 directly interacts with either SOX1 or BMX. An interaction between SOX1 and STAT3 was observed (Figure 5D), however not between STAT3 and BMX (Figure 5D). In addition, a significant decrease in the expression of activated pSTAT3 was seen in both sub-cellular fractions of the BMX and SOX1 shRNA infected cells (Figure 5E). However, there was no change in total expression of STAT3. Additionally, a significant decrease in STAT3 DNA binding activity was observed in both BMX and SOX1 shRNA infected cells (Figure 5F). Overall, we see an interaction between SOX1 and STAT3, and upon loss of either BMX1 or SOX1 expression we observe a loss of STAT3 activation.

To further elucidate the connection between the SOX1 and STAT3, a decrease in the STAT3 target gene *Mcl-1* and *Stat3* itself were observed by qRT-PCR in shSOX1 clone #7 cells (Figure 6A). However, no change was observed for the STAT3 targets genes *Survivin* or

*Myc* (Figure 6A). Finally, since prostatospheres are also a model for generating aggressive populations of cells in culture, we generated them from LNCaP cells and asked if STAT3 genes were affected. qRT-PCR analysis was performed and compared to adherent LNCaP cells, expression of *Stat3* and *Stat3* target genes *Mcl-1*, *Myc*, and *Survivin* were increased as well as *Bmx* and *Sox1* (Figure 6B).

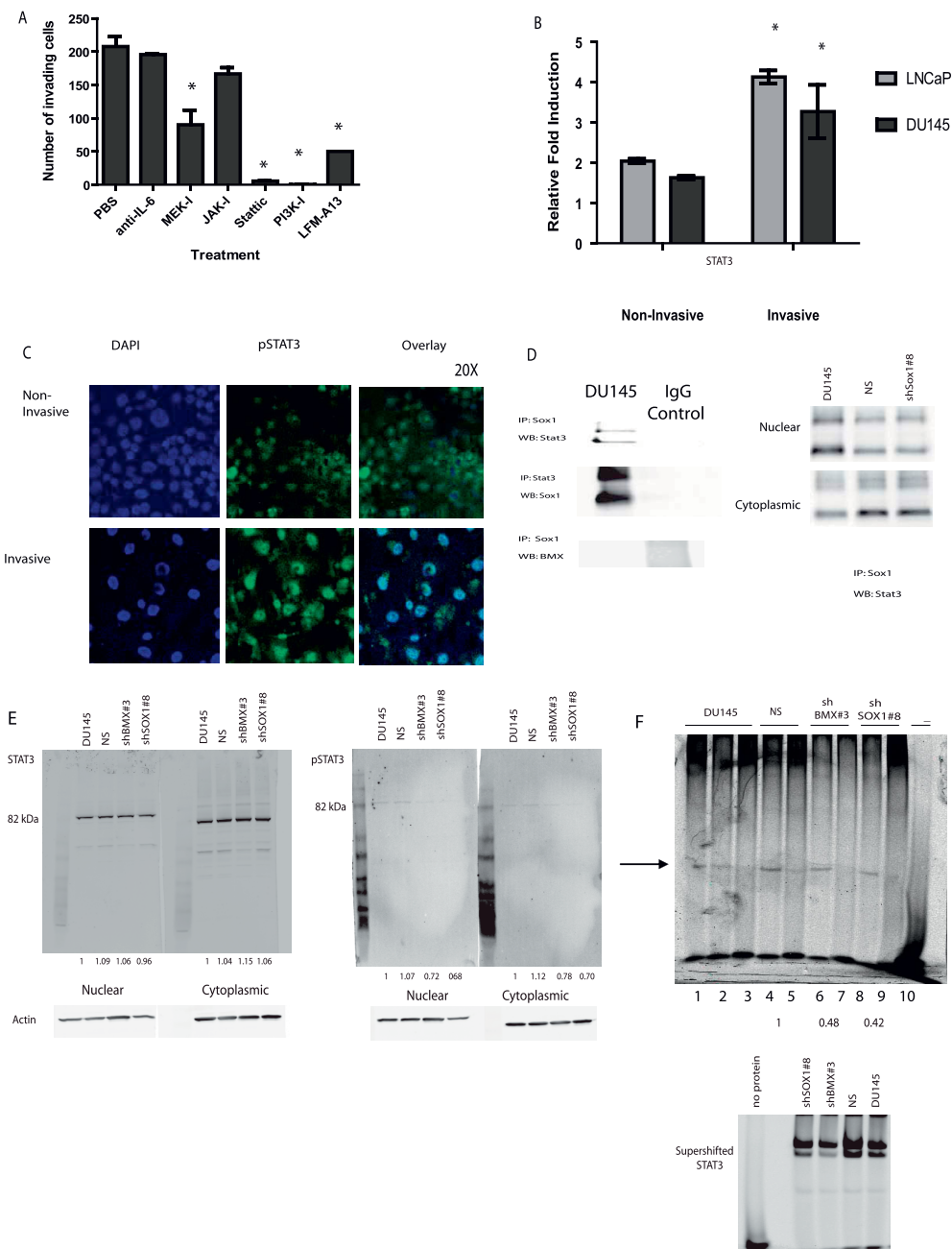
In order to determine what might be regulating the increased expression of *Stat3* and *Sox1*, transcription factor binding sites were analyzed using Genomatix software. In both the *Stat3* and *Sox1* promoters there are a number of overlapping binding sites for transcription factors with a significant matrix value such as GATA-binding factors, RNA polymerase II transcription factor IIB (TFIIB), NeuroD/Beta2, TALE homeodomain class recognizing TG motifs, TCF11 transcription factor otherwise known as Nrf2, Nkx homeodomain factors, and finally the Zinc finger transcription factor RU49 also called Zipro1 (Additional File 7, Table S4). With this information, we can begin to understand why the methylation of *Sox1* could serve as a master regulator of CSC invasion, thereby controlling its potential to undergo EMT and further metastasize.

Additional analysis using the GEO database determined that both *Sox1* and *Stat3* are expressed at higher levels in metastatic prostate cancer tissues and not *Bmx* (Figure 6C and 6D). Overall, we demonstrate that SOX1 is an epigenetically regulated target involved in the progression of prostate cancer, and is involved in signaling via the STAT3 pathway.

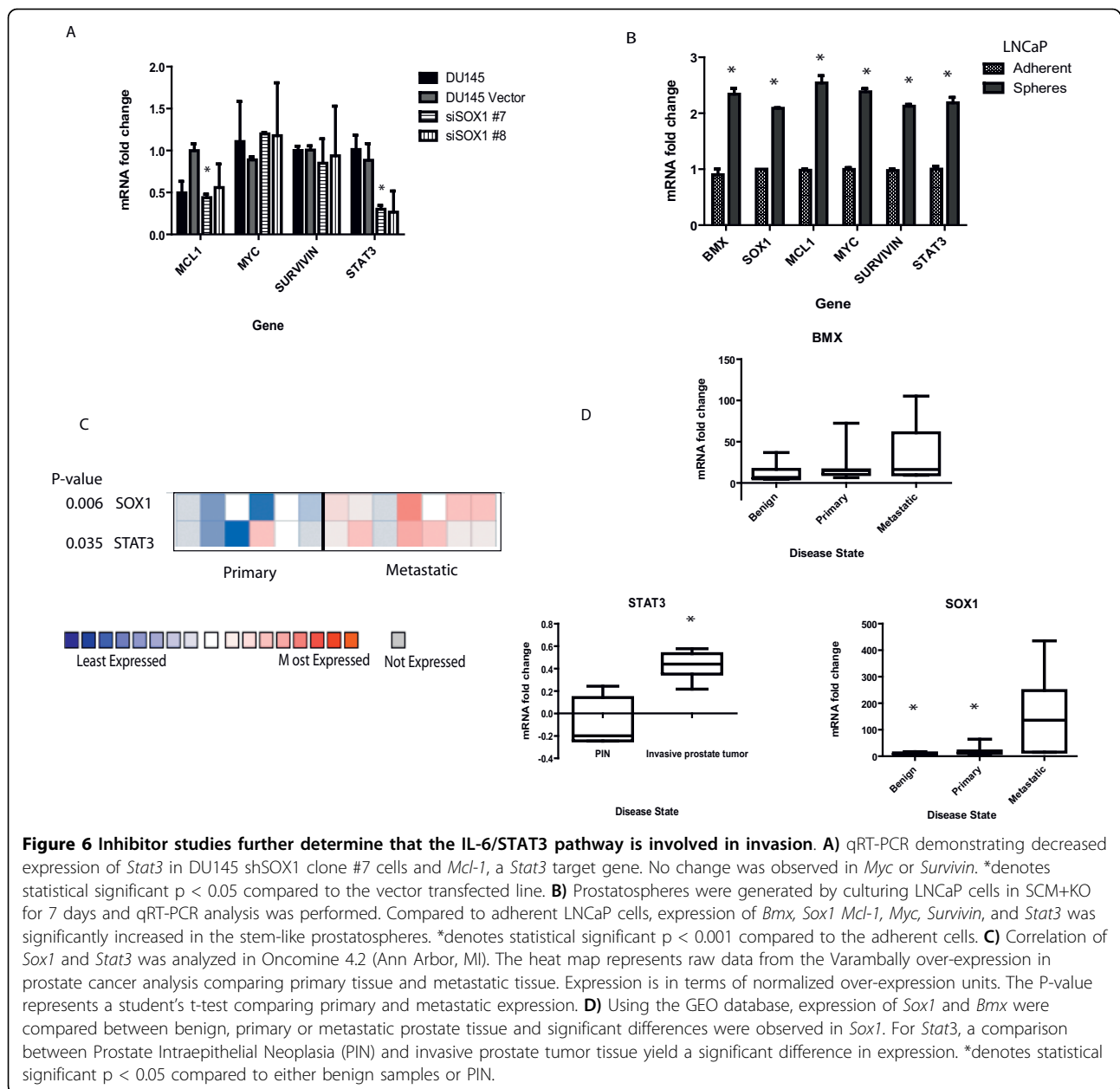
#### Discussion

The process of epigenetic regulation by DNA methylation involves covalent modification of cytosine nucleotides at the C5 position in specific areas of CpG dinucleotides. The majority of methylated CpG dinucleotides are present in heterochromatic regions, and thus are unexpressed in the genome [41]. The process of methylation in mammals evolved as a method of silencing genes when their expression is not required. For example, the process of genomic imprinting involves DNA methylation where one allele of a gene, either maternal or paternal, is silenced [42]. This process only affects a few hundred genes within the genome, most of which encode for genes that regulate embryonic and neonatal growth [43]. Likewise, a number of CpG islands on one X chromosome are methylated during a process called X-chromosome inactivation [44]. This process ensures an equal amount of gene expression between males and females.

Using this model of invasion, we currently have developed a method to analyze differences in global CpG promoter methylation between total prostate cancer



**Figure 5 Direct interaction between SOX1 and STAT3.** **A)** Matrigel invasion assays were performed for 24 hours toward SCM using DU145 cells in the presence of the anti-IL-6, the PI3K inhibitor LY294002, a small molecular inhibitor of MEK called U0126 (thus downstream inhibition of extracellular-related kinase (ERK1 and ERK2) mediated responses), a small molecule inhibitor JAK called AG490 (Janus Kinase) and an inhibitor of its partner signal transducers and activators of transcription-3 (STAT3) called Stattic or the Tec kinase family inhibitor LFM-A13. Significant differences were observed between control cells and those cells treated with U0126, Stattic, LY294002 and LFM-A13. **B)** qRT-PCR analysis was performed as mentioned in Figure 3. \*denotes statistical significant  $p < 0.05$  compared to the non-invasive cells. Increased levels of *Stat3* are seen in invasive LNCaP and DU145 cells compared to the parental lines. **C)** Staining of pSTAT3 in invasive or non-invasive DU145 cells was performed directly on the Matrigel membrane and carried out as previously described in Figure 3. **D)** DU145 lysates were incubated with either SOX1, STAT3 or BMX overnight at 4°C with rotation. Samples were then incubated with Protein A-agarose beads to isolate complexes. Membranes were then incubated overnight at 4°C using primary antibodies for STAT3 or SOX1. The membrane was developed using the Odyssey from Licor. Protein loading was normalized using actin as a control. **E)** Western blotting for STAT3 and pSTAT3 in sub-cellular protein extracts from DU145, NS, shBMX#3 or shSOX1#8. **F)** STAT3 EMSA: Each lane contains WT-IR STAT3 oligos. Lane 1-3 DU145, 4 and 5 NS, 6 and 7 shBMX#3, 8 and 9 shSOX1 #8 and 10 contains no protein. Lane 2 contains excess MU-IR STAT3 and lanes 3, 5, 7 and 9 contain excess unlabeled WT probe. Supershifted samples appear below and only contain WT-IR STAT3.



cells and their invasive population using promoter tiling arrays from Agilent. We identified a small subset of genes which were found to be differentially methylated between non-invasive and invasive LNCaP and DU145 cell lines. The results were highly intriguing because the majority of the genes normally function during human development (Additional File 4, Table S3). Based on previous data, these invasive cells demonstrated characteristics of true cancer stem cells (CSCs) [7]. It is becoming more evident that CSCs are not governed by the same type of genetic regulation as normal stem cells, and arguably may be an epithelial cell that has up-regulated pathways that have been previously observed

in true stem cells. To determine the epigenetic profile of these invasive prostate cancer cells and putative TICs, we determined which genes are differentially methylated.

The appearance of *Sox1* as one epigenetically regulated target presented the most interesting finding of this investigation. SOX proteins are transcription factors that are key regulators of determining neuronal cell fate, not only mammals, but also in *Drosophila*, *Xenopus*, and avian models [36]. Recently, much attention has been focused on these transcription factors since ectopic expression of *Sox2* along with *Oct3/4*, *Klf4* and *Myc* have been shown to reprogram murine fibroblasts to pluripotency, which in turn yields induced pluripotent

stem (iPS) cells [45]. In our model, when expression of SOX1 was decreased in DU145 cells using shRNA, there was a significant reduction in invasion toward our stem cell media termed SCM (Figure 4D). Although SOX1 has yet to be implicated as a regulator of aggression in prostate cancer, it has been implicated as a marker of CSCs in breast cancer. Using either CD44<sup>+</sup>/CD24<sup>-</sup> or CD133<sup>+</sup> cells isolated from *Brcal1-deficient* mouse mammary tumors, expression of *Sox1* was found to be significantly higher in these cells when compared to their counterparts [46]. In fact, expression of *Sox1* was found to be 19.2-fold higher in CD44<sup>+</sup>/CD24<sup>-</sup> compared to CD44<sup>-</sup>/CD24<sup>+</sup> cells, which represented the greatest change in any gene from this analysis [46].

The appearance of *Bmx* (also referred to as *Etk*) as a differentially methylated target was also interesting, yet not surprising, since this protein is a well-known regulator of prostate cancer. BMX is a family member of the Tec family of non-receptor tyrosine kinases that are predominately expressed in cells of hematopoietic origin, yet recently has also been shown to be expressed in arterial endothelium and a variety of epithelial cells [21,39,47,48]. Although BMX has a role in the formation of leukemia [21,49], our research is the first to demonstrate that BMX may play a significant role in the regulation of prostate cancer invasion and TICs. Although our shRNA studies against BMX did not demonstrate significant differences in invasion toward SCM, we were able to inhibit invasion of DU145 cells using the Tec family kinase inhibitor LFM-A13 without affecting normal cell proliferation (Additional File 6, Figure S3B), suggesting that this family of kinases may be indeed involved in metastasis.

After uploading our extensive list of differentially methylated genes into the Ingenuity pathway analysis software, we observed that a number of the genes were members of the IL-6/STAT3 pathway. We tested a number of inhibitors of the IL-6 pathway for their ability to block invasion toward SCM. Small and non-significant effects of invasion were seen when inhibitors for MEK and JAK pathways, as well as a neutralizing antibody to IL-6 itself (Figure 5A). However, significant effects were seen using a PI3K inhibitor and a STAT3 inhibitor (Figure 5A). The role of PI3K signaling in prostate CSC regulation has been characterized, thus this observation is not too surprising [50]. The most pronounced effect, however, was observed with the STAT3 inhibitor Stattic. This drug inhibits binding of a phosphotyrosine-containing peptide derived from the gp130 receptor to the STAT3 SH2 domain with IC<sub>50</sub> value of 5.1 ± 0.8 μM after 1 hr of incubation at 37°C [51]. The role of STAT3 in cancer progression has been known for sometime [52-56], and its role in CSC regulation has only recently been investigated. Higher levels of STAT3 have been demonstrated

in CSCs isolated from liver, bone, cervical and brain cancers [26,27,29,57-59], and furthermore treatment of putative glioblastoma stem cells (GBM-SC) with Stattic results in a dramatic reduction in their formation [27]. Although the *Stat3* gene itself was not methylated in any of our studies, qRT-PCR analysis demonstrated that compared to non-invasive cells, the invasive cells had a significant increase in expression of *Stat3* (Figure 5B) and ICC detected an increase in active protein as well (Figure 5C). However, as seen in Figure S3B, there was a significant reduction in cell proliferation with Stattic treatment. To determine if this was the reason why we observed such a significant reduction in invasion, we took the remaining cells which survived treatment and further placed them through an invasion assay. The cells were unable to invade toward SCM, indicating that the cells resistant to Stattic-induced apoptosis were still sensitive at inhibiting invasion by lowering STAT3 (data not shown). A similar result was observed in the GBM-SCs, since different isolates of the cells responded differently to treatment with Stattic. The authors concluded that GBM-SCs derived in serum respond to Stattic by undergoing apoptosis, however in those derived using stem cell media they do not [27]. They state that this could be a result of certain GBM-SC lines being more differentiated, and are thus more sensitive to STAT3 inhibition.

Since inhibition of SOX1 with shRNA and BMX ultimately with LFM-A13 (data not shown, but LFM-A13 inhibited IL-6 mediated activation of BMX in LNCaP cells) decreased invasion toward SCM, we sought to determine if an interaction might be occurring between these differentially methylated genes and STAT3. To test this, an IP was performed to see if either BMX or SOX1 directly interact with STAT3. We found that only SOX1 could directly interact with STAT3 and not BMX (Figure 5D), and this interaction occurs in both the cytoplasm and the nucleus. In these sub-cellular fractions, we still see an association between SOX1 and STAT3 in shSOX1 cells since expression of the protein was not fully ablated (Figure 4B). Interestingly, decreased expression of either BMX or SOX1 does result in significantly less active STAT3 (Figure 5E) and a decrease in its DNA binding activity (Figure 5F). This observation is not too surprising since BMX has been shown to regulate such cellular processes as differentiation, motility, invasion, apoptosis, and more recently, when inhibited, a delay in tumor growth [22,60-66]. Specifically, within the prostate, BMX is up-regulated in tumors from both mouse and human specimens compared to benign tissues, and when over-expressed in cell lines, led to an increase in proliferation and elevated levels of AKT and STAT3 [22]. Albeit having a role in the formation of leukemia [21,49], our research is the

first to demonstrate that BMX may play a significant role in the regulation of prostate CSCs.

Both STAT3 and SOX1 are transcription factors that regulate cell fate and differentiation; however a direct interaction between these proteins has never been identified. Future studies will be needed to determine what protein domains of each molecule are important for this interaction, as well as which promoters these transcription factors are regulating. However, the OncoPrint and GEO data further support the observation that expression of both *Sox1* and *Stat3* are key genes regulating the progression of prostate cancer (Figure 6C and 6D). Regulation of *Sox1* and *Stat3* expression could occur coordinately since within their promoters they both contain transcription factor binding sites for NeuroD, TALE containing proteins, TCF11, and Nkx5 (Additional File 7, Table S4). The TCF family of transcription factors regulates many patterns of development and activation of the TCF/LEF promoters. Recently, the Wnt proteins have been shown to regulate the 'stemness' of CSCs [67-70]. Additionally, expression of Nkx factors are required for neuronal cell fate, and interestingly, *Nkx2.2*, *Nkx6.1* and *Irx3*, a NKX target, are also methylated in our study (Table 1) [71].

## Conclusions

Overall, our data demonstrates that *Sox1* is methylated in two prostate cancer cell lines, LNCaP and DU145, and two short-term primary prostate cancer cultures, PCSC1 and PCSC2, yet not methylated in the invasive compartment of these cells. The expression of *Sox1* was found to be correlated with increased levels of *Stat3* in our invasive cells, and to directly interact with the protein product as well. Finally, both *Sox1* and *Stat3* were found to have increased expression in relation to the progression of prostate cancer in humans. Using our *in vitro* method to investigate invasion we can begin to understand which genes are epigenetically regulated in the invasive putative CSC population. The process of epigenetic regulation is complex, but we have begun to unravel it in these invasive cells from the prostate.

## Additional material

**Additional file 1: Table S1: Total methylated gene lists generated by Chip Analytics software.** A) LNCaP B) DU145. Data represents those changes with a p-value  $\leq 0.05$  using nearest neighbor analysis. Genes are listed in alphabetical order according to their gene name.

**Additional file 2: Table S2: Total methylated gene lists generated by Chip Analytics software.** A) PCSC1 B) PCSC2. Data represents those changes with a p-value  $\leq 0.05$  using nearest neighbor analysis. Genes are listed in alphabetical order according to their gene name.

**Additional file 3: Figure S1: Prostatosphere formation and differentiation of DU145 and shSOX1 #7 cells.** DU145 cells were seeded 1000 cells per mL in replacement media SCM and supplemented with B27 in non-adherent 6 well plates coated with Hydrogel. The prostatospheres were generated for 5-7 days and then quantified. A)

Comparison of DU145 spheres and those from clone #7 using the shRNA against SOX1. B) Number of spheres generated from DU145, NS and shSOX1 clone #7 cell lines. C) Ability of DU145 NS invasive cells to differentiate after addition of 1% human serum for 96 hours in culture and morphologically resemble DU145 NS cells. D) Comparison of differentiation potential of invasive cells isolated from DU145, NS and shSOX1 #7 cell lines.

**Additional file 4: Table S3: Summary of significant functional gene pathways generated by Ingenuity software analysis for genes not methylated within the invasive cells.**

**Additional file 5: Figure S2: Ingenuity analysis of methylation data demonstrating significant changes in functional gene pathways.** A) DU145 B) LNCaP.

**Additional file 6: Figure S3: A)** Ingenuity analysis of methylation data demonstrating significant changes in canonical gene pathways in LNCaP. **B)** Proliferation assay for 24 hours using inhibitors from Figure 5A measured using CellTiter-Glo. Significant differences were seen between control cells and cells treated with Stattic. \*denotes statistical significant  $p < 0.05$  compared to the adherent cells.

**Additional file 7: Table S4: Predicted transcription factor (TF) binding sites common to both the *sox1* and *stat3* promoters.** The potential TF factors sites were generated using the Genomatix software with full length promoter sequences for both genes.

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## Authors' contributions

LAM contributed to the conception and design, collection and/or assembly of data, data analysis and interpretation and manuscript writing. EMH contributed to the conception and design, data analysis and interpretation, final approval of manuscript and other (extensive editing). XZ contributed significantly to the collection and/or assembly of data. WLF contributed to the conception and design, financial support, final approval of manuscript and other (extensive editing).

All authors read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

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