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**Expression of Pluripotent Stem Cell Reprogramming Factors by
Prostate Tumor-Initiating Cells**

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Table I. Patient demographics for Tissue Arrays

Sample	Age	Gender	Diagnosis	Stage	Gleason Score
TMA1	76	M	Adenocarcinoma	Organ Confined	3+3
TMA2	76	M	Adenocarcinoma	Organ Confined	3+3
TMA3	75	M	Adenocarcinoma	Organ Confined	3+2
TMA4	75	M	Adenocarcinoma	Organ Confined	3+2
TMA5	38	M	Hyperplasia of prostate	-	-
TMA6	38	M	Hyperplasia of prostate	-	-
TMA7	70	M	Adenocarcinoma	Organ Confined	3+3
TMA8	70	M	Adenocarcinoma	Organ Confined	3+3
TMA9	66	M	Adenocarcinoma	Organ Confined	3+4
TMA10	66	M	Adenocarcinoma	Organ Confined	3+3
TMA11	75	M	Adenocarcinoma	Locally Advanced	3+4
TMA12	75	M	Adenocarcinoma	Locally Advanced	3+4
TMA13	60	M	Adenocarcinoma	Organ Confined	3+3
TMA14	60	M	Adenocarcinoma	Organ Confined	3+3
TMA15	54	M	Adenocarcinoma	Organ Confined	3+3
TMA16	54	M	Adenocarcinoma	Organ Confined	3+3
TMA17	64	M	Adenocarcinoma	Organ Confined	3+3
TMA18	64	M	Adenocarcinoma	Organ Confined	3+3
TMA19	80	M	Adenocarcinoma	Organ Confined	3+3
TMA20	80	M	Adenocarcinoma	Organ Confined	3+3
TMA21	72	M	Adenocarcinoma	Organ Confined	2+4
TMA22	72	M	Adenocarcinoma	Organ Confined	3+3
TMA23	56	M	Adenocarcinoma	Organ Confined	3+3
TMA24	56	M	Adenocarcinoma	Organ Confined	3+3
TMA25	64	M	Adenocarcinoma	Organ Confined	3+3
TMA26	64	M	Adenocarcinoma	Organ Confined	3+4
TMA27	65	M	Adenocarcinoma	Organ Confined	3+4
TMA28	65	M	Adenocarcinoma	Organ Confined	3+4
TMA29	69	M	Adenocarcinoma	Organ Confined	3+3
TMA30	69	M	Adenocarcinoma	Organ Confined	3+3
TMA31	70	M	Adenocarcinoma	Organ Confined	4+4
TMA32	70	M	Adenocarcinoma	Organ Confined	4+4
TMA33	73	M	Adenocarcinoma	Organ Confined	3+3

TMA34	73	M	Adenocarcinoma	Organ Confined	3+3
TMA35	74	M	Adenocarcinoma	Organ Confined	3+4
TMA36	74	M	Adenocarcinoma	Organ Confined	3+4
TMA37	69	M	Adenocarcinoma	Locally Advanced	3+5
TMA38	69	M	Adenocarcinoma	Locally Advanced	3+5
TMA39	82	M	Adenocarcinoma	Organ Confined	3+3
TMA40	82	M	Adenocarcinoma	Organ Confined	3+4
TMA41	81	M	Adenocarcinoma	Organ Confined	3+3
TMA42	81	M	Adenocarcinoma	Organ Confined	3+3
TMA43	70	M	Adenocarcinoma	Organ Confined	4+4
TMA44	70	M	Adenocarcinoma	Organ Confined	4+4
TMA45	20	M	Adenocarcinoma	Organ Confined	3+3
TMA46	20	M	Adenocarcinoma	Organ Confined	3+3
TMA47	68	M	Adenocarcinoma	Organ Confined	3+3
TMA48	68	M	Adenocarcinoma	Organ Confined	3+3
TMA49	70	M	Adenocarcinoma	Organ Confined	3+4
TMA50	70	M	Adenocarcinoma	Organ Confined	3+3
TMA51	73	M	Adenocarcinoma	Organ Confined	3+3
TMA52	73	M	Adenocarcinoma	Organ Confined	3+4
TMA53	70	M	Adenocarcinoma	Organ Confined	3+4
TMA54	70	M	Adenocarcinoma	Organ Confined	3+4
TMA55	51	M	Adenocarcinoma	Organ Confined	3+3
TMA56	51	M	Adenocarcinoma	Locally Advanced	3+4
TMA57	76	M	Adenocarcinoma	Locally Advanced	4+4
TMA58	76	M	Adenocarcinoma	Locally Advanced	4+4
TMA59	40	M	Adenocarcinoma	Metastatic	Not Reported
TMA60	40	M	Adenocarcinoma	Metastatic	4+4
TMA61	73	M	Adenocarcinoma	Locally Advanced	3+4
TMA62	73	M	Adenocarcinoma	Metastatic	4+4
TMA63	64	M	Adenocarcinoma	Metastatic	4+4
TMA64	64	M	Adenocarcinoma	Metastatic	4+4
TMA65	70	M	Adenocarcinoma	Metastatic	Not Reported
TMA66	70	M	Adenocarcinoma	Locally Advanced	4+4
TMA67	60	M	Adenocarcinoma	Locally Advanced	3+4
TMA68	60	M	Adenocarcinoma	Locally Advanced	4+4
TMA69	65	M	Adenocarcinoma	Metastatic	4+4
TMA70	65	M	Adenocarcinoma	Metastatic	4+4
TMA71	72	M	Hyperplasia of prostate	-	-
TMA72	72	M	Hyperplasia of prostate	-	-
TMA73	73	M	Hyperplasia of prostate	-	-
TMA74	73	M	Hyperplasia of prostate	-	-
TMA75	70	M	Benign prostate tissue	-	-
TMA76	70	M	Benign prostate tissue	-	-

TMA77	78	M	Benign prostate tissue	-	-
TMA78	78	M	Benign prostate tissue	-	-
TMA79	65	M	Benign prostate tissue	-	-
TMA80	65	M	Benign prostate tissue	-	-

Table II. Patient demographics for PCR analysis

Sample	Age	Gender	Diagnosis	Stage	Gleason Score
PCR1	53	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3
PCR2	48	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3
PCR3	68	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3
PCR4	76	Male	Adenocarcinoma of prostate	Organ Confined	4 + 3
PCR5	53	Male	Adenocarcinoma of prostate	Organ Confined	4 + 3
PCR6	68	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3
PCR7	74	Male	Adenocarcinoma of prostate	Organ Confined	4 + 4
PCR8	72	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3
PCR9	62	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3
PCR10	54	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3
PCR11	56	Male	Adenocarcinoma of prostate	Organ Confined	3 + 4
PCR12	56	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3
PCR13	55	Male	Adenocarcinoma of prostate	Organ Confined	3 + 4
PCR14	63	Male	Adenocarcinoma of prostate	Organ Confined	3 + 4
PCR15	53	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3
PCR16	64	Male	Adenocarcinoma of prostate	Organ Confined	3 + 4
PCR17	68	Male	Adenocarcinoma of prostate	Organ Confined	3 + 4
PCR18	63	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3
PCR19	66	Male	Adenocarcinoma of prostate	Organ Confined	3 + 4
PCR20	70	Male	Adenocarcinoma of prostate	Organ Confined	3 + 4
PCR21	65	Male	Adenocarcinoma of prostate	Organ Confined	3 + 4
PCR22	64	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3
PCR23	54	Male	Adenocarcinoma of prostate	Organ Confined	4 + 3
PCR24	64	Male	Adenocarcinoma of prostate	Organ Confined	3 + 4
PCR25	62	Male	Adenocarcinoma of prostate	Organ Confined	4 + 3
PCR26	62	Male	Adenocarcinoma of prostate	Organ Confined	4 + 3
PCR27	61	Male	Adenocarcinoma of prostate	Organ Confined	3 + 4
PCR28	62	Male	Adenocarcinoma of prostate	Organ Confined	3 + 5
PCR29	53	Male	Adenocarcinoma of prostate	Organ Confined	3 + 4
PCR30	58	Male	Adenocarcinoma of prostate	Organ Confined	4 + 3
PCR31	57	Male	Adenocarcinoma of prostate	Locally Advanced	4 + 5
PCR32	65	Male	Adenocarcinoma of prostate	Locally Advanced	3 + 3

PCR33	53	Male	Adenocarcinoma of prostate	Locally Advanced	3 + 3
PCR34	61	Male	Adenocarcinoma of prostate	Locally Advanced	3 + 4
PCR35	73	Male	Adenocarcinoma of prostate	Locally Advanced	3 + 4
PCR36	52	Male	Adenocarcinoma of prostate	Locally Advanced	4 + 4
PCR37	64	Male	Adenocarcinoma of prostate	Locally Advanced	4 + 3
PCR38	54	Male	Adenocarcinoma of prostate	Locally Advanced	5 + 4
PCR39	61	Male	Adenocarcinoma of prostate	Locally Advanced	3 + 2
PCR40	61	Male	Adenocarcinoma of prostate	Locally Advanced	4 + 3
PCR41	54	Male	Adenocarcinoma of prostate	Locally Advanced	4 + 3
PCR42	62	Male	Adenocarcinoma of prostate, metastatic	Metastatic	Not Reported
PCR43	64	Male	Adenocarcinoma of prostate	Metastatic	4 + 3
PCR44	87	Male	Adenocarcinoma of prostate	Not Reported	5 + 4
PCR45	76	Male	Adenocarcinoma of prostate	Not Reported	3 + 4
PCR46	71	Male	Adenocarcinoma of prostate	Not Reported	2 + 3
PCR47	77	Male	Adenocarcinoma of prostate	Not Reported	4 + 4
PCR48	83	Male	Adenocarcinoma of prostate	Not Reported	5 + 4
PCR49	-	Male	Adenocarcinoma of prostate	Not Reported	Not Reported
PCR50	70	Male	Adenocarcinoma of prostate	Organ Confined	3 + 4
PCR51	63	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3
PCR52	69	Male	Adenocarcinoma of prostate	Organ Confined	4 + 5
PCR53	81	Male	Hyperplasia of prostate	-	-
PCR54	82	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3
PCR55	64	Male	Adenocarcinoma of prostate	Organ Confined	3 + 3

SUPPLEMENTAL METHODS

Histological and Immunohistochemical Analysis

TMA slides containing duplicate cores from 40 prostate tissues (detailed information in Table I) were purchased from Cybrdi (MD, USA) and stored at 4°C until use. After air drying and equilibrating at room temperature for 2 hrs, the slides were sequentially deparaffinized in 2 changes of xylene, rehydrated through a series of graded alcohols and blocked for endogenous peroxidase activity for 10 mins in 3% hydrogen peroxide diluted in methanol. Optimal staining required 25 mins of heat antigen retrieval in 10mM Citrate buffer pH 6.0 using a microwave oven. Slides were blocked in 2% normal rabbit serum for 30 mins, and then with Avidin and Biotin solutions (Vector Labs) for 30 mins each.

Anti-human SOX2 (R&D Systems) and OCT3/4 (Abcam) were applied to the sections at 4µg/ml and incubated overnight at 4°C. Slides were washed twice for 5 mins in TBS buffer and stained using the ABC-Elite kit (Vector Labs) following the manufacturer's instructions. Positive staining was detected with DAB (Vector Labs) as the chromogen and hematoxylin 560 (SurgiPath) as the counterstain. Slides were cleared in xylene and mounted using Cytoseal (Richard-Allan Scientific).

Western Blotting

Whole cell lysates were prepared by incubating cells in a modified RIPA buffer [50mM HEPES (pH7.5), 150mM NaCl, 1% Triton X-100, 2mM EDTA, 2mM EGTA, 50mM sodium β-glycerophosphate, 5mM sodium pyrophosphate, 50mM sodium fluoride, 1mM sodium orthovanadate, 1mM DTT, 1mM PMSF, 10µg/ml leupeptin, and 10µg/ml aprotinin] at 4°C for 1 hr, followed by centrifugation at 13,000×g for 10 mins to remove insoluble particles. Protein concentrations were determined by the BCA protein assay (Pierce). Equal amounts (100ug) of total cellular extracts were separated by SDS/PAGE and transferred to nitrocellulose membranes. The membranes were blocked in TBST [20mM Tris-HCl (pH 7.5), 500mM sodium chloride, and 0.05% Tween 20] containing 5% nonfat dry milk at room temperature for 2 hrs and then incubated with primary antibodies (OCT3/4 1:1000, c-Myc 1:1000 and β-actin 1:2000 from Cell Signaling, SOX2 1:1000 and Klf4 1:1000 from Santa Cruz, Nanog 1:1000 from BioLegend, E-cadherin 1:1000 from BD Bioscience) in TBST containing 1% BSA (fraction V), followed by incubation for 2 hrs at room temperature with an HRP-conjugated secondary antibody (Rabbit secondary antibody 1:2000, Mouse secondary antibody 1:5000) in TBST containing 5% nonfat dry milk. Visualization was by the enhanced

chemiluminescence detection system (Amersham). Approximate time of development was 30 secs to 3 mins.

Isolation of RNA and RT-PCR Analysis

Total RNA was isolated from cells using an RNeasy Mini Kit from Qiagen. The concentration of the RNA was assessed spectrophotometrically. Reverse transcription was performed on a 1µg aliquot of total RNA using a Verso cDNA Kit (Thermo) and oligo-dT primers according to the manufacturer's recommendations. PCR was performed on 50ng cDNA using 0.2µM each gene specific primer (see Table II for sequences) and GoTaq Green Master Mix (Promega) according to the manufacturer's instructions. For all genes, PCRs were initially denatured for 1 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at gene specific temperature for 45 sec. (see Table III for gene specific temperatures), and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min. PCR aliquots were visualized on 2% agarose gels by ethidium bromide staining. Actin was used as an internal control.

Semi-quantitative RT-PCR was also performed by using a panel of first-strand cDNAs from 48 human prostate samples commercially available from Origene (TissueScan Prostate Cancer II, see Table III for details), according to the manufacturer's instructions. A pool of normal human prostate RNA was used to make cDNA as described above (Clontech). Images were captured on an AlphaImager 2200 (AlphaInnotech) and band intensities were calculated using AlphaEase software (AlphaInnotech). Relative transcript levels were normalized to Actin levels for each case. In order to detect pseudogenes of Oct4A we followed the procedure of Panagopoulos et al.¹⁰ Briefly, the Oct4A PCR product was purified using the Wizard SV Gel and PCR Clean-Up System

(Promega). ApaI was used to digest purified products corresponding to 20µl of the amplified volume. Digestion yields two fragments, 224- and 399-bp long corresponding to isoform 1 of Oct4A while undigested products represent pseudogenes.

PCR Primer specifics

GENE NAME	PRIMER SEQUENCE	ANNEALING TEMPERATURE	REFERENCE
Actin	F5'-CAGCCATGTACGTTGCTATCCAGG -3' R5'-AGGTCCAGACGCAGGATGGCATG -3'	55°C	Origene (Rockville, MD)
Oct 4A	F5'-ACACCTGGCTTCGGATTTGCCT-3' R5'- GCTTCCTCCACCACTTCTGCAGC-3'	60°C	Panagopoulos et al. Genes, Chromosomes Cancer 2008; 47:521-529.
Sox2	F5'-CCCCCGGCGGCAATAGCA -3' R5'-TCGGCGCCGGGAGATACAT -3'	55°C	Pal and Ravindran. Cell Prolif 2006; 39:585-598.
Nanog	F5'-CCTCCTCCATGGATCTGCTTATTCA-3' R5'-CAGGTCTTCACCTGTTGTAGCTGAG-3'	55°C	Pal and Ravindran. Cell Prolif 2006; 39:585-598.
cMyc	F5'-TACCCTCTCAACGACAGCAG-3' R5'-TCTTGACATTCTCCTCGGTG-3'	55°C	This study
Klf4	F5'-GAGAGAGACCGAGGAGTTCA-3' R5'-CCTTGCTGACGCTGATGAC-3'	55°C	This study

FACS Analysis and Cell-sorting

DU145 and PC3 cells were detached from culture vessels with 0.05% trypsin in PBS. Cells were incubated for 10 hrs in fresh medium on a rocker platform, to enable regeneration of cell adhesion molecules. Cells were then washed, suspended in buffer (PBS containing 1% BSA and 1mM CaCl₂), and stained with either PE or FITC conjugated primary antibodies (E-cadherin 0.25µg/3x10⁵, PODXL 1µg/3x10⁵, SSEA1 0.5µg/3x10⁵, SSEA4 0.5µg/3x10⁵ from R&D Systems, CD44 10µl/5x10⁵, CD9 0.25µg/3x10⁵, CD24 2µg/3x10⁵ from BD Pharmingen, EpCAM 10ul/5x10⁵ from Biomeda, CD133 10µl/1x10⁶ from Miltenyi Biotech) and the appropriate isotype-matched control for 30 min at 4°C. Cells were stained with propidium iodide (Sigma) and

analysis was done (FACSCalibur flow cytometer, Becton Dickinson). Live single cells were gated for analysis and sorted (FACS AriaSOP Cell Sorter with Diva 6.1 software, Becton Dickinson). Cells were either used immediately for experiments or were cultured exactly as for parental unsorted cells.

Prostate spheroid culture

The prostate spheroid cultures were made from primary prostate tumor tissue, DU145 and PC3 cells according to the method of Shi et al.¹¹ Briefly, cells were grown in suspension culture using ultralow attachment plates (Corning) to generate spheroids. The cells were cultured at a density of 1,000cells/ml in serum free DMEM/F12-50/50 supplemented with 20ng/ml EGF (Biosource), 10ng/ml bFGF (Invitrogen), 5µg/ml heparin (Sigma), 2nM Glutamate, 1% penicillin-streptomycin (50 IU penicillin and 50µg/ml streptomycin), 0.2% BSA, 1x B27 without Vitamin A and 1x Insulin-Transferrin-Selenium-A (Gibco). The culture media was changed every 3 to 4 days.